ON THE ECOLOGY AND EVOLUTION OF FUNGAL SENESCENCE

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Contents

Chapter 1

General introduction

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All organisms that have a unitary body plan appear to age and die sooner or later. Organisms like plants and fungi that have modular body plans appear to be virtually immortal, though also they do not invariably escape the aging process. This thesis focuses on several typical aspects of the biology of filamentous fungi like modular growth, and their relation to the fungal senescence process.

A definition of aging and senescence

The definition of the word ‘aging’ varies widely throughout scientific literature. The word applies both to processes that involve development and maturation, and to processes that involve deterioration. The word ‘senescence’ has less wide a range of association and often applies to processes that involve deterioration only. Here, the two are considered equivalent: Aging or senescence is defined as a progressive age-related decline in the fitness of an individual, due to inherent factors, and ultimately culminating in death. This definition is relatively straightforward when applied to unitary organisms, but not so when applied to modular organisms. This is because it heavily depends on the concept of individuality, which is not particularly straightforward in the latter. Modular organisms like fungi and plants are typically composed of multiple genetically identical, but physically independent modules or vegetative clones. In plant biology these are frequently referred to as ‘ramets’. The ramets that make up one genetic entity are together referred to as a ‘genet’. The unit of selection will generally coincide with a ramet rather than with a genet, and a point could thus be made that each single ramet should be considered an individual. Here however, largely because this has been customary in previous work, a fungal individual is provisionally defined as one genetic entity. Fungal senescence is defined accordingly, as the age-related decline in fitness of one such genetic entity, due to inherent factors, ultimately culminating in the irreversible growth arrest and death of all the vegetative modules or clones of which that entity consists. The term ‘fungal senescence’ historically comprises a seemingly heterogeneous set of phenomena, some of which can be called aging according to its former definition, though some of which should probably be classified as systemic diseases. There is in fact only a thin line between disease and aging. In one respect, the entire process of aging may even be seen as a collection

of inborn diseases that have accumulated in an organism over the course of its evolutionary past.

On the evolution of aging

Evolutionary adaptations occur via natural selection of heritable traits that increase fitness (Darwin, 1859). Aging however seems a highly maladaptive trait: It is not immediately clear why aging would have evolved in the first place. Darwin surprisingly never addressed this paradox. One of the earliest evolutionary explanations of aging was one made by Alfred Russel Wallace, the co-discoverer of evolution by natural selection. In a footnote to the English translation of the 1881 essays of the German theoretician August Weismann, he essentially suggested that immortality would be sacrificed for the sake of reproduction (Wallace, 1889), an idea that was embraced by Weismann and would be echoed many years later in the seminal works of Medawar (1952), Williams (1957) and Kirkwood (1977).

Paramount to the latter idea of immortality being sacrificed is the Weismannian **distinction between germ line and soma**. Weismann stated that whereas germ cells are able to transmit hereditary information, somatic cells are not. In other words: There is a unidirectional flow of hereditary information from germ line to soma, but not the other way round. This idea is in obvious contrast to Lamarckian evolution, and is commonly known as the **Weismann barrier**. Aging is a property of the soma and simply cannot be a property of the germ line: The germ line must be immortal. In line with this, modular organisms ranging from fungi to plants and even some animals like colonial invertebrates, that do not have a clear distinction between germ line and soma, indeed appear not to senesce. Like Wallace, Weisman essentially suggested that aging evolves because organisms must invest resources in reproduction rather than in somatic maintenance.

Darwin and his contemporaries did not have a modern-day understanding of the exact mechanism by which traits are inherited. Though the principles of Mendelian genetics had already been discovered in the nineteenth century, it took until the early twentieth century for them to be rediscovered and incorporated into evolutionary theory. The Neo-Darwinistic awareness that genetic variation is the basis upon which natural selection acts obviously also greatly changed the way in which evolutionary biologists dealt with the problem of aging. Greatly influenced by the approach of theoreticians like Haldane (1927), Norton (1928) and Fisher (1930), Peter Medawar (1952) ultimately formulated the idea that **aging evolves in the shadow of natural selection**. This is now the central tenet of the evolutionary biology of aging. Most organisms experience an ever increasing, cumulative risk of death. This is because of predation, disease or other accidental causes of mortality. Any trait that would

manifest itself only after every individual of a certain population had died from extrinsic causes, would not be under selection. This means that regardless of the nature of this trait, which may be deleterious or even lethal, it would be able to spread through the population. Aging thus evolves as it is not counteracted by natural selection. This idea is generally known as the **mutation accumulation theory of aging** (Medawar, 1952). Deleterious traits with pleiotropic, beneficial effects early in life may even be favored by natural selection. Although this was in fact also pointed out by Medawar, the latter idea was more fully developed by George Williams and is now commonly known as the **antagonistic pleiotropy theory of aging** (Williams, 1957). Both theories are not mutually exclusive and their relative importance is in fact still an issue of debate. A special case of the mutation accumulation theory of aging is the **disposable soma theory** put forward by Thomas Kirkwood (1977). The latter is based on the premise that somatic maintenance and repair are costly. Resource allocation between maintenance and repair will be optimized so that the total reproductive output of an organism is maximized. This means that given a particular regimen of extrinsic mortality just enough metabolic resources will be invested to maintain an organism in a proper state only for the duration of its expected life time.

A large body of associative as well as experimental evidence exists to support the evolutionary theories on aging (Rose, 1991). Many studies use a comparative approach. The results of these studies are frequently in line with the evolutionary theories. Taxa that are characterized by a high level of extrinsic mortality generally adopt a strategy of early and copious reproduction and relatively short life span. Taxa that are characterized by a low level of extrinsic mortality generally adopt a strategy of late reproduction and long life span. Many animals for example have evolved mechanisms to escape predation risk, like large body size or protective shells, and these are relatively long-lived (Austad, 1997; Kirkwood, 2002). There is also more direct, experimental evidence. Selection for postponed reproduction in the fruit fly *Drosophila melanogaster* for example leads to a delay in the aging process and *vice versa*. (Luckinbill and Clare, 1985). Stearns *et al.*, (2000) showed in an even more direct test, that when populations of *D. melanogaster* were subjected to different adult mortality rates, life history parameters like life span and age at maturity evolved in the predicted direction.

Proximate mechanisms of aging

There are numerous proximate, mechanistic explanations of aging, perhaps as many as there are researchers in the field. Only several of the most important ones will be discussed here. In 1908 the physiologist Rubner discovered a link between metabolic rate, body size and life span. Life span and metabolic rate appear to be inversely correlated. Longer lived animals are

generally bigger and they have a lower metabolic rate. This became known as the **rate of living hypothesis (**Pearl, 1928). Life span can be modulated by numerous external factors that influence metabolic rate, including temperature and nutrition. Numerous though not all observations are in line with the rate of living hypothesis. Birds for example have higher metabolic rates than mammals of a similar size, but on average they live longer (Austad, 1997). A similar positive correlation between metabolic rate and life span was also found within outbred laboratory populations of mice (Speakman *et al.,* 2004). The empirical data is thus seemingly contradictory, and as discussed also further on, this is probably because not just the rate but the efficiency of metabolism is a key parameter. In the case of the mice investigated by Speakman *et al.* (2004), the individuals that had a higher metabolic rate all showed an increase in the proton conductance of the inner mitochondrial membrane. The mitochondria of longer-lived mice were less well coupled and consequently showed increased but less efficient metabolism.

Dietary conditions obviously greatly influence metabolic rate and efficiency. **Calorie restriction** (CR) refers to a dietary regimen that is low in calories, without malnutrition. It has a life span extending effect, and this was first noted in rodents by McCay *et al*. (1935). Since its discovery in rodents, the life span extending effect of CR has received notable attention and has been documented in many different organisms, ranging from yeast (Lin *et al.,* 2000) to primates (Lane *et al.,* 2001; Ingram *et al*., 2004). In *D. melanogaster*, the life span extending effect of CR is observed when starting a diet at any age (Mair *et al*., 2003). It is associated with increased resistance to various kinds of stress including for example heat and oxidative stress, and appears to forestall many late-onset diseases including cancer (Weindruch and Walford, 1988; Sohal and Weindruch, 1996; Hursting *et al*., 2003; Berrigan *et al*., 2002). CR allows organisms to postpone reproduction and survive unfavorable conditions. When food intake is restored, calorie restricted individuals are typically still able to reproduce whereas controls that are fed *ad libitum* are post-reproductive or no longer alive. CR is thus of clear selective value (Holliday, 1989). Recent work has unraveled that it probably acts through a conserved regulatory program that induces a wide array of physiological changes, rather than by simply delaying the overall metabolic rate (reviewed by Guarente and Picard, 2005).

Aerobic metabolism uses oxidative processes to maximize the energy yield that can be obtained from food. As the terminal acceptor of electrons molecular oxygen is used. As a byproduct of this process though, reactive oxygen species (ROS) are formed. Harman (1956) proposed that aging results from free radical associated damage to the cell, inducing irreversible damage to the cell its constituents, an idea referred to as the **free radical theory of aging**. This idea has since then received notable attention and experimental support.

Comparative studies have for example shown that there is an inverse correlation between longevity and the amount of mitochondrial free radicals produced in mammalian mitochondria (Barja, 2002). Furthermore, longevity is correlated with the induction of antioxidant defenses like superoxide dismutase and catalase. This has been found for example in Drosophila (Rose, 1989) and in Caenorhabditis (Larsen and Clarke, 2002). Experimental manipulation of these antioxidant defenses, like the overexpression of CuZn Sod (Sod1, the cytoplasmic form of superoxide dismutase) and catalase, for example increase longevity in *D. melanogaster* (Orr and Sohal 1994).

On the evolution and ecology of fungal senescence

As mentioned earlier, modular organisms like fungi do not have a clear distinction between germ line and soma. Because of this, systemic senescence is expected to be absent or greatly delayed in fungi. Parts or modules like individual fruiting bodies may outlive their usefulness and die, but the fungal mycelium is generally expected to be long-lived. Moreover, intrinsic to their way of growing, fungi have a fecundity that increases exponentially with age. Fungi typically show radial growth and their size consequently increases exponentially with age. Assuming that their total size is roughly proportional to their fecundity, their fecundity thus also increases exponentially with age. This means that there is a disproportional advantage to attaining old age and selection will consequently oppose senescence. This type of argument was already made by Hamilton in 1966, and applies for example also to organisms like fish (Reznick *et al*., 2002), that do not stop growing after reaching the age of maturity, and benefit from a disproportional increased fecundity with old age. Thus only under very stringent conditions aging or senescence is expected to evolve in fungi. Most fungi indeed appear to be extremely long-lived. One famous example is the Honey mushroom *Armillaria bulbosa,* nowadays known as *Armillaria gallica.* In a seminal paper, Smith and coauthors (1992) reported a single clone of this fungus that was approximately 15 hectares in size. They estimated it to be at least 1500 years old and about ten tons heavy, which is about the size of an adult blue whale. There are nonetheless important exceptions found that do appear to senesce in well-studied fungal genera like Neurospora and Podospora.

One of the exceptional fungi that senesce is the pseudo-homothallic, coprophilic ascomycete *Podospora anserina.* All natural isolates of this species age and die within a matter of weeks (Rizet, 1953). Why? The answer probably lies within the ecology of the species. Many fungi occupy ecological niches in which they are limited neither spatially nor temporally. The above mentioned species of Armillaria for example is usually found as an innocuous saprophyte, living on organic matter in the soil. It can spread over large distances

via specialized bundles of hyphae called rhizomorphs. Spatially as well as temporally it is virtually unlimited and thus expected to be extremely long-lived. Fungi that grow on more ephemeral substrates on the other hand may be expected to be short-lived. *P. anserina* grows on herbivore dung and this of course is an extremely ephemeral substrate. Its life cycle is illustrated in figure 1-1. Podospora does not spread via vegetative spores like for example species from the related genus Neurospora (Figure 1-2), and all vegetative modules of an individual Podospora colony hence share the same fate. This means that the unit of selection coincides with the genetic individual as a whole. Other coprophillic fungi may similarly be expected to be short-lived. In line with this, cases of senescence have for example been reported from other species of Podospora like *P. curvicolla* (Bockelman and Esser, 1986), isolates of coprophillic Ascobolus species (Marcou, 1961) as well as from *Sordaria macrospora* (Gagny, 1997). Senescence may also be expected in other fungi that live on a temporally as well as spatially limited substrate and have a strategy of early and copious reproduction. Particularly in many pathogenic fungi the necessary conditions for aging to evolve appear to be met. Notably, natural isolates of Phomopsis, a fungal pathogen of Plantago species, also appear to senesce (A. van Harn, unpublished observation).

Fungi, as discussed below, age in a different way than unitary organisms do. For example, whereas the telomeres progressively shorten in somatic cell lines of animals due to a decrease in telomerase activity, this does not appear to be the case in the senescence process of *P. anserina* (Schwartz and Osiewacz, 1996). Senescence in fungi is almost invariably associated with the somatic accumulation of defective mtDNA, a process that also is involved in human degenerative syndromes and male sterility in plants. Taylor *et al.*, (2002) recently showed that mitochondrial defects accumulate when the importance of within cell selection for replication increases relative to that among cells for cellular functionality. In their case the mitochondrial defects were '*petite*' mutations in yeast that have a within-cell replicative advantage, but render the cells unable to respire. They manipulated the effective population sizes of the yeast cultures and showed that the petite mutations only accumulated in small populations, where selection between cells was relatively relaxed, thus increasing the importance of within cell selection. Similarly, the senescence phenomena in filamentous fungi can be viewed as a multilevel selection problem. Selection operates on mitochondrial genomes within a single mitochondrion, on mitochondria within a cell, on cells within an organism, and so on. Consistent with this is for example the observation that the serial passage of *P. anserina* in liquid culture causes longevity (Turker and Cummings, 1987). Normally *P.anserina* is grown on solid agar slants. Though this was never formally proven, serial passage in liquid culture probably allows for strong somatic selection. This underlines the importance of the modular body plan.

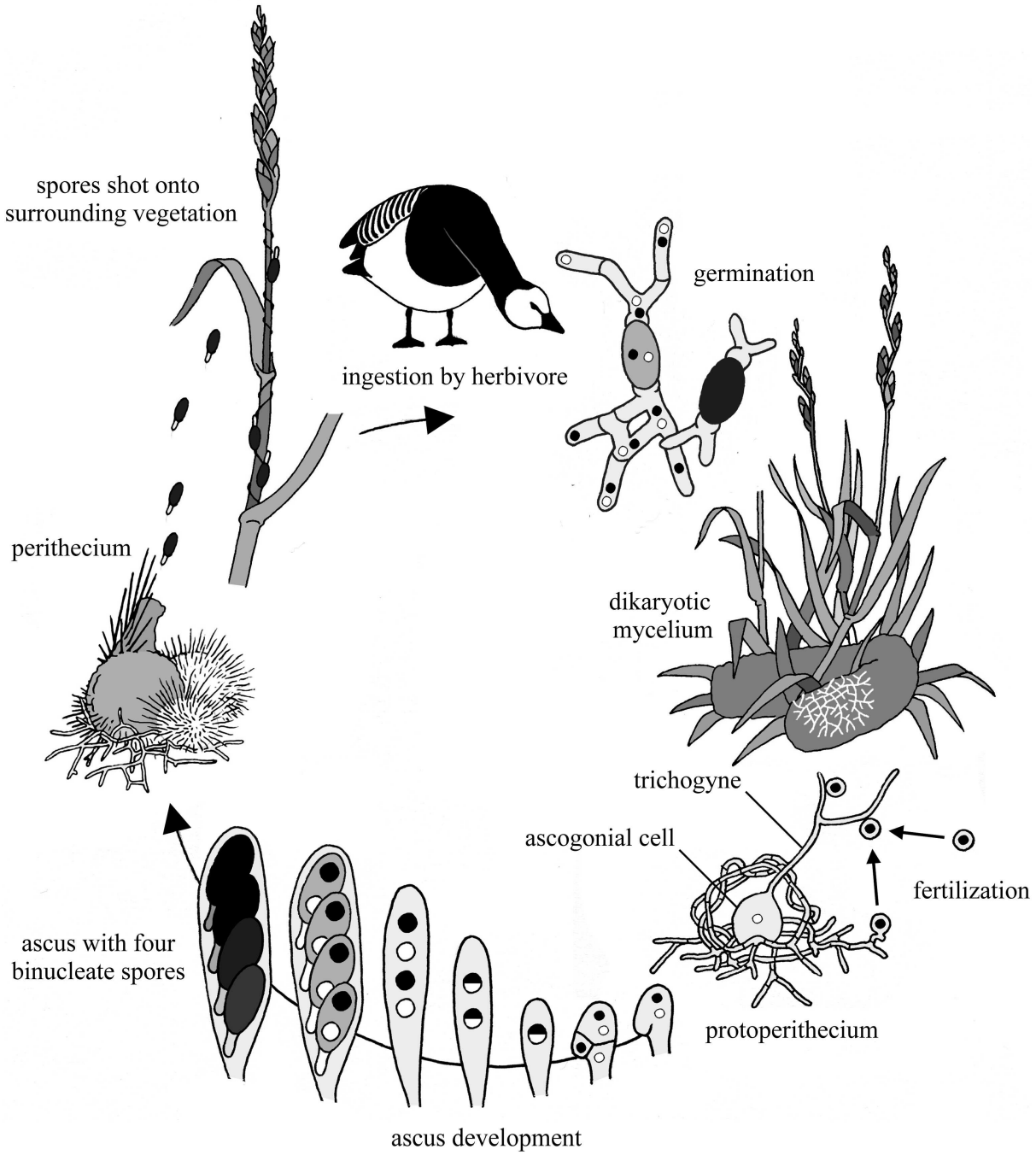

Figure 1-1. Life-cycle of *Podospora anserina*. Although monokaryotic derivatives can be found, mycelia are usually dikaryotic, containing nuclei of both mating types. Colonies form protoperithecia containing ascogonial cells from which trichogynes extend. These trichogynes fuse with male gametes that contain a nucleus of the opposite mating type. Fertilization leads to the formation of an ascus with four binucleate ascospores. The spores are shot onto the surrounding vegetation and ingested by herbivores. After passing the intestinal tract they germinate, giving rise to novel dikaryotic mycelia.

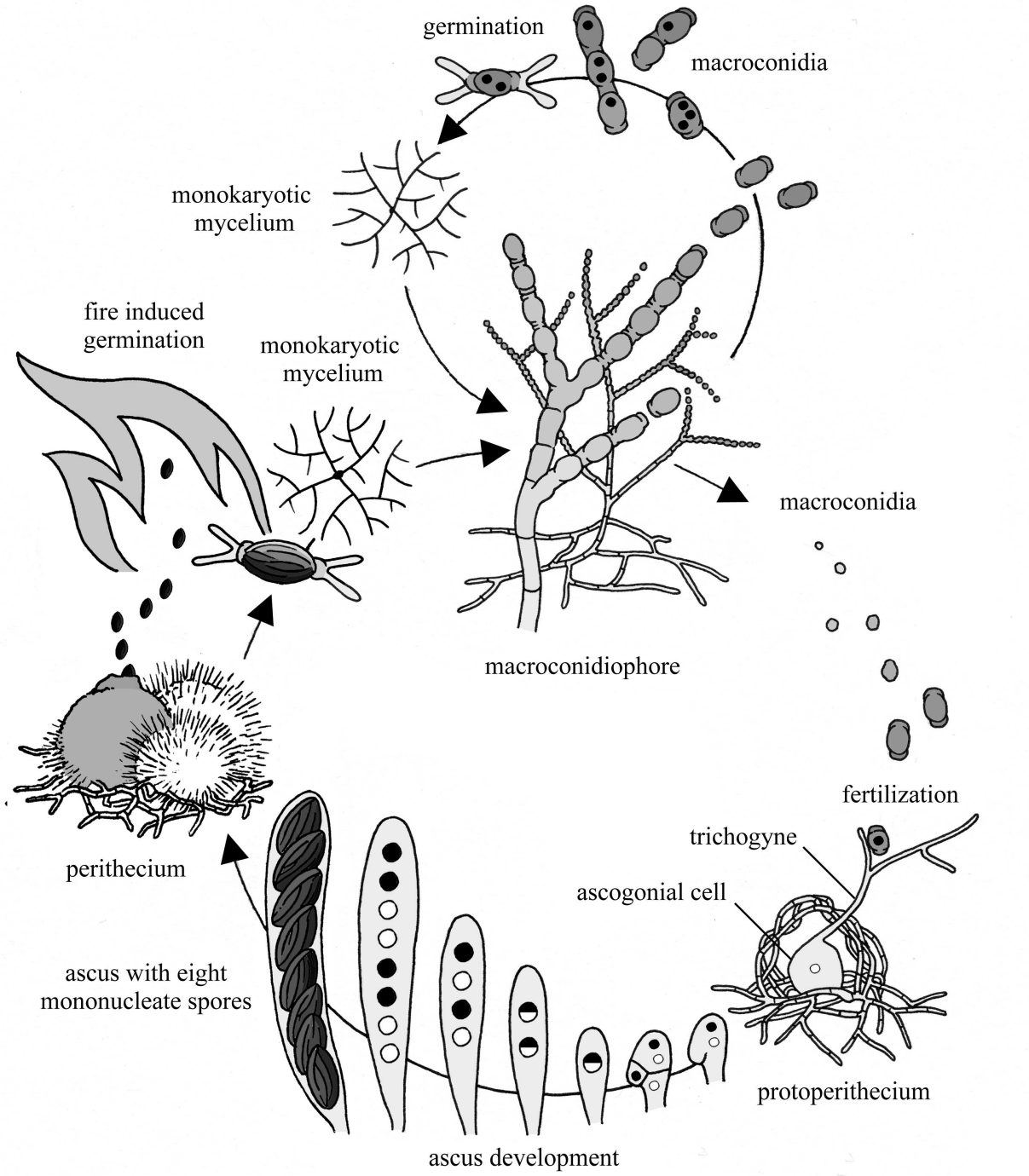

Figure 1-2. Life-cycle of *Neurospora intermedia.* Mycelia are monokaryotic. They give rise to macroconidiophores that produce mono- or multinucleate macroconidia that may serve as vegetative spores or as male gametes. Colonies form protoperithecia that contain ascogonial cells from which trichogynes extend. These trichogynes fuse with male gametes containing a nucleus of the opposite mating type. Fertilization leads to the formation of an ascus with eight mononucleate ascospores. These are shot onto the surrounding vegetation and germinate after induction by fire, giving rise to new mycelia.

All natural isolates of *P. anserina* that have thus far been described senesce. Senescence is characterized by a progressive deterioration of the growing mycelium. Cultures decline both in growth rate and fertility, ultimately culminating in irreversible growth arrest. At the dying front of the mycelium, hyphae show morphological aberrations and typically accumulate large amounts of dark pigment. Life span is usually expressed in centimeters of growth and is a strain specific trait that is inherited largely maternally (Rizet, 1953; Rizet, 1957; Marcou, 1961). The demise of a culture starts with the appearance, after a strain-specific time, of an infectious 'senescence determinant' that can transform a non-senescent culture into a senescent one. When two vegetative compatible cultures fuse, one of which is still young and one of which is in the senescent state, the mixed culture typically adopts the senescent state. The senescent state can be inherited maternally, though the life expectancy of a culture is typically reset in the sexual cycle by a hitherto unknown mechanism (Marcou, 1961), a process often referred to as 'rejuvenation'.

The appearance of the 'senescence determinant' coincides with the amplification of covalently closed circular derivatives of the mitochondrial genome. These derivatives are referred to as senDNAs (Jamet-vierny *et al.,* 1980; Belcour *et al.* 1981; Wright *et al.,* 1982). Loss of senDNA can be accomplished by treatment with ethidium bromide and appears to be associated with a reset of the life expectancy (Koll *et al.*, 1984), whereas gain is associated with a transformation into the senescent state (Tudzynski *et al.,* 1980). The most frequently observed senDNA is senDNA α, also referred to as pl-DNA for plasmid-like DNA because of its resemblance to bacterial plasmids. SenDNA α is a multimer consisting of a number of 2.5 Kb units that correspond exactly to intron α, a self-splicing group II intron of the mitochondrial *cox1* gene. The latter intron is present in all natural isolates thus far collected (Kück *et al.* 1985). It is an active retrotransposon (Sellem *et al.*, 1993), and hence associated with major rearrangements of the mitochondrial genome. Other senDNAs than senDNA α (also indicated using Greek letters) do not correspond to introns, but to certain regions of the mitochondrial genome, and these may be variable in size (Koll *et al*., 1985; Dujon and Belcour, 1989).

Many spontaneous long-lived mutants of *P. anserina* have been described. These were all either female sterile or showed reduced fertility. Many of them showed large deletions within the mitochondrial genome partially or entirely covering the intron α region, and all lacked amplified senDNA α (Osiewacz *et al.,* 1989; Vierny *et al.,* 1982; Koll *et al*., 1985; Belcour and Vierny, 1986; Kück *et al.*, 1985 Schulte *et al.,* 1988). Ultimately intron α

however proved to be just one of many factors in the senescence syndrome of *P. anserina*. A more precise deletion of the intron from the mitochondrial genome (in strain *mid26,* for mitochondrial intron deletion) for example did not abolish senescence (Begel *et al.,* 1999). The latter clearly indicates that it is not just the presence of the mobile intron that is responsible for senescence.

More recent work has indicated that the respiratory chain has a pivotal role in the senescence process (Dufour and Larsson, 2004), either via the production of cytotoxic free radicals or via an energy level mediated pathway. Disruption of cytochrome c oxidase or respiratory complex IV causes the induction of an alternative respiratory pathway, and this is associated with a reduced level of oxidative stress, a stabilization of the mitochondrial genome, and complete female sterility, the latter presumably indicating a severe deficiency in cellular energy production (Dufour *et al.,* 2000). Alterations in copper metabolism can have similar effects, because the cytochrome c oxidase route is copper dependent. A loss-of-function mutation in the nuclear gene *grisea* for example, that encodes a copper-activated transcription factor regulating the expression of the copper permease *PaCrt,* also leads to reduced levels of oxidative stress, a stabilization of the mitochondrial genome, reduced fertility and longevity (Osiewacz and Nuber, 1996; Borghouts *et al.,* 2002). The mitochondrial genetic instability that is generally observed in senescent isolates may thus well result directly from the oxidative damage associated with respiration. The instability may even promote additional oxidative damage itself, effectively resulting in a positive feedback loop. This has however never formally been proven. The mitochondrial electron transport chain (ETC) is composed of several protein complexes that couple electron transport to the translocation of protons out of the mitochondrial matrix, over the inner mitochondrial membrane, into the inter-membrane space. The proton translocating complexes are NADH:ubiquinone oxidoreductase or complex I, ubiquinol:cytochrome c oxidoreductase or complex III, and cytochrome c oxidase or complex IV. The electrochemical gradient that is built-up by these complexes is generally referred to as $\Delta\psi_m$. The energy that is harnessed in $\Delta\psi_m$ is used by the mitochondrial F_0-F_1 ATP synthase or respiratory complex V to phosphorylate ADP. In Podospora additional ETC components exist that are typical for most fungal and plant ETCs, but not for animal ETCs. One of these components is the alternative oxidase (AOXp). Though the latter is not expressed in wild-type cultures that are in exponential phase, it typically is constitutively expressed in the long-lived mutants that are deficient in cytochrome c oxidase (Dufour *et al.,* 2000). AOXp allows electron transport to bypass the cytochrome c oxidase dependent pathway when blocked. AOXp is itself not associated with proton translocation and thus uncouples electron transport downstream of ubiquinone from phosphorylation, thereby potentially lowering both energy and

mitochondrial free radical production. Mild uncoupling is known to have this effect (Miwa and Brand, 2003; Merry, 2002). Consistent with this, cytochrome c oxidase deficient mutants typically show reduced fertility indicative of a reduced cellular energy level, as well as reduced levels of oxidative stress. Lorin *et al.* (2001) however showed that over-expression of the alternative pathway can antagonize the effect of complex IV dysfunction, restoring both the original levels of cellular energy and oxidative stress to that of the original wild type isolate, consequently restoring both senescence and fertility. This may be explained by an indirect effect of AOXp on the functioning of respiratory complex I. Krause *et al*. (2004) recently showed that the respiratory chain of Podospora, like that of mammals, is organized in a large super-molecular structure called the respirasome. The individual components of the chain are thus assembled not entirely independently, which could be consistent with the former observation. Whatever the exact etiology behind it, there are ultimately at least three factors consistently associated with senescence in *P. anserina*: The level of cellular oxidative stress, the stability of the mitochondrial chromosome, and the cellular energy level.

Belcour *et al.* (1991) described a phenomenon in Podospora called *premature death.* The latter is a growth arrest that occurs very rapidly after germination of the ascospores and is under the control of the nuclear genome. It is probably an equivalent of the *natural death* phenomenon in Neurospora (discussed further on). Premature death is associated with a specific mtDNA deletion somehow caused by the interaction between a mutant allele of the *AS4-1* gene that encodes a cytosolic ribosomal protein, and the rmp gene (for *régulateur de la mort prématurée*, Sellem *et al.* 1993). The latter has two known natural alleles that are coupled to the mating type locus. *Premature death* may result from erroneous cytosolic protein synthesis.

Senescence in Neurospora

In contrast to Podospora isolates, Neurospora isolates normally do not senesce. Laboratory strains like the standard Oak Ridge *Neurospora crassa* wild type are typically capable of indefinite vegetative growth (Griffiths, 1992). Some natural isolates however do. Senescence can for example be found in about a third of all the Hawaiian *N. intermedia* wild types (Rieck *et al*., 1982; Griffiths and Bertrand, 1984; Debets *et al.,* 1995). It is usually demonstrated by using serial transfers of macroconidia. After a number of transfers, the fertility of the cultures, their conidial production and growth rates decline. The last cultures typically still show conidiation, though none of the spores produced are able to germinate. Like in Podospora, death often but not invariably coincides with morphologically aberrant hyphae and increased pigmentation. Reminiscent of the ‘senescence determinant’ from Podospora, the demise of a

culture typically starts after a strain-specific time. When young cultures are mixed with compatible senescent cultures, the mixed cultures adopt the set life expectancy of the latter. Though sectors sometimes escape the dying front of a mycelium (a phenomenon that is called the *Lazarus* effect), they ultimately always permanently stop growing (Griffiths *et al.*, 1986). This indicates that senescence is probably different from the *stopper* phenomenon of Neurospora that is characterized by continuous cycles of growth and growth arrest (Bertrand and Pittenger, 1969). Like in Podospora, senescence is inherited maternally and life expectancy is reset in the sexual cycle.

The senescence phenotype found in the *N. intermedia* strains was termed *Kalilo*, after the Hawaiian word for "dying" or "hovering between life and death" (Griffiths, 1992). Comparison of senescing and non-senescing cultures showed that it is associated with an extragenomic element: an 8.6 Kb linear mitochondrial plasmid with long, perfect terminal inverted repeats (TIRs) (Chan et al., 1991). This plasmid contains two main open reading frames (ORFs) that are transcribed from a promoter located within its terminal region (Vickery and Griffiths, 1993). The latter ORFs encode two viral-like DNA dependent polymerases: a DNA and an RNA polymerase. The plasmid probably replicates via a strand displacement mechanism like that of adenoviruses, thus involving a panhandle or racquet shaped ssDNA intermediate (Griffiths *et al.*, 1992). Over time, the plasmid typically increases in titer and integrates into the mitochondrial genome by a yet unknown mechanism, effectively acting as an insertional mutagen (Bertrand *et al.*, 1985). Plasmids allegedly integrate via a form of homologous recombination between a short sequence within the tip of the plasmid and a short sequence that may be anywhere in the mitochondrial genome. Integration typically results in large, inverted duplications of mtDNA on either side of the plasmid, which is probably due to the involvement of the ssDNA replication intermediate. For a reason that is not yet understood, the mtDNA molecules that contain an integrated plasmid copy are 'suppressive': Over time they gradually replace the wild-type molecules until the latter are no longer detected (Myers *et al.*, 1989). They may thus have a replicative advantage over the wild type mtDNA molecules. Senescing cultures typically show aberrant cytochrome spectra and ribosomal profiles. They become deficient in functional mitochondria and ultimately die.

The *N. intermedia* senescence plasmid, here referred to as pKALILO, is normally inherited maternally, which is consistent with its mitochondrial location (Griffiths and Bertrand, 1984). Also its integrated copies can be inherited maternally, though this occurs only in cultures that are close to death (Bertrand *et al.*, 1985). In addition, pKALILO can be transmitted paternally (Yang and Griffiths, 1993) or horizontally (Debets *et al.*, 1994). Horizontal transmission occurs via transient hyphal fusion and is highly effective. Though the

transmission of the senescent state is effectively blocked between incompatible wild types, the plasmid itself and its potential to induce senescence is transmitted with relative ease: Frequencies of horizontal transmission between incompatible wild types are in the order of ten percent (Debets *et al*., 1994). Not just within, but also between Neurospora species the plasmid can be horizontally transmitted. The latter can be achieved for example by introgression and subsequent heterokaryosis (Bok *et al.,* 1999). The relative ease with which it is transmitted among different hosts, either within or between species, probably reflects the parasitic nature of the element.

Several studies have focused on the distribution of pKALILO among natural isolates from around the world (Yang and Griffiths, 1993b; Arganoza *et al.,* 1994). The prototypic plasmid has thus far however only once been found outside the natural population of *N. intermedia*: In an isolate of *N. tetrasperma* from Moorea-Tahiti (He *et al.*, 2000). The latter authors did not report whether in *N. tetrasperma* it was associated with senescence as it was in *N. intermedia*. Several homologues of pKALILO were found in other Neurospora species (Marcinko-Kuehn *et al.,* 1994) and even within the related genus Gelasinospora (Yuewang *et al*., 1996), but all of these were different from the prototypic plasmid at the DNA sequence level, and not unambiguously associated with senescence.

A senescence syndrome like that of the *N. intermedia* isolates from Hawaii was also found in *N. crassa* isolates from Aarey, India (Court *et al.*, 1991). It was called *Maranhar,* a Sanscrit word meaning roughly the same as *Kalilo* (Griffiths, 1992). Like the *Kalilo* strains, the *Maranhar* strains from India also contained a linear mitochondrial plasmid. The latter plasmid, here referred to as pMARANHAR, is structurally similar to pKALILO (with large, perfect TIRs, and containing two main ORFs that encode two viral-like DNA dependent polymerases), though at the DNA sequence level it is completely different (Court and Bertrand, 1992). PMARANHAR appears to induce senescence in the exact same way as pKALILO, via an increase in titer and its concomitant integration into the mitochondrial genome.

Senescence is also observed in Neurospora isolates that carry one of the mitochondrial retroplasmids pVARKUD and pMAURICEVILLE (Akins *et al.*, 1986; Akins *et al.,* 1989). Like pKALILO and pMARANHAR, also these plasmids interfere with mitochondrial function. Both pVARKUD and pMAURICEVILLE encode a reverse transcriptase (RT) that can generate hybrid mitochondrial cDNA via RNA template switching, an activity commonly known from retroviral and retroviral-like RTs, for example also from the RT encoded by intron α (Sellem *et al.,* 2000). The plasmids are able to initiate cDNA synthesis *de novo* (without a primer) at the 3’ cca sequence of the plasmid transcript (Wang and Lambowitz, 1993), but they are able to do so also at the 3’ cca of tRNAs (Chiang and Lambowitz, 1997).

The plasmids hence often incorporate mitochondrial tRNAs. They may integrate into the mitochondrial genome via subsequent homologous recombination (Chiang *et al.,* 1994). The variant and integrated forms of pVARKUD or pMAURICEVILLE can be found in senescing as well as in non-senescing cultures (Chiang *et al.,* 1994; Fox and Kennel, 2001), so integration itself is perhaps not directly the cause of senescence. The latter has in fact also never formally been demonstrated for pKALILO or pMARANHAR. It is probably the over-replication of these plasmids or one of their variant forms that leads to a loss of respiratory function and ultimately death (Stevenson *et al.*, 2000).

Senescence in Neurospora is thus prominently associated with mitochondrial plasmids. However also nuclear elements have been described in Neurospora that are associated with senescence phenomena, like the phenomenon called *natural death* (Lewis and Holliday, 1970), a nuclear mutation that leads to accelerated senescence under both growing and stationary conditions. *Natural death* is highly reminiscent of *premature death* in *P. anserina*. Lewis and Holliday initially proposed that senescence in the *natural death* strains results from errors in protein synthesis. Munkres and Minssen (1976) later showed that *natural death* could be alleviated with antioxidants. Like the *premature death* strains (Munkres and Rana, 1978), the *natural death* strains accumulated a pigment called lipofuscin, which is the end product of lipid peroxidation. Munkres and Minssen also showed that *natural death* was associated with an upregulation of the activities of antioxidant enzymes like superoxide dismutase, gluthathione peroxidase and glutathione reductase. The natural death strains typically die within several subcultures, often much earlier than strains that die from plasmid associated senescence. They die within maximally six subcultures at 26 °C, or within one or two at 34 °C. The natural death mutation is associated with cytochrome deficiencies, ribosomal deficiencies, reduced oxygen uptake and extremely low ATP/ADP ratios (Seidel-Roger *et al.*, 1989; Pall, M. L., 1990). Concomitantly, the strains show a high level of mtDNA recombination (Bertrand *et al.*, 1993).

Outline of this thesis

The main aim of this thesis is to expand our knowledge of the fungal senescence phenomenon in order to gain a better understanding of its possible proximate and ultimate causes. It deals with a comparative analysis of the existing variation in life span that is found between and within fungal species, but also with the experimental manipulation of factors like dietary or calorie restriction. The work is done with the two major systems in which fungal senescence has been described, the ascomycetous genera Podospora and Neurospora.

Chapter 2: In chapter 2 a comparative analysis is made of invertron-based senescence in two sympatric sister species of the genus Neurospora, one of which has a heterothallic and one of which has a pseudo-homothallic life cycle. Both the frequency and severity of the invertron-based senescence syndromes appear to be host-specific traits. A possible relationship with the different life histories of the two hosts is discussed.

Chapter 3: Though most Neurospora isolates that carry the mitochondrial plasmid pKALILO senesce and die within a matter of weeks, some natural isolates do not appear to senesce at all. This chapter describes a genetic and molecular characterization of these latter strains. In two of the non-senescing pKALILO carrying isolates of *N. intermedia,* variant or integrated copies of the plasmid were found that arose via recombination involving a novel mitochondrial retroelement. This element was also present in the other long-lived isolates. A possible mechanism is suggested for the mode by which senescence is suppressed in these isolates.

Chapter 4: In chapter 4 the effects of calorie restriction (CR) are tested on the senescence syndrome of *P. anserina*. CR extends the life expectancy of natural isolates up to five-fold, much more than normally observed in unitary systems. Natural variation largely corresponds to the presence or absence of pAL2-1 homologues, a group of invertron-type plasmids structurally similar to the pKALILO homologues of Neurospora. The presence of a pAL2-1 homologue drastically reduces the effect of CR. This was substantiated by constructing near-isogenic lines with and without the plasmid. A model is proposed to explain how these plasmids affect CR.

Chapter 5: In chapter 5 a novel spontaneous longevity mutant of *P. anserina* is characterized. Unlike most previously described longevity mutants, the strain was not completely female sterile. Longevity was inherited maternally and corresponded to the integration of a plasmid into the promoter region of the mitochondrial *nad2/nad3* dicistron, which had repercussions throughout the respiratory chain leading to a faster but energetically less efficient form of respiration. The latter was associated with a reduced level of oxidative stress and an increased stability of the mitochondrial genome.

C**hapter 6:** Although the mitochondrial invertrons and their integrative potential have been known for years, the exact mode by which they integrate into the mitochondrial genome has remained elusive. The structure and distribution of pAL2-1/mtDNA or pKALILO/mtDNA recombination junctions were analyzed in an attempt to clarify the integration mechanism.

Integrated copies were found mainly within the mitochondrial rDNA regions of the two respective hosts. Plasmid/mtDNA recombination junctions often but not invariably contained additional insertions and/or deletions. A model for the integration mechanism is proposed.

Chapter 7: In the general discussion and summary, I review the most important findings of this thesis in the light of what was known and make several suggestions for future research.

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Chapter 2

Polymorphism for pKALILO based senescence in Hawaiian populations of *Neurospora intermedia* and *Neurospora tetrasperma*

M. F. P. M. Maas, A. van Mourik, R. F. Hoekstra and A. J. M. Debets

Abstract

The natural population of the heterothallic ascomycete *Neurospora intermedia* from Hawaii is polymorphic for the presence of the linear mitochondrial senescence plasmid pKALILO. Although inter-specific horizontal transmission is at least experimentally possible, pKALILO based senescence has thus far never been found outside the natural population of *N. intermedia*. Here we demonstrate that it is not limited to *N. intermedia,* but also present in the sympatric population of its close pseudo-homothallic relative *Neurospora tetrasperma*. As in its heterothallic congener, senescence in *N. tetrasperma* was associated with the integration of plasmid copies into the mitochondrial genome. The frequency of the senescence plasmid however was substantially lower in *N. tetrasperma* than in *N. intermedia,* and its phenotypic effects were substantially less severe. These results are discussed in the light of the respective life histories of the two hosts.

Introduction

Mitochondrial plasmids are ubiquitous among filamentous fungi (Griffiths, 1995). Though most of them do not have any clear effects on their hosts, some do. PKALILO of *Neurospora intermedia* for example (Bertrand *et al*., 1985, Bertrand *et al.,* 1986), pMARANHAR of *Neurospora crassa* (Court *et al*., 1991) and pAL2-1 of *Podospora anserina* (Maas *et al*., 2004) are all three associated with a senescence syndrome.

PKALILO naturally occurs in the Hawaiian population of *N. intermedia,* where it is found in almost half the isolates (Debets *et al.,* 1995). Cultures that contain this plasmid typically show a progressive decline in vigor, ultimately culminating in death (Griffiths, 1992, Bertrand, 2000). In aging cultures, the plasmid inserts into the mitochondrial genome and is often found at sites located within or near the *rnl* gene that encodes the large mitochondrial rRNA (Bertrand *et al.,* 1985, see also chapter 6). For a reason not yet understood, mtDNA molecules containing an integrated copy are “suppressive”: They gradually replace the wild-

type molecules (Myers *et al*., 1989). Senescing cultures become deficient in functional mitochondrial ribosomes and die.

Because of its mitochondrial location, pKALILO and its concomitant senescence phenotype are usually transmitted maternally. The plasmid can however also be transmitted paternally (Yang and Griffiths, 1993a) or horizontally (Debets *et al.*, 1994). Horizontal transmission occurs via transient hyphal fusion and is strongly restricted by somatic incompatibility. Somatic or vegetative incompatibility is a multi-locus self/non-self recognition system that results in cell death when hyphae of incompatible isolates fuse. The loci involved in this process are called *het*-loci and are usually highly polymorphic within a population (Glass *et al*., 2000). Despite the fact that vegetative incompatibility constitutes a successful barrier against the infectious spread of the senescent state though, it does not completely prevent cytoplasmic exchange, and the plasmid itself can be transmitted with relative ease even among incompatible wild types (Debets *et al.*, 1994). Its rate of intra-specific horizontal transmission is in the order of five percent. This relatively high rate can be seen as a hallmark of virulence and may partly explain the prevalence of pKALILO in the *N. intermedia* population from Kauai.

Experimental evidence indicates that pKALILO can be transmitted not just intra- but also inter-specifically. The latter is possible for example via introgression and subsequent heterokaryosis (Bok *et al.*, 1999). However, despite several large-scale surveys among isolates from around the world (Yang and Griffiths, 1993b; Arganoza *et al.*, 1994), the prototypic plasmid has only once been found outside *N. intermedia*: In an isolate of *N. tetrasperma* originating from Moorea-Tahiti (He *et al.*, 2000). The authors did not report whether the latter isolate showed any signs of senescence. Several pKALILO homologues were found in a range of different hosts including *N. discreta*, *N. crassa* and even a species of the related genus Gelasinospora (Marcinko-Kuehn *et al.*, 1994; Yuewang *et al*., 1996; He *et al.*, 2000). However, all of these plasmids proved substantially different from pKALILO at the DNA sequence level. Besides, those tested neither integrated into the mitochondrial genome nor showed a convincing correlation with senescence. Thus, the pKALILO associated senescence syndrome itself has formally never been described from natural populations outside the Hawaiian one of *N. intermedia.* The majority of pKALILO homologue containing isolates has however never been tested for senescence and the overall picture is thus incomplete.

To explore the inter-specific distribution of pKALILO based senescence, the Hawaiian Neurospora population was screened for novel cases. PKALILO and its associated senescence phenotype were not restricted to the heterothallic species *N. intermedia*. A large number of cases were found in the closely related pseudo-homothallic species *Neurospora tetrasperma*.

Both in *N. intermedia* and in *N. tetrasperma*, senescence was associated with the integration of pKALILO into the mitochondrial genome. Compared to that in *N. intermedia* however, the frequency of the senescence plasmid was substantially lower in *N. tetrasperma*. Also, its phenotypic effects were substantially less severe.

Materials and Methods

Collection, identification and culturing of Neurospora isolates.

Soil samples were collected from Hawaii in June and July 1998. Six of them were taken from three different locations on the isle of Oahu, and 36 of them were taken from eleven different locations on the isle of Kauai. Soil was pasteurized to obtain Neurospora isolates (Perkins and Turner, 1988), by incubating the samples for 30 minutes at 60 ˚C in modified Westergaard's Medium (WM), pH 6.3. Chloramphenicol was used to suppress bacterial growth; 2-furylmethanol (furfural) was used to improve ascospore germination. Following pasteurization, soil was incubated at 25 ˚C for up to two weeks. As soon as they appeared, Neurospora colonies were isolated and subcultured on Vogel's Minimal Medium (VMM), pH 5.8 (Davis and de Serres, 1970). From each isolate, conidia were stored at -80 ˚C, or as silica-gel stocks as previously described (Perkins and Turner, 1988).

For species identification, all isolates were crossed with known reference strains as described by Perkins and Turner (1988). *N. intermedia* strains FGSC #3416 (mating type A) and FGSC #3417 (mating type a), and *N. crassa* strains FGSC #5798 (mating type A), FGSC #4317 (mating type A) and FGSC #4347 (mating type a) were used as reference. Self-fertile isolates producing four-spored asci that could not be crossed with any of these were classified as pseudo-homothallic *N. tetrasperma* isolates.

Senescence was demonstrated using serial subculturing as previously described by Griffiths and Bertrand (1984). Life span was expressed either in days or in number of subcultures. Standard working protocol for Neurospora was as given by Davis and DeSerres (1970).

Detection of pKALILO and pHANALEI-2 homologues

Isolates were tested for the presence of mitochondrial plasmids by PCR and Southern analysis. For the prototypic senescence plasmid pKALILO, this was done using both techniques; for the allegedly neutral mitochondrial plasmid pHANALEI-2, this was done using Southern analysis only. The latter plasmid served as an internal control.

Cultures were grown in liquid VMM, pH 5.8, for approximately 24 hours at 25 ºC. Mycelium was harvested, dried between filter paper and ground using liquid nitrogen. Total DNA was extracted using standard procedures (Sambrook *et al.,* 1989). Prior to the addition of phenol and chloroform, samples were treated for one hour with proteinase K (100 μg/ml final concentration). Electrophoresis was done in 40 mM Tris-HCl, 20 mM acetic acid and 1 mM EDTA (TAE), pH 8.0 using 1% (w/v) agarose, and DNA was visualized using ethidium-bromide staining according to standard procedures (Sambrook *et al*., 1989).

PCR analysis was done using primers located within the large open reading frame (ORF) encoding the DNA polymerase of pKALILO: 5' GGT GGA ATC TGT GAG CTA TA 3' and 5' TGC ATC TCC CTC TTC TTC AC 3'. Primer sequences were based on the sequence of the prototypic plasmid (Chan *et al.*, 1991; GenBank database accession number X52106). Per reaction approximately 100 ng of genomic DNA was used as template. 0.2 μM of each primer, 200 μM of each dNTP, 10mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) Triton X-100, 0.01% (w/v) gelatin and 1 unit of SuperTaq DNA polymerase (HT Biotechnology) was used in a final reaction volume of 25 μl. Amplification was done in a Progene thermal cycler (Techne) using an elongation time of one and a half minutes and an annealing temperature of 58 ºC. Amplification was additionally done at more permissive temperatures. Electrophoresis and visualization of the products was done as described above.

Southern analysis was done according to Sambrook *et al.* (1989). Electrophoresis was done in TAE using 0.8% (w/v) agarose. Per lane, between two and five μg of either (*PstI* and/or *MvnI*) digested or undigested total DNA was used. DNA was transferred onto positively charged nylon membranes (Hybond^{tm} N^+, Amersham Biosciences) and probed either with the large internal 8,202 bp *KpnI* fragment of pKALILO or with pHANALEI-2 in its entirety, each cloned in a pUC18 vector (Vickery and Griffiths, 1993). Probes were labelled using random primed digoxigenin-11-dUTP (DIG-dUTP) labeling and detection was done using the chemi-luminescent substrate CSPD® (Boehringer Mannheim).

Sequencing of the terminal inverted repeat (TIR)

A 631 bp region of the terminal inverted repeat (TIR) of pKALILO was sequenced. Within this region, the plasmid can be distinguished from any of its known, avirulent family members (He *et al.*, 2000). This was done for two isolates of both Neurospora species collected. For the amplification of this region, PCR was done using the following primers: 5' CAG TCT ACC CTC TAC AAT TG 3' and 5' CAC TAT TGA AGG CGC AGG GC 3'. These were based on the sequence of the prototypic plasmid (Chan *et al.*, 1991; GenBank database accession number X52106). Reaction conditions were as described above. Electrophoresis and

visualization was done as also described above. PCR products were purified using a High Pure PCR Product Purification Kit (Roche) and cloned in *E. coli* DH5-α using the pGEM®-T Easy Vector system (Promega). Plasmid DNA was recovered using a High Pure Plasmid Isolation Kit (Roche), and sequenced according to the methods described by Sanger (1977) using Big Dye™ terminators (Applied Biosystems). Sequences thus found were compared with that of the prototypic plasmid (Chan *et al.*, 1991; GenBank database accession number X52106).

Semi-random two-step PCR (ST-PCR) based analysis of plasmid integration sites

To identify the exact plasmid integration sites, a 'plasmid-tagging' approach was devised, based on a previously described, semi-random, two-step PCR (ST-PCR) procedure (Chun *et al.*, 1997). The latter is a way of amplifying unknown flanking sequences. First, a reaction was done using a primer pointing outwards from within the TIR region of pKALILO (5' GAA ATG ATA AAA AGA TCA CAA AGG G 3'), in combination with the partially degenerate primer given in the original protocol (5' GGC CAC GCG TCG GAC TAG TAC $N_{(10)}$ GAT AT 3'). Next, a nested reaction was done using a second primer pointing outwards from within the TIR region of the plasmid (5' GGG TTA AAA TAG GAA CAA AAG GGG 3'), in combination with the other primer given in the original protocol, which comprises the 5' specific part of the partially degenerate primer (5' GGC CAC GCG TCG GAC TAG TAC 3'). Reaction conditions were as originally described. Electrophoresis and visualization was done as described earlier.

ST-PCR products were (gel-) purified, cloned and sequenced as described above. Based on sequence similarity of the products with sequences from the GenBank database (using BLASTn version 2.2.9; Altschul *et al.*, 1997), PCR primers were designed to amplify the corresponding DNA regions from young subcultures of the same isolates and for comparison, these were purified, cloned and sequenced as described above.

Statistical analysis

Statistical analyses were done using the software package SPSS® version 11.0.1 (SPSS).

Results

Senescence in N. intermedia and N. tetrasperma isolates from Hawaii

The Hawaiian population of Neurospora was sampled in search of pKALILO based senescence. Neurospora isolates were obtained from soil samples following heat shock and furfural treatment. Out of 42 soil samples collected in June and July 1998, 30 contained Neurospora. Samples taken from inside or near sugarcane fields typically yielded a large number of isolates. One sample for example yielded 25 isolates per gram of soil. In total, 128 isolates were obtained. All were pure cultures and produced brightly orange colored conidia. Based on crosses with known reference isolates, exactly half of these cultures were classified as single mating type isolates of the heterothallic species *N. intermedia*. Of these 64 isolates, 24 were of mating type A and 40 were of mating type a (a marginally significant deviation from the expected 1:1 ratio; Likelihood Ratio test, G=4.0, df=1, P=0.044). The other half of the isolates was self-fertile and produced four-spored asci; these 64 were classified as double mating type isolates of the pseudo-homothallic species *N. tetrasperma*. Both Neurospora species often occurred in the same soil sample and we did not observe any spatial patterns in their distribution. Since no effort was made to isolate large numbers of cultures from every sample, it may very well be that all soil samples contained ascospores from both species.

All isolates were tested for senescence in a serial subculturing test. Every two or three days, vegetative subcultures were made using bulk conidial transfers as mentioned in the materials and methods section. Cultures were termed dead when vegetative subculturing was no longer possible. Numerous isolates of both species collected showed senescence. Compared to *N. intermedia* though, substantially less isolates of *N. tetrasperma* senesced within the time-course of the experiment: Whereas 39% (25/64) of the *N. intermedia* isolates senesced within 3 months, the same was true for only 19% (12/64) of the *N. tetrasperma* isolates (a marginally significant difference; Likelihood Ratio test, G= 6.53, df=1, P=0.011). In all cases, senescence was associated with a progressive decline in growth rate and female fertility. The final subcultures of senescing isolates typically still showed large amounts of conidiospores, whereas none of the spores that had been produced were able to germinate. Both in *N. intermedia* and in *N. tetrasperma*, the senescence trait was contagious: Senescent subcultures were mixed in ratios of approximately 1:1 with young subcultures of the same isolate. These mixtures typically adopted the life expectancy of the senescent subcultures (This was tested for eight isolates from both species; data not shown). This was not the case when they were mixed with young subcultures from incompatible wild-type isolates (Ten combinations were tested for each of the same eight isolates; data not shown).

Senescence is associated with the presence of pKALILO in both species

All isolates were tested for the presence of pKALILO homologues. This was done using PCR experiments and Southern analysis. Results obtained with these two methods were in agreement. All isolates were additionally tested for the presence of pHANALEI-2 homologues. The latter is a circular mitochondrial plasmid that is allegedly neutral. This was done using Southern analysis only. Detailed results can be found in the appendix and these have been summarized in Table 2-1. Senescence was correlated with the presence of pKALILO homologous plasmids (Likelihood ratio test, G=115.4, df=1, P<0.0001; Table 2-2), but not with that of pHANALEI-2 homologues. This was true for both species.

Five out of 30 *N. intermedia* isolates (*NI05.01*, *NI15.02*, *NI28.01*, *NI30.4* and *NI31.02*) and two out of 14 *N. tetrasperma* isolates (*NT23.06* and *NT35.02*) containing a pKALILO homologue did not show any signs of senescence within the time course of the experiment.

Table 2-1. Frequencies of senescence among plasmid free and plasmid containing isolates. See appendix for detailed information per isolate.

Species	pKALILO	pHANELEI-2	Senescent	Non-senescent
N. intermedia	-	-	0	22
	-	+	0	12
	+	-	18	5
	+	+	7	0
N. tetrasperma	-	-	0	49
	-	+	0	1
	+	-	11	2
	+	+	1	0

Table 2-2. Results of a hierarchical log-linear analysis of the frequency data using a backward elimination process. The final, most parsimonious model is given. The likelihood ratio tests show whether removing the variable listed will significantly change the explanatory value of the model. Note that table 2-1 is only marginally populated and the analysis may therefore suffer from low power.

Variable	Likelihood Ratio test		
	G	df	P
Model	5.2	8	0.735
pKALILO * senescence	115.4	1	0.000
pKALILO * species	9.0	1	0.003
pHANALEI-2 * species	18.6	1	0.000

Although the correlation between the presence of pKALILO homologues and senescence was thus not perfect, isolates lacking a pKALILO homologue never showed signs of senescence in either species.

Within the pKALILO family of mitochondrial plasmids, only the prototypic form has been associated with senescence. Therefore, a region within the TIR was analyzed by which the prototypic form can be distinguished from its three avirulent family members (He *et al.,* 2000). We cloned and sequenced a 631 bp DNA fragment located within this region, from a short- and a long-lived pKALILO containing isolate of each species (*N. intermedia* isolates *NI08.04* and *NI05.01,* and *N. tetrasperma* isolates *NT18.01* and *NT23.06,* respectively). All TIR sequences thus obtained were identical to that of the prototypic plasmid (Chan *et al.* 1991; GenBank accession number X52106; sequence data not shown).

PKALILO integrates into the mitochondrial genome

In senescent subcultures of both its non-native host *N. crassa* and its native host *N. intermedia,* pKALILO integrates into the *rnl* gene that encodes the large subunit mitochondrial *rRNA* (Bertrand *et al*., 1985, see also chapter 7). Thus far, integration has only been found at that specific location. This could be due to site- or region-specificity of the integration process. Alternatively, this could be due to the selective accumulation of molecules that contain these integrated copies. In the case of its avirulent homologues, integrated copies of the plasmid were never found. We thus tested whether integration would also occur in *N. tetrasperma* isolates, using restriction fragment length polymorphism (RFLP) analysis and hybridization. Blots were hybridized with a pKALILO specific probe. In addition to the 8.6 Kb autonomously replicating plasmid, senescent isolates frequently showed RFLP patterns that were consistent with rearrangements involving the plasmid. These presumably were copies that had integrated into the mitochondrial genome. These putative inserts were often similar in parallel series of subcultures from the same isolate, but typically varied among isolates (data not shown).

Two isolates, one from each species of Neurospora, were studied in more detail. Using a ‘plasmid-tagging’ approach, we determined the exact sites of integration. DNA fragments were amplified containing plasmid/mtDNA recombination junctions. These fragments were cloned and sequenced. In both cases an integration site was found within *rnl*: In *N. intermedia* isolate *NI21.05* an integrated copy was found in exon 1 of this gene, at position 12.244/45; in *N. tetrasperma* isolate *NT39.03* one was found also in exon 1 of this gene, but somewhat further downstream, at position 12.501/02 (both corresponding to the mitochondrial genome sequence of standard laboratory strain *74-OR23-1A* of the *N.*

intermedia sister species *N. crassa* as determined by the Neurospora sequencing project; Whitehead Institute/MIT Center for Genome Research). The sequences flanking these pKALILO/mtDNA recombination junctions are given in Figure 2-1.

In both cases we were able to find only one side of each of the integrated plasmids. Formally this may have been due to a deletion of the other side, but more likely this is due to the formation of inverted target site duplications as was previously shown to occur (Bertrand *et al.*, 1985). The inserts were not detected in young subcultures, thus confirming that integration occurred *de novo.* Unlike in earlier work, there was no obvious homology between the mitochondrial target sequence and the distal end of the plasmid. When compared to the sequence of the autonomous element (Court and Bertrand, 1991), the termini of the integrated copies were incomplete: In both cases two base pairs were missing. These may formally reflect existing variation or *de novo* deletions.

In addition to the integrated plasmid copies, we observed age-related nucleotide substitutions in the mitochondrial genome. A comparison of the region flanking the integrated plasmid in isolate *NT39.03* with the corresponding wild-type mtDNA region from young subcultures for example showed that two substitutions were present, located 63 and 549 bp downstream of the exact insertion site (Data not shown).

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pKALILO, 3’ terminus of the autonomous element (according to Court and Bertrand, 1991)

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                    5’ AGGAACAAAA GGGGTCAGTG GTGCCCCTTA CAC 3’
```

N. intermedia MtDNA, isolate *NI21.05*

```
Young       5’ AGGTATGAAA TGCATTTTTT TTTGTATACA TCGGGGGATC GTGAAGATTT 3’
                |||  |  |||  |  |  |  |       |  ||||||||| ||||||||||
Senescent   5’ AGGAACAAAA GGGGTCAGTG GTGCCCCTTA CCGGGGGATC GTGAAGATTT 3’
```

N. tetrasperma MtDNA, isolate *NT39.03*

```
Young       5’ GCACTTGTTA AATCTTAAAA GCATCGAAAA TTTAACGGAT TAAATAATAT 3’
                          |     | |    |   |    |  ||||||||| ||||||||||
Senescent   5’ AGGAACAAAA GGGGTCAGTG GTGCCCCTTA CTTAACGGAT TAAATAATAT 3’
```

Figure 2-1. PKALILO/mtDNA recombination junctions as found in senescent subcultures of *N. intermedia* isolate *NI21.05* and *N. tetrasperma* isolate *NT39.03*, plus a sequence alignment with the mtDNA sequence as found in young subcultures of these same two isolates. The pKALILO sequence is indicated in grey. Note that in both cases the two distal base pairs appear to be missing. There is no apparent homology between the plasmid and the mtDNA target site.

pKALILO is more frequent and more virulent in N. intermedia than in N. tetrasperma

The frequencies of both pKALILO and pHANALEI-2 homologues are species dependent. Both plasmid types are more frequent in *N. intermedia* than in *N. tetrasperma.* PKALILO is present in 47% (30/64) of the *N. intermedia* isolates, whereas it is present in only 22% (14/64) of the *N. tetrasperma* isolates (Likelihood Ratio test, G=9.0, df=1, P=0.0027; Tables 2-1 and 2-2). Similarly, pHANALEI-2 homologues are present in 30% (19/64) of the *N. intermedia* isolates, whereas they are present in only 3% (2/64) of the *N. tetrasperma* isolates (Likelihood Ratio test, G=18.6, df=1, P<0.0001; Tables 2-1 and 2-2).

In addition, compared to *N. intermedia* isolates, *N. tetrasperma* isolates were longer-lived (Log-Rank test, Z=7.81, df=1, P=0.005). This difference is largely due to the fact that the frequency of pKALILO is lower in *N. tetrasperma* than in *N. intermedia.* When only the senescing pKALILO isolates were taken into account, the *N. tetrasperma* isolates were still (though only marginally) longer-lived (51 ± 3 days or 22 ± 2 subcultures for *N. tetrasperma* isolates versus 33 ± 8 days or 15 ± 2 subcultures for *N. intermedia* isolates; Log-Rank test, Z=4.4, df=1, P=0.037). The difference is mainly in the early cohorts and it is thus the onset of senescence that is delayed, as can also be deduced from the graph presented in figure 2-2.

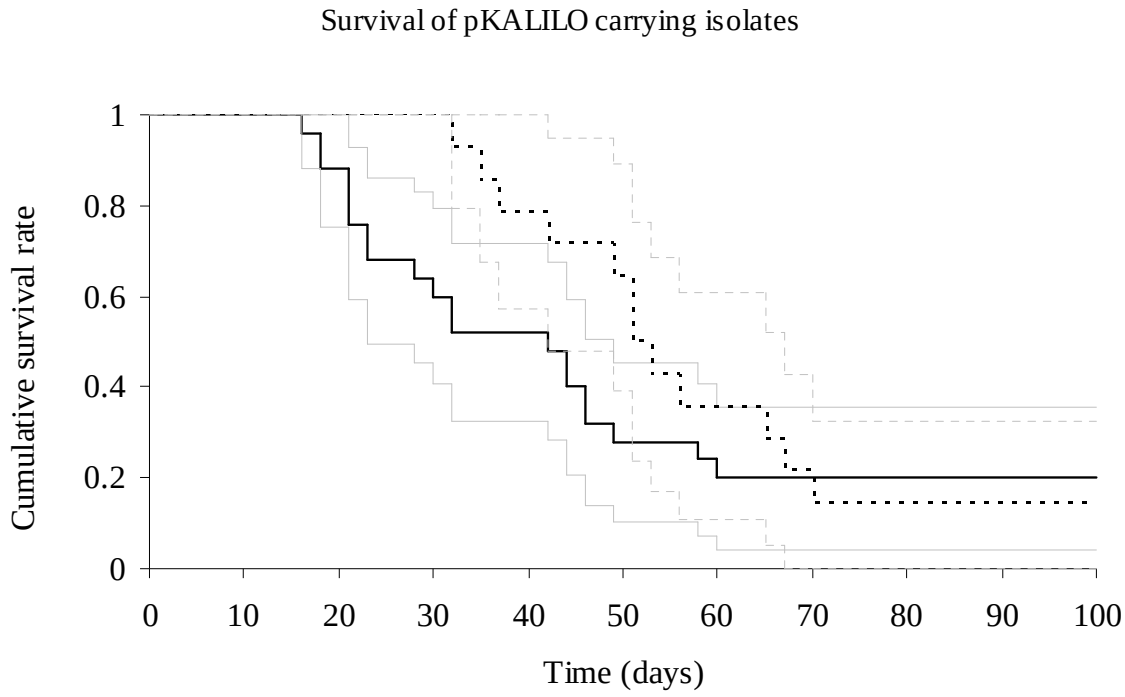

Figure 2-2. Kaplan-Meier survival functions of *N. intermedia* (solid line) and *N. tetrasperma* (dotted line) isolates carrying pKALILO. The 95% confidence intervals are indicated using grey lines.

Given the aforementioned and unexpected bias in the ratio of mating types that was found among *N. intermedia* isolates, we also tested for mating type specific effects. We tested whether or not pKALILO was equally distributed among isolates of the two mating types and whether or not it had a different effect on life span among isolates of the two mating types. Neither its frequency nor the severity of its effect differed.

Discussion

PKALILO based senescence in the pseudo-homothallic species N. tetrasperma

Experimental work showed that pKALILO can be transmitted horizontally among Neurospora isolates of the same species with relative ease (Debets *et al.*, 1994). Also inter-specific transmission proved experimentally possible, at least via introgression and subsequent heterokaryosis (Bok *et al.*, 1999). As might consequently be expected, pKALILO homologous plasmids are distributed across species (Arganoza *et al.*, 1994; He *et al.*, 2000), and can even be found outside the genus of Neurospora (Yuewang *et al*. 1996). Although the phylogeny of the plasmids is by and large congruent with that of the nuclear genome of their hosts, at least two of the pKALILO homologous plasmids are found across species (He *et al*., 2000): pLA-KALILO that had originally been found in *N. tetrasperma* isolates from Louisiana, was also found in an isolate of *N. crassa* from Haiti; pKALILO that had originally been found in *N. intermedia* isolates from Hawaii, was also found in an isolate of *N. tetrasperma* from Moorea-Tahiti. This shows that inter-specific horizontal transmission is probably also a natural phenomenon. The authors of the latter study (He *et al*., 2000) however did not report whether the *N. tetrasperma* isolate from Moorea-Tahiti senesced, and if so, whether it did in the same way as *N. intermedia* isolates do. Thus, despite the fact that pKALILO has been found outside its original host, the senescence syndrome associated with it has formally only been described from the population of *N. intermedia.* Though pKALILO also causes senescence in *N. crassa,* in which it was experimentally introduced via forced heterokaryosis (Griffiths *et al.* 1990), its effect in other species is not at all self-evident. Here we have shown that pKALILO is present at a considerable frequency in the natural population of *N. tetrasperma*, in which it leads to senescence in a similar way as it does in its heterothallic congeners. The plasmids present in *N. tetrasperma* appear to be identical to the prototypic plasmid at the DNA sequence level and the associated senescence phenotype and molecular phenomenology is similar to that in *N. intermedia* and *N. crassa*. As discussed below though, the frequency of the plasmid was substantially lower in *N. tetrasperma* than in *N. intermedia,* and its phenotypic effects less severe.

Plasmid frequencies are lower in N. tetrasperma than in N. intermedia

PKALILO is much more frequent in *N. intermedia* than it is in *N. tetrasperma*: Whereas the senescence plasmid is present in almost half the *N. intermedia* isolates as observed in earlier work (Debets *et al*., 1995), it was present in less than a quarter of all the *N. tetrasperma* isolates collected. The allegedly neutral pHANALEI-2 homologues are also much less frequent in *N. tetrasperma* than they are in *N. intermedia*. This suggests that plasmid frequencies are a species specific property that is largely independent of the plasmids their phenotypic effects. Frequencies of mitochondrial plasmids appear to be even lower in truly homothallic species, suggesting that this has to do with the mating system of the host. In homothallic isolates from the genus Gelasinospora for example, only two out of eighteen isolates collected were found to contain pGEL-KALILO (Yuewang *et al.,* 1996). There thus appears to be a relationship between the frequency of these plasmids and the degree of outcrossing in their hosts, with high frequencies attained in an exclusively outcrossing, heterothallic host, low frequencies in an exclusively self-fertilizing, homothallic host, and intermediate frequencies in a facultative outcrossing, pseudo-homothallic one.

The different plasmid frequencies may reflect natural rates of horizontal transmission, which likely vary between species. Horizontal transmission is one of the most important criteria for a plasmid with deleterious effects on its host to be maintained within a population. Even with perfect vertical transmission, such a plasmid would disappear from the population if it would not have something like horizontal transmission to compensate for its deleterious effects. pKALILO is not expected to be lost from the population due to its high rate of horizontal transmission. Indeed, it is still found in the population 25 years after it was first sampled. Horizontal transmission presumably depends on the degree of within-host replication. It will consequently be linked to virulence. Therefore, pKALILO is expected neither to disappear nor to become fixed in the population and a species specific polymorphism may be expected.

The rate of horizontal transmission depends on the amount of physical contact between individual colonies. This is different between (pseudo-)homothallic and heterothallic hosts, because self-fertilization in (pseudo-)homothallic hosts basically eliminates the need for physical contact. Although outcrossing is experimentally possible in *N. tetrasperma*, there is a large degree of sexual dysfunction associated with it (Jacobson, 1995), so in nature *N. tetrasperma* presumably mostly relies on self-fertilization. In a heterothallic background, although the plasmid is inherited mainly maternally, pKALILO can be transmitted via the paternal route (Yang and Griffiths, 1993a) with frequencies of up to fifteen percent depending on the isolates used. Paternal transmission probably occurs by invasion of the maternal culture

of the paternal cytoplasm prior to ascospore formation (Debets and Griffiths, 1998) and this can be seen as a form of horizontal transmission. If this is indeed a naturally important mode of transmission, the plasmid's spread would surely be affected by the amount of self-fertilization because fertilization by another isolate, as required for outcrossing, heterothallic species, increases the risk of infection. These effects of the mating system have been substantiated by population genetic modeling and appear to be in line with natural frequencies of mitochondrial plasmids found in plants (Bengtsson and Andersson, 1997). If the effect of self-fertilization is indeed the explanation of the observed frequency differences, it would suggest that the rate of paternal transmission is in fact much more important than currently conceived. An important observation in this matter is that dead cultures still contain a lot of conidia whereas these do not germinate at all, or die shortly after germination. Remarkably, in some crosses a considerable fraction of these 'dead' spores still have the capacity to serve as male gametes and transmit the plasmids in the process of fertilization (unpublished data).

In short, mitochondrial plasmids thus appear to be more common in a heterothallic than in a pseudo-homothallic host because the former is more susceptible to infection. This appears to be irrespective of the phenotypic effects they have on their hosts: Both the senescence plasmid pKALILO and the neutral plasmid pHANALEI-2 have a lower frequency in *N. tetrasperma* than in *N. intermedia*. Depending on the mating system, plasmids may be expected to have not only a different frequency, but also a different phenotypic effect.

pKALILO has a less severe effect in N. tetrasperma than in N. intermedia

In general, when a parasite would be transmitted only vertically, it would rapidly coevolve with its host towards a more benign relationship: commensalism or possibly even mutualism. This is because when a parasite is vertically transmitted as opposed to horizontally, its interests will align with the interests of its host. Between a heterothallic and a pseudo-homothallic host, pKALILO is expected to diverge accordingly. The plasmids from *N. intermedia* and *N. tetrasperma* did not appear to differ at the DNA sequence level but did have different phenotypic effects. PKALILO carrying isolates thus seem to show host-specific senescence. This strongly suggests that the differences are an incidental effect of the host and that there may be frequent inter-specific transmission of pKALILO. These results are counterintuitive, as plasmids are expected to evolve more rapidly than their hosts.

In summary, the Hawaiian population of Neurospora was screened for cases of pKALILO based senescence outside of its natural host *N. intermedia*. It was found also in *N. tetrasperma*. The hallmarks of senescence were similar in both species: Senescence was

contagious and associated with the presence of the prototypic senescence plasmid and its integration into the mitochondrial genome. However, pKALILO was less frequent in *N. tetrasperma* than in *N. intermedia* and its phenotypic effects less severe. It is appealing to invoke a role of the mating system to explain the differences observed.

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Chapter 3

Cytoplasmic suppression of pKALILO based senescence in natural isolates of *N. intermedia*

M. F. P. M. Maas and A. J. M. Debets

Abstract

The mitochondrial plasmid pKALILO causes senescence in *N. intermedia.* Though never formally proven, this is allegedly due to the fact that it integrates into the mitochondrial genome of its host. Little is known about suppressors of this type of senescence. Recently we found several natural isolates that carried the plasmid but did not show any signs of senescence. We analyzed these. Their ability to tolerate the plasmid was inherited maternally, indicating a cytoplasmic origin of the trait. The plasmid titer was highly stable within, though variable between the isolates. In two out of five cases, stably integrated or variant copies were found. These were characterized using semi-random two-step PCR (ST-PCR) and sequencing, which led to the discovery of a novel mitochondrial retro-element with which pKALILO had recombined. A possible mechanism by which senescence is suppressed, is discussed.

Introduction

Aging or senescence in fungi has been a long-standing matter of debate because fungi, being typical modular organisms and thus lacking a clear distinction between germ line and soma, are not expected to age and die. However, even though most fungi indeed appear to be extremely long-lived, some exceptions exist. Cases of senescence have for example been reported within the well-studied genera Neurospora and Podospora (Griffiths, 1992).

Senescence in Neurospora can almost always be ascribed to the presence of a single mitochondrial plasmid, like one of the linear mitochondrial invertrons pKALILO (Bertrand *et al.*, 1985; Bertrand *et al.*, 1986; Myers *et al.*, 1989) and pMARANHAR (Court *et al.*, 1991), or one of the circular mitochondrial retroplasmids pVARKUD and pMAURICEVILLE (Akins *et al.*, 1989). These plasmids integrate into the mitochondrial genome and interfere with mitochondrial function, which is allegedly the cause of death of the cultures that carry them. Often they are found close to or within the *rns* or *rnl* genes that encode the mitochondrial rRNAs (Bertrand *et al.*, 1985; Bertrand and Griffiths., 1989; Chiang *et al.*,

1994; Myers *et al.*, 1989; Maas *et al.*, 2005). PKALILO and pMARANHAR possibly integrate via a form of non-homologous end-joining (NHEJ, see chapter 6); PVARKUD and pMAURICEVILLE integrate via a reverse transcription step. The latter two both contain an open reading frame (ORF) encoding a reverse transcriptase (RT) that can generate hybrid mitochondrial cDNA via RNA template switching. Template switching is also known from the RT encoded by intron α of *P. anserina* (Sellem *et al.*, 2000). Following hybrid cDNA formation, the plasmids may integrate via homologous recombination (Chiang *et al.*, 1994).

Little is known about suppressors of plasmid based senescence in Neurospora. Based on the analogy with senescence in *P. anserina*, one might expect that modifications of the respiratory chain could act as suppressors of senescence (Dufour and Larsson, 2005). Thus far however, there is scarcely any evidence to support this. In the case of pKALILO, there are reports of nuclear encoded suppressors. In octads collected from crosses between senescing and non-senescing laboratory strains of Neurospora, several cases were found in which senescence and 'immortality' segregated in a four to four ratio (Griffiths *et al.*, 1992). In one case the titer of pKALILO was reduced to barely detectable levels, whereas in another case the titer of the plasmid was high but stable and associated with copies of the plasmid stably integrated into the mitochondrial chromosome. These nuclear encoded suppressors of pKALILO based senescence have not been characterized any further. A previous search for suppressors among natural *N. intermedia* isolates was purportedly unsuccessful (Debets *et al.*, 1995), though not every pKALILO carrying isolate senesced and the population that was screened thus contained potential suppressors.

Recently, we resampled the Hawaiian population of *N. intermedia* (Maas *et al.*, 2005, see also chapter 2). Several isolates that carried the prototypic senescence plasmid did not senesce. We analyzed these. The ability to tolerate the plasmid was inherited maternally, indicating a cytoplasmic origin of this trait. The plasmid titer was highly stable within, though variable between the isolates. In two out of five isolates, stably integrated or variant copies of the plasmid were found. These were characterized using semi-random two-step PCR (ST-PCR) and sequencing, and this led to the discovery of a novel mitochondrial retro-element that had recombined with pKALILO. A possible mechanism of suppression is discussed.

Materials and Methods

Isolates and culturing methods.

Isolates were from a recent collection of Hawaiian *N. intermedia* wild types (Maas *et al.*, 2005). 30 out of 64 isolates from this collection carry pKALILO. Five of those that carry it

however, did not appear to senesce. We reciprocally crossed these five with short-lived natural isolates from the same collection. Long-lived isolates *NI05.01*, *NI15.02* and *NI28.01*, which are of mating type a, were each crossed with short-lived natural isolate *NI16.07*, which is of mating type A. Long-lived isolates *NI30.04* and *NI31.02*, which are of mating type A, were each crossed with short-lived isolate *NI16.04*, which is of mating type a. Crosses were done at 25 ˚C, on Westergaard's Medium (WM), according to the protocol given by Davis and DeSerres (1970). From each cross, perithecia were dissected in order to collect asci. Of each complete, eight-spored ascus, every individual spore was collected and allowed to ripen for one week on Vogel's Minimal Medium (VMM), after which germination was induced by a 30 minute heat shock at 60 ˚C. Senescence was routinely tested by serially sub-culturing the isolates at 25 ˚C, on VMM, as previously described by Griffiths and Bertrand (1984). Growth rates were tested on VMM using 50 cm long tubes.

DNA analysis.

Cultures for DNA extraction were grown in liquid VMM for approximately 24 hours at 25 ºC. Mycelium was harvested, dried between filter paper and ground using liquid nitrogen. Total DNA was extracted according to standard procedures (Sambrook *et al.*, 1989). Prior to the addition of phenol and chloroform, samples were incubated for 1 hr at 37 ºC with proteinase-K (100 µg/mL final concentration), save those to be used in an semi-random two-step PCR (ST-PCR, described further on). Electrophoresis was done in 40 mM Tris-HCl, 20 mM acetic acid and 1 mM EDTA (TAE), pH 8.0 using 1% (w/v) agarose, and DNA was visualized using ethidium-bromide staining (Sambrook *et al.*, 1989).

Southern analysis was done using standard procedures (Sambrook *et al.*, 1989). Electrophoresis was done in 45 mM Tris-HCl, 45 mM boric acid and 1 mM EDTA (TBE), pH 8.0 using 0.8% (w/v) agarose. Per lane, we used between two and five µg of either digested (using restriction enzymes *PstI*, *MvnI* or both) or undigested total genomic DNA. DNA was transferred onto positively charged nylon membranes (Hybond^{tm} N+, Amersham Biosciences) using capillary blotting, and probed with the large internal 8.2 Kb *KpnI* fragment of pKALILO cloned in a pUC18 vector (Vickery and Griffiths, 1993). Probes were labeled using random primed Digoxigenin-11-dUTP (DIG-dUTP) labeling, and detection was done using the chemi-luminescent substrate CSPD® according to the manufacturer's instructions (Boehringer Mannheim).

To test for the presence of the putatively circular progenitor of the retro-element, PCR was done using primers located within the respective 5' and 3' untranslated regions (UTRs) of the putative reverse transcriptase (RT) gene of the element: 5' TCC CCG TTC GCC ACA

AGT T 3' and 5'CGA CGG GGG AGG AAG GAT TA 3'. Primers were based on the sequence derived from isolate *NI28.01* and yield an expected PCR product size of at least 180 bp. We used an annealing temperature of 60 ˚C and an elongation time of three minutes. Electrophoresis and visualization was done as described above.

To test the rest of the *N. intermedia* wild types for the presence of the novel mitochondrial retro-element, PCR was done using primers located within the putative reverse transcriptase (RT) gene of the element: 5' TAA CTT AAC CGC CAA CGT AT 3' and 5' GAC CCC TTT CTT CCA GTT TAT 3'. These were based on the sequence derived from isolate *NI28.01* and yield an expected PCR product size of 341 bp. We used an annealing temperature of 54 ˚C and an elongation time of one minute. Electrophoresis and visualization was done as described above.

ST-PCR based analysis of pKALILO/mtDNA recombination junctions.

To amplify pKALILO/mtDNA recombination junctions, a 'plasmid-tagging' approach was used, based on the semi-random two-step PCR (ST-PCR) procedure given by Chun *et al.* (1997). The modified procedure was done as previously described (Maas *et al*., 2005). Electrophoresis and visualization was done as described above. ST-PCR products were (gel-) purified using a High Pure PCR Product Purification Kit (Roche) and cloned in *E. coli* DH5-α using the pGEM®-T Easy Vector system (Promega). Plasmid DNA was recovered using a High Pure Plasmid Isolation Kit (Roche) and sequencing was done according to the methods described by Sanger (1977), using Big Dye™ terminators version 3 (Applied Biosystems). We compared the sequences of the ST-PCR products with sequences from the GenBank database using BLAST version 2.2.9 (Altschul *et al.*, 1997).

Results

Maternally inherited suppression of pKALILO based senescence

Whereas vegetative cultures of pKALILO carrying isolates normally senesce within a time frame of approximately one month, five out of 30 pKALILO carrying isolates from our previous collection of *N. intermedia* wild types did not appear to senesce: We were able to maintain these five for at least four or five months (which is approximately equivalent to 50 subcultures) without any clear signs of senescence. To determine whether this trait would be stably inherited and whether it would be of nuclear or cytoplasmic origin, we analyzed several linearly ordered octads obtained from crosses with the long-lived isolates.

The long-lived isolates were reciprocally crossed with short-lived ones from the same collection. Three out of five long-lived natural isolates (*NI15.02*, *NI28.01* and *NI30.04*) produced viable progeny when crossed with a wild-type isolate of the opposite mating type. The other two long-lived isolates (*NI05.01* and *NI31.02*) did produce viable ascospores when used as a male, but only had barren perithecia when used as a female partner in a cross. Note that infertility also commonly occurred in crosses using random combinations of short-lived natural isolates. It is therefore not necessarily associated with the longevity trait: Formally it may also have been due to general sexual incompatibility. As an additional measure of possible pleiotropy, we tested the growth rates of short- and long-lived isolates. These did not significantly differ (ANOVA; F=1.83, df=1, P=0.190).

From each fertile cross two complete, eight-spored asci were collected. Cultures derived from the eight spores of each ascus were individually tested for senescence by means of serial sub-culturing. For practical reasons the experiment was terminated after 30 vegetative subcultures (equivalent to approximately 10 weeks) and longevity was therefore defined as having a 'life span' of more than 30 subcultures. Results are given in Table 3-1.

Table 3-1. Octad analysis of the longevity trait found in several natural *N. intermedia* isolates. Reciprocal crosses were made between long- (LL) and short-lived (SL) isolates and perithecia were dissected to obtain linearly ordered octads. Per cross, two of these octads were analyzed. 'Life span' is indicated in number of subcultures until death. One subculture is equivalent to two or three days.

Cross	'Life span' in number of subcultures of progeny from linearly ordered octads							
NI05.01 x *NI16.07* (LL♀ x SL♂)	(sterile)							
NI16.07 x *NI05.01* (SL♀ x LL♂)	19	10	12	8	12	16	6	9
	13	19	15	14	18	16	16	16
NI28.01 x NI16.07 (LL♀ x SL♂)	>30	>30	>30	>30	>30	>30	>30	>30
	>30	>30	>30	>30	>30	>30	>30	>30
NI16.07 x *NI28.01* (SL♀ x LL♂)	14	14	9	11	11	11	8	6
	11	9	14	16	16	14	7	17
NI15.02 x *NI16.07* (LL♀ x SL♂)	>30	>30	>30	>30	>30	>30	>30	>30
	>30	>30	>30	>30	>30	>30	>30	>30
NI16.07 x *NI15.02* (SL♀ x LL♂)	6	6	8	8	13	13	13	8
	20	18	10	13	9	9	14	14
NI30.04 x *NI16.04* (LL♀ x SL♂)	>30	>30	>30	>30	>30	>30	>30	>30
	>30	>30	>30	>30	>30	>30	>30	>30
NI16.04 x *NI30.04* (SL♀ x LL♂)	9	17	13	13	9	8	10	9
	14	12	12	12	9	8	8	8
NI31.02 x *NI16.04* (LL♀ x SL♂)	(sterile)							
NI16.04 x *NI31.02* (SL♀ x LL♂)	13	10	14	20	13	10	11	12
	14	16	17	9	17	19	12	16

Although life span was inherited at least partly in a Mendelian fashion (as for example clearly observed in the second ascus from the cross using short-lived isolate *NI16.04* as a female and long-lived isolate *NI30.04* as a male; Table 3-1), the longevity trait itself showed a clear pattern of maternal inheritance: When a long-lived isolate was used as a female, all eight cultures derived from an ascus were long-lived; when the same isolate was used as a male, all eight were typically short-lived. Although isolates *NI05.01* and *NI31.02* appeared to be female sterile, the longevity trait of these two isolates was not inherited paternally. We concluded that in all cases examined the longevity trait was cytoplasmic.

Although the longevity trait could correspond to differences within the plasmid sequence itself, this is unlikely: Plasmids from short- and long-lived isolates did not show any sequence variation within the virulence associated terminal inverted repeat (TIR) region (Maas *et al.*, 2005). Thus, although this formally remains a possibility, we assumed that suppression of pKALILO based senescence is caused not by differences within the plasmid, but by some other cytoplasmic factor.

Longevity is associated with a stable titer of the senescence plasmid

In senescing isolates, pKALILO typically increases in titer and integrates into the mitochondrial genome. MtDNA molecules that carry an integrated copy of pKALILO are suppressive: They gradually replace the wild-type molecules, perhaps by competitive exclusion (Myers *et al.*, 1989). To see whether this process would be different in the five long-lived natural isolates, we compared these to several of our short-lived ones using restriction fragment length polymorphism (RFLP) and hybridization. In the case of short-lived isolates, we compared DNA from the first with that from the last viable subculture of each isolate. In the case of long-lived isolates, we compared DNA from the first with that from approximately the 30^{th} subculture of each isolate. Short-lived isolates invariably showed a progressively increasing titer of the autonomously replicating, 8.6 Kb form of the senescence plasmid. This was associated with the appearance of putative plasmid insertions as observed also previously. A typical example is shown in Figure 1. In some of these isolates, the putative insertions appeared to be present already in the first subcultures (For example in isolates *NI08.04* and *NI21.05*). In contrast to the short-lived isolates, long-lived isolates did not show an age-related increase in plasmid titer: All five maintained a stable titer of the autonomously replicating form. The titer was nonetheless variable between isolates: It was either suppressed to a barely detectable level (in isolates *NI15.2* and *NI31.02*), or tolerated at a considerable level (in isolates *NI05.01, NI28.01* and *NI30.04*). From the long-lived isolates, only in *NI05.01* a putative integration was visible by Southern analysis (Figure 3-1).

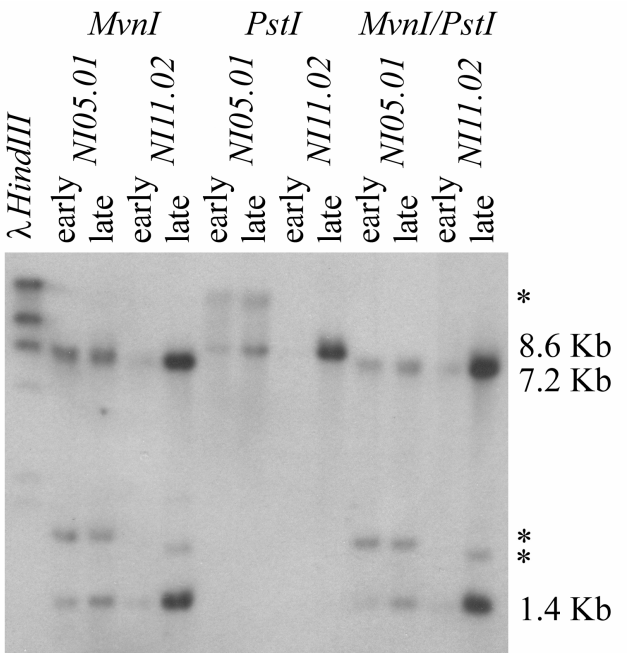

Figure 3-1. Example of the Southern analysis. In long-lived isolate *NI05.01* putative integrated and/or variant copies (marked with an asterisk) are observed both in early and in late subcultures. In short-lived isolate *NI11.02* they are observed only in late, senescent subcultures. Total genomic DNA was digested with *MvnI*, *PstI* or both, and probed with the large internal 8.2 Kb *KpnI* fragment of pKALILO. The 8.6 Kb fragment corresponds to the complete, autonomously replicating plasmid; the 7.2 and 1.4 Kb fragments correspond to the *MvnI* digested autonomously replicating plasmid; *PstI* does not cut inside the plasmid.

KALILO/mtDNA recombination junctions from long-lived isolates

We analyzed pKALILO/mtDNA recombination junctions from short- and long-lived isolates. A modified, semi-random, two-step PCR (ST-PCR) procedure (Chun *et al*., 1997) was used to amplify the sequences flanking the putatively integrated plasmid copies. Recombination junctions were found in subcultures of both short- and long-lived isolates (see also chapter 6). ST-PCRs typically gave either one or two distinct products with template DNA from senescent cultures, but often none with that from young cultures. ST-PCRs done with template DNA from long-lived isolates only gave products in the case of isolates *NI05.01* and *NI28.01*. Products were found both in early and in late subcultures of the latter two isolates. These were purified, cloned and sequenced for comparison. Integrated copies of pKALILO found in senescent subcultures of short-lived isolates were often located within or near to the *rnl* gene that encodes the large subunit mitochondrial rRNA (see chapter 6). Often, deletions were present at the plasmid/mtDNA recombination junctions. The recombination junctions found in the two long-lived isolates *NI05.01* and *NI28.01* appeared to be different though. They were located in a different region of the mitochondrial chromosome. Also, they did not all show clear deletions. Moreover, as described below, the recombination junctions that were found in the two long-lived isolates precisely marked the termini of known mitochondrial RNA species, suggesting that they came about through a reverse transcription step.

In long-lived isolate *NI05.01*, pKALILO was flanked by a short (65 bp) region of

mtDNA corresponding to the 3' untranslated region (UTR) of the *nd5* gene that encodes a subunit of respiratory complex I (The pKALILO/mtDNA recombination junction corresponded to position 32,735/36 of the mitochondrial genome sequence of *N. crassa* strain 74-OR23-1A as determined by the Neurospora Sequencing Project; Whitehead Institute/MIT Center for Genome Research). This region was followed by an element of at least 1 Kb in size, corresponding to a cryptic retro-element as discussed further on. In line with the RFLP analysis in which only one additional fragment was observed whereas more might have been expected (see figure 3-1), we were able to find only one ST-PCR product in isolate *NI05.01*, which probably implies that the sequence flanking pKALILO consisted of a large (> 1 Kb) inverted repeat as also described previously (Bertrand and Griffiths, 1989). The sequence of the flanking sequence is given in Figure 3-2a.

In long-lived isolate *NI28.01*, two recombination junctions were found involving pKALILO. In one case, the region flanking pKALILO corresponded to the 5' UTR of a putative reverse transcriptase (RT) gene; the terminus of the integrated plasmid copy was incomplete. In the other case, the region flanking pKALILO corresponded exactly to the 5' end of the mitochondrial *trna*^{Arg} gene; the terminus of the integrated plasmid copy was complete. The entire, 74 bp long, mature tRNA sequence was present including the CCA triplet that is added post-transcriptionally by the mitochondrial tRNA nucleotidyl transferase. The formation of this recombination junction must therefore have involved a RNA intermediate. The tRNA sequence was followed by a short (51-56 bp) fragment corresponding exactly to the 5' end of *rns*, the small subunit mitochondrial rRNA gene. These sequences were joined head-to-tail and followed by a sequence with high similarity to the cryptic retro-element that was found also in isolate *NI05.01*. Additional PCRs and sequencing showed that the recombination junctions were present on one molecule. The hybrid molecule mapped as a circle composed of pKALILO, mtDNA and the novel retroelement. The sequence is given in Figure 3-2b.

A novel mitochondrial retroelement from long-lived isolates of N. intermedia

As noted above, *NI05.01* and *NI28.01* both contain a novel retro-element that might have been involved in the recombination events that gave rise to the sequences given in figure 3-2. Over a length of 1,020 bp, the novel element is 87% similar between the two isolates. It has an AT-biased nucleotide content (approximately 60% AT), consistent with its presumed mitochondrial location. Though it does not share significant DNA sequence homology with any element from the GenBank database, at the protein level it does. It contains a 1,989 bp long putative open reading frame (ORF) that has similarity to the reverse transcriptases (RTs)

3-2a. Plasmid/mtDNA derived hybrid sequence from long-lived *N. intermedia* isolate *NI05.01*

```
GGCCACGCGT CGACTCGTAC GGACTGCTCG GATATGCAAG TTCCGCTAGA ATTACAGGAG   0,060
AGACAAGACT ACGAGCTAGA ATATCCCTGA AAGAGGGTAG AAAAGCCCTG CGTCTGAAAT   0,120
ATTCTGTAAT CAAAGCGGAA AATCACTACG CAGTCCCAGC AAGTAACTGG CGTGACTGAC   0,180
TTGAAGCTAC AATTAAATAC TTTGGGGTAA AAAGAAACGT ACGGAAGTGC GTTAGACCTA   0,240
CACCACAAGA CATAGCGGAA AGCGTACCAT TAAACATGGA GTCAGCTTCC GGACCCACTA   0,300
TTACAAAAAA TACCTCCAAT GGCAGTCGAC CCAGCACTCG CCAGGCCGGA CCAACATCCG   0,360
GTTTCCCTGT TATTAGCATA TCACAGCAAA GCGCGCGGCG GAGGCGCGTG CTTCCGGAAT   0,420
CGAAAGCACT TAAACAAGCA ATTAGAGAAA GAAACACAGT CTTGACAGAA AGCAAAGATC   0,480
TGAAAAGGCG TATAAAGGCG CGTAGAGCAA TAAAACTGAG CTGTAAATAG CAATTGGAAA   0,540
AGGGAATCTA ATCTGCAAAT TAAACTAACA TCTAACGTTG TCAGTTCAAT TAGCCTTAAT   0,600
ACGTACGGTA GCGAGCAACA CCTATACGGA ATGACTGAAC ACGTGATGCG TAACCAGTCA   0,660
TTCTTAATCT GTATAAAGCT GCAATAATCT ACCTGTACAG GCAAATTAAA CCAGCAACCT   0,720
CAGTTGCCAG TTCAATACGC CCAAAGGGGG ATATCCAGCG GGCGAGCAAC TATCTGTTAG   0,780
CAGTCAACTA CCGTTGCTGC TCCGCTTTGC GGACACAGAT GGAGCTATCA GCACTGACAA   0,840
TTAGACCTTT GGACAATTGT TACAGGCCTC AAGCTCTCGT GTTTCCAGTC GCATTCGTAA   0,900
GGCCAATTCC TTGCGACGGG GGAGAAAGGA TTAAAATGGT TTCAGCTTTG AAACCATTTT   0,960
TTATTCTTCC TCCTGGAAAC CCGGATGGTG ATTCCGAGCA AGTCACATTT ACTTAAAATA   1,020
TAATTAGAGC AAATGTGCCT TTGCGGTCAT CATCATCCTA TAGGCCCCAT TTAGTAGGAA   1,080
ATTAACAGCT GGAGGTAAAA AAGGTCTGAG AATCGGTAAG tGTGTAAGGG GCACCACTGA   1,140
CCCCTTTTGT TCCTATTTTA ACC                                           1,163
```

3-2b. Plasmid/mtDNA derived hybrid sequence from long-lived *N. intermedia* isolate *NI28.01*

```
 GGTTAAAAT AGGAACAAAA GGGGTCAGTG GTGCCCCTTA CATAAATATT TACAGAAACT   0,060
TGTGGCGAAC GGGGAGTTAA ACAAATTCTG GAAAGTAAGC TGAAGATTAA TGGAAAGATC   0,120
CATTGCCTTC AGAACTGCTG CCTTCAACTA CTGCTTCTAT GGTTGGTATA AATACCGTAC   0,180
TGTAAATGAA GTGGTGAGAA TTAACTTAAC CGCCAATCGT ATAATCAAAG AGCGCTGAGA   0,240
TAAAATTGAC TTCATGAGAG TGTTCATCCC AAAAGCCAAC GGTAAACTGC GTCCATTAGG   0,300
AGTACCAACT TGCGCTTGAC GTCTTGTACT TCATATGTAC CCAAATTTTC TCGGGATTTT   0,360
ATTAAGAGGG GAACTTTCCG CATTTAATCA CGCTTACCTC CCCAAAAAAG GTACTCTAAC   0,420
CTGTATAACG GAATGAGTGA CGAAAGTCCT CAAAACGAAG TATGTGTATG AGTTCGATAT   0,480
 TAAAGGATTC TTTGATAACG TGAGCGTTGA AGATACTCTT AATAAACTGG AAGAAAGGGG   0,540
TCTTCCAGTT AATATGACAT CACACTGGAT CTCCTTATTG GAATCTACAC CATGAAATAT   0,600
TGACTGAAAA AAAGGAGAAT ACAAAGGAAT GAAAGATGAT CAAGGAAAAC CGGTACCATT   0,660
AATACCTATG GATGAATGCT TAGGTGAAAG ACAGATGTGA AAAGACGAAT TAAACAAGAC   0,720
CGCGCTAGAA ATCGTCGCAT CATGGGAAGA AAACGAAGAA GACTATAACG ATGGTAACTA   0,780
CGTCGAACAC CCATATTATA CTCAAATTGC GGATGATCCA AACTATGTCG AGAAAATCCA   0,840
GGAGTTAGAA TCAATTCTTA TCAAATACAG AAACCCGCAT GACTGAATGG CTATGCCAGG   0,900
AACTCAAGAG TTAATTAAAG GGCTCCCACA AGGGGCCGCA ATTTCACCAG TCTTAAGTCT   0,960
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ACTAGCTTTA GTAGACTGAA GGCGCAAATT ACTGGACCAG GGAATCGGCC TGCTTATGTA 1,020
CGCAGATGAC GGGATACTAT ATAGTAATGA GCCATTTACC GAGTTCCCCC CGGATGGATT 1,080
TGAAATAGCA CAAGAAAAAT CTGGGTGGGT GAAAAGAAAT GGGAGTTGAG AAAAAGACTC 1,140
TCAAAAATTC TTAGGAGTTC GTTATCACCA TAAAACTGAT TTAATCTCAG GTGAAACGCG 1,200
TAGTGGAAAA ACACTGGAAA TTCGGAAGAA CCCAGCTAAA TGTATGGAGT TGATTAGATC 1,260
AATTCTTCCC ACTTCGTGAA GCTCTCCACC ACCGACTAGA CTAGATGCTT TAATGCGTAG 1,320
TTCACTGAAA AGGTCAGGCA CTCAAGATTG TATAACGGTA TTTGAGATGC AATTGAATGA 1,380
CCTGAAAGTG GCATATCAAA ATGAAAGACG ATCCTGATGA TGTATCTTCA GAGGACCCAA 1,440
AGAATCGTTG ATCGCTCGTA AGAAGCTGCA GACAACGGCT TCATCTTACG CTTGCCATTG 1,500
ATTAGGAGGT TTACTAACCT CCATAATAAA TCCCAAGCTG CTAAAGCACG GAAAACAATG 1,560
GGAGAACGCG GGAAATTACG GCAGCCCATC AGATATGCAG GTACCAATTA AATTGCAAGA 1,620
GAAACAGGAT TATGAGCTGG AATATCCCTT GATTGAGGGA AGAAAGGCAC TACGTCTGAG 1,680
ATACTCGGTA ATCAAAGCGG AAAATCACTA CGCAGTCCCA GGAAGTAATT GGCGTGAATG 1,740
ACTCGAAGCA ACAATCAAAT ACTTCGGAGT AAAAAGAAAT ATGCGTAAGC GTACTAAACC 1,800
TACTCCTCAG GACATTGCAG AGAGCATACC ACTAAACATG GAATCAGCTT CTGATCCCAC 1,860
TATTACAAAT GATACCTCCA ATGGCAGTCA ACCTAGCACT CGCCAGGCCG GACCAACATC 1,920
CGGTTTCCCA GTAATTAGCA AAGTACACAG CAGCGCACGG CGAAGGCGCG TGCTTCCGGA 1,980
ATCGAAAGCA CTGAAAAGGG CAATTAGAGA AAGAAACACA GTCTTAACAG AAAGTAAAGA 2,040
TCTGAAAAGG CGTATAAAGG CGCGAAGGGC AAGAAACCGA GCTGCAAAGA ACAATTAGAA 2,100
GAAGTTATCT AAGCTGCAAA TTAAACTATC AACTCACGTT GTTAGTTCAA TTAGCCTTAA 2,160
TCTTTGTACA ACAGCGAGCA AACACCTATA CGGAATGACT GAACGCGTGA GCGCAACCAG 2,220
TCATTCTTAA TTTCGTATAA AGCTACAAGC GTCTACCTGT ACCGGCTAAT TAAGCCAGCA 2,280
ACCTACGTTG CAAGTTCAAT TCGCCAAAAG GTGAATACCT TGCAGGTGAG CAACTATCTG 2,340
TTAGCAGTCA ACTACCGTTG CTGCTCCGCT TTGCGGACAC AGATGGAGCT TTCAGCATTG 2,400
ACAATTAGAC CTTCGGACAA TTGTTACGGG CCTCAAGCTC TCGTGTTTCC AGTCGCATTC 2,460
GTAAGGCCAA TTCCTTGCGA CGGGGGAGGA AGGATTAAAA TGGTTTCAGC TTTGAAACCA 2,540
TTTTTTATTC TTTCTCCTGG AAACCCGGAT GATGATTCCG AGCAAGTCAC ATTTACTTAA 2,600
AATATAATTA CAGCAAATGT GCCTTTGCGG TCATCATCAT CATCTATTTT TATTGATGTA 2,660
ATATAAAATT AAAAAAAATT TTTTTTTATT ACATC*TGGAT TCTCATTTTA GATTCGAACT* 2,720
*AAAATCGGAA TATTAGAAGT ATCCTGCTCT TCCTTTGAGC TAATGAGAA*G TGTAAGGGGC 2,780
ACCACTGACC CCTTTTGTTC CTATTTTAAC C 2,811

Figure 3-2. Mitochondrial sequences that were found in long-lived *N. intermedia* isolates *NI05.01* and *NI28.01*, consisting partly of pKALILO (indicated in black), sequences derived from the mitochondrial genome (indicated in grey with possible similarity to the terminal 3' trinucleotide repeat sequence of the novel element in dark grey), and a novel, cryptic retro-element. The initiation- and termination-codons of the putative RT gene are boxed. The $tRNA^{Arg}$ sequence is indicated in italics (with the cca triplet that is post-transcriptionally added by the mitochondrial tRNA nucleotidyl transferase underlined). Bases 1120 and 1121 in the sequence from isolate *NI05.01* may represent an addition to the terminal dinucleotide repeat sequence of pKALILO.

that are encoded by several mitochondrial retro-plasmids. The predicted 663 amino acids long protein is most similar to the RT from the retro-plasmid pTHR1 from *Trichoderma harzianum* (GenBank accession nr. AAF89327; Antal *et al.*, 2002), with 48% similarity and 35% identity over a length of 395 amino acids (E value = $4 \cdot 10^{-44}$). Similarity to pTHR1 was followed by that to the RTs encoded by pVARKUD from *N. intermedia* and pMAURICEVILLE from *N. crassa* (GenBank accession nrs. AAA70286 and AAA70287 respectively; Nargang, 1986), with 46% similarity and 29% identity over a length of 354 amino acids for both RTs (E value = $1 \cdot 10^{-26}$ for both RTs). The novel element thus presumably corresponds to a novel retro-plasmid. PVARKUD and pMAURICEVILLE from Neurospora as well as intron α are able to incorporate mitochondrial sequences via the formation of hybrid cDNAs. As known also from certain retroviruses, this probably occurs by erroneous template switching of the RT they encode (Akins *et al.*, 1989; Chiang et al., 1994; Chiang and Lambowitz, 1997, Sellem *et al.*, 2000). In the process often mitochondrial tRNAs are incorporated, as was found in isolate *NI28.01* (see figure 3-2b). We used PCR to see if we could find the putatively circular progenitor element (lacking any additional mtDNA or pKALILO sequences) in *NI28.01* and *NI05.01*. Primers were based on the sequence found in isolate *NI28.01*, extending outward from within the 5' and 3' UTR of the predicted RT gene. We were unable to find the exact sequence corresponding to the progenitor (lacking any additional mtDNA or pKALILO sequences), though a form of the plasmid was found with the sequence of pKALILO nearly entirely deleted. The molecule consisted almost entirely of the novel retro-element, a 125 bp region of mtDNA including the tRNA sequence, and a 29 bp region derived from the distal end of pKALILO. The latter sequence was found in both orientations.

Using PCR, with primers located within the RT gene, we tested for the presence of the novel retro-element in the rest of the Hawaiian *N. intermedia* wild types. From the pKALILO free isolates, none contained the element. From the pKALILO containing isolates, all five that were long-lived contained it, suggesting that the presence of this element was involved in the suppression of the senescence trait. Additionally, we tested for its presence among the cultures from the octad analysis, which showed that it was maternally inherited, confirming its cytoplasmic location.

Discussion

The fungal senescence phenomenon of *P. anserina* appears to be causally linked to the activity of the respiratory chain: Disruption of the normal respiratory pathway results in a switch to alternative modes of respiration, with a concomitant reduction in the levels of reactive oxygen species (ROS) produced and a strikingly longer life span (Dufour *et al.*, 2000;

Dufour and Larsson, 2004). The spontaneous longevity mutants that have been found in *P. anserina* typically have major deletions in the mitochondrial genome and these all suffer from a loss of female fertility, which is presumably due to a severe cellular energy deficit. Unlike the senescence process in Podospora however, the fungal senescence trait caused by pKALILO appears to be suppressed in our isolates neither at the cost of fertility nor that of the mycelial growth rate. This should not be surprising, because these were not laboratory isolates, but isolates derived from ascospores collected directly from the field (Maas *et al.*, 2005, see also chapter 2) that have thus been subject to natural selection.

Although previous work did suggest pleiotropic effects of pKALILO on the reproductive fitness of its host (Bok and Griffiths, 2000), we have not been able to reproduce these results using isogenic combinations of isolates with and without pKALILO (unpublished results). Moreover, also the invertron pAL2-1 from *P. anserina* shortens life span without any noticeable beneficial effects on reproduction (Maas *et al.*, 2004). There thus does not appear to be a trade-off between life span and reproduction with regard to invertron based senescence, contrary to expectation from ultimate, evolutionary explanations of aging. Furthermore, invertron based senescence does not appear to respond to calorie restriction in a life span extending manner as true aging does (Maas *et al*., 2004) and is thus fundamentally different from the fungal senescence phenomenon as originally described by Rizet (1953). Also a different mode of suppression may be expected.

Cytoplasmic suppression of pKALILO based senescence

Here we have shown that suppression of pKALILO based senescence, as found in the natural population of *N. intermedia* from Hawaii, is maternally inherited and associated with a stable titer of the autonomously replicating senescence plasmid. Short-lived isolates typically accumulated variant mtDNA molecules with pKALILO integrated in or close to one of the mitochondrial rRNA genes (see also chapter 6), whereas this was not the case in long-lived isolates. The isolates that were able to tolerate pKALILO were co-infected with a novel mitochondrial retro-element. In at least two of these isolates, stable recombinants of the two were found, including short intervening stretches of sequence derived from the mitochondrial genome. The latter molecules were possibly generated via hybrid cDNA formation by erroneous template switching of the RT encoded by the novel element, as also known for example from the RTs encoded by retro-plasmids of the *Mauriceville* group (Akins *et al*., 1989, Chiang *et al*., 1994, Chiang and Lambowitz, 1997).

Whereas integrated copies found in senescing isolates are usually located within the mtDNA region that contains the mitochondrial rRNA genes (see chapter 6), the copies found

in isolates *NI05.01* and *NI28.01* were located in a different region. Unlike most integrated copies that are found in senescing isolates, they had complete termini. Furthermore, as mentioned above, they had recombined with the novel retroelement. Either one or a combination of these three may account for the suppression of pKALILO based senescence in natural isolates. The intact termini are most likely a side-effect of the reverse transcription mediated integration process, as is the site of integration, and the novel retro-element itself is thus probably in some way responsible for the suppression of senescence.

Although similar effects of pTHR1 from Trichoderma have not been reported (Antal *et al.,* 2002), both pVARKUD and pMAURICEVILLE have been associated with a senescence phenotype in Neurospora (Akins *et al.,* 1989): They integrate into the mitochondrial genome, generating suppressive mtDNA variants that gradually replace the wild-type mitochondrial genome. This presumably causes senescence by interfering with mitochondrial function. The newly discovered retro-element may similarly be a cryptic senescence plasmid. Testing this would require an isolate containing the retro-element but devoid of pKALILO. Unfortunately we have not yet obtained such an isolate; of the 64 natural isolates tested only the long-lived pKALILO carrying isolates contained the element. Since the novel retro-element may thus be a senescence plasmid itself, our observation is puzzling. The simplest explanation would be that the element is not associated with a virulent phenotype and that this is dominant over the effect of pKALILO. Alternatively, pKALILO may be suppressed by means of interference competition with the novel element. Although parasitic virulence is generally driven by resource competition within hosts (Ebert, 1998), interference competition is believed to attenuate virulence rather than enforce it (Massey *et al.,* 2004). This is in fact a well known phenomenon in viruses. It would thus be relevant to experimentally test the latter hypothesis, by combining cytoplasms from different isolates carrying different types of senescence plasmids. As far as we know, despite their relatively high frequencies, none the senescence plasmids have ever been found in one common cytoplasm in nature.

Acknowledgements

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Chapter 4

The linear mitochondrial plasmid pAL2-1 reduces calorie restriction mediated life span extension in *Podospora anserina*

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Abstract

Calorie restriction is the only life span extending regimen known that applies to all aging organisms. Although most fungi do not appear to senesce, all natural isolates of the modular filamentous fungus *Podospora anserina* have a limited life span. In this paper, we show that calorie restriction extends life span also in *Podospora anserina*. The response to glucose limitation varies significantly among 23 natural isolates from a local population in The Netherlands, ranging from no effect up to a five-fold life span extension. The isolate dependent effect is largely due to the presence or absence of pAL2-1 homologous plasmids. These mitochondrial plasmids are associated with reduced life span under calorie restricted conditions, suggesting a causal link. This was substantiated using three isogenic combinations of isolates with and without plasmids. A model is proposed to explain how pAL2-1 homologues influence the response to calorie restriction.

Introduction

For over half a century calorie restriction (CR) has been known to extend life span, although the molecular basis for this has remained largely unsolved (Koubova and Guarente, 2003). CR refers to a dietary regimen low in calories, but without starvation. Since the life span extending effect of CR was first noted in the 1930s in rodents (McCay *et al*., 1935), it has been studied in many other organisms. It extends life span for example in yeast, rotifers, spiders, worms, fish, mice, and rats (Weindruch and Walford, 1988) and probably also in nonhuman primates (Lane *et al*., 2001). The life span extending response to CR has clear selective value because it allows organisms to postpone reproduction until food is available. When food intake is restored, the calorie restricted individuals are still capable of producing progeny, even when well-fed controls are post-reproductive or no longer alive (Guarente and Kenyon, 2000). Other external conditions under which life span extension has been observed include heat stress (Shama *et al*., 1998), superoxide dismutase and catalase mimetics (Melov

et al., 2000), hypergravity (Le Bourg and Minois, 1997) and high external osmolarity (Kaeberlein *et al*., 2002). Different hypotheses have been put forward to explain the mechanism by which CR works. One model proposes that it slows down metabolism, thereby slowing the production of potentially cytotoxic reactive oxygen species (ROS) consequently slowing the aging process (Harman, 1981). An alternative model proposes that it induces a wider range of responses that ultimately lead to life span extension (Koubova and Guarente, 2003).

A broadly accepted theory on the evolution of ageing is the disposable soma theory (Kirkwood, 1977). This theory considers the 'Weismannian' distinction between germ line and soma. The germ line is the cell line that is set apart from the soma for sexual reproduction. Whereas the germ line must be immortal, the soma is not required after successful reproduction and is therefore allowed to senesce. As opposed to unitary organisms, modular organisms like hydroids, corals and bryozoans lack a clear distinction between germ line and soma and are therefore expected to be long-lived. In addition, modular organisms have a reproductive value increasing with time and senescence is consequently often delayed and directed at the module (Andrews, 1998).

Filamentous fungi as typical modular organisms are therefore generally expected to be extremely long-lived. Some remarkable exceptions however exist, like for example the coprophilic ascomycete *Podospora anserina*. *P. anserina* has a strain-specific life span and exhibits a distinctive senescence phenotype (Rizet, 1953). This phenotype is characterized by an age-related decline in mycelial growth rate and fertility, a progressive reduction in the amount of aerial hyphae that are formed, and an increased pigmentation of the distal mycelium. Senescence occurs in all natural isolates of this fungus (Rizet, 1953). Other examples of senescence in fungi exist within the genus Neurospora where some populations appear to show polymorphism for senescence (Debets *et al*., 1995, Maas *et al.*, 2005). These fungal senescence syndromes all have in common that they are associated with an accumulation of defective mitochondrial DNA (for reviews see Dujon and Belcour 1989; Griffiths 1992) and this may be causally linked to the activity of the respiratory chain and its concomitant production of ROS (Dufour *et al*., 2000).

Linear mitochondrial plasmids have been shown to affect life span both in Podospora and in Neurospora. These plasmids typically have an invertron structure (with long terminal inverted repeats, TIRs) similar to that found for example in linear dsDNA viruses. In Neurospora the presence of linear mitochondrial plasmids is typically correlated with mitochondrial instability culminating in death. These mitochondrial plasmids, called pKALILO and pMARANHAR, have therefore been termed senescence plasmids (Griffiths, 1992). In *P. anserina* on the other hand, the presence of a linear mitochondrial plasmid called

pAL2-1, was initially associated with an increased life span rather than with senescence. The latter plasmid was first discovered in strain *AL2*, where it had integrated into the third intron of *cob*, the apocytochrome b gene which is a component of respiratory complex III. It was associated with longevity and consequently, pAL2-1 has historically been termed a life span-prolonging plasmid (Hermanns *et al*., 1994). The life span prolonging effect of pAL2-1 however seems an exception rather than a rule. The majority of all natural isolates containing pAL2-1 homologous plasmids do not show this effect (van der Gaag *et al*., 1998).

The data presented here show that senescence is significantly delayed by CR in *P. anserina*, and that the natural variation that exists for the response to CR corresponds largely to the presence or absence of pAL2-1 homologous mitochondrial plasmids in the isolates. Analysis of near-isogenic lines with or without a pAL2-1 homologue confirmed that these mitochondrial plasmids reduce the life span extending effect of CR in *P. anserina*. Presumably they interfere with the efficiency of cellular energy production, thus blocking the response to CR as the latter requires a conservative use of resources.

Material and Methods

Strains and culturing conditions

Properties and growth conditions of the pseudo-homothallic ascomycete *P. anserina* have previously been reviewed by Esser (1974). All isolates used in this study (Table 6-1) are from a collection of wild types originating from the surroundings of Wageningen, The Netherlands. About twenty percent of these wild types contain an autonomously replicating mitochondrial plasmid homologous to pAL2-1 (van der Gaag *et al*., 1998).

Isolates were grown on cornmeal agar with either regular or 100-fold reduced D-glucose content. Regular cornmeal agar contained 1.7% (w/v) Difco™ cornmeal agar, 0.7% (w/v) D-glucose, 1.0% (w/v) sucrose, 0.1% (w/v) yeast extract, 0.1 ‰ KH_2PO_4 and 0.6% (w/v) agar. Where D-glucose was 100-fold reduced, sucrose was used to correct the osmotic value of the medium. *P. anserina* is unable to utilize sucrose as a carbon-source.

Construction of near-isogenic lines

Loss of pAL2-1 homologues can occur during the sexual cycle (van der Gaag *et al*., 1998). It was thus possible to obtain plasmid-free and plasmid-containing progeny from self-fertilized isolates. This was done for isolates *Wa26, Wa32* and *Wa119*; progeny obtained by self-fertilization were tested for the presence of pAL2-1 homologues using PCR analysis. DNA

was extracted according to standard procedures (Sambrook *et al.*, 1989) and PCR was done using oligonucleotide primers (5' GGA GGA TCG AGG GGA GAT TTT G 3' and 5' GGT TGT TTT ATG TCG CCT ATG AGA 3') located within the terminal inverted repeat (TIR) of the plasmid. PCR amplification was done using 35 cycles with an annealing temperature T_a of 49 ºC and an elongation time of one minute. The resulting sets of near-isogenic siblings, hereafter referred to as 'isogenic', were used to analyze the effect of the plasmid.

Survival analysis

Life span was measured in hours of growth using 50 cm long glass 'race tubes' containing eighteen ml of either regular or glucose limited medium. Tubes were inoculated using young mycelium and incubated at 27 ºC. Growth was monitored daily.

23 natural isolates were used to examine the natural variation for the response to CR, ten of which contained a pAL2-1 homologue and thirteen of which did not. Per isolate, ten replicate tubes were used: five for each condition (either normal or calorie restricted). Growth rates were determined by calculating the slopes of the linear parts of the individual growth curves (in mm/day). These data were compared using two-way analysis of variance (Two-way ANOVA). Life span was measured in hours until growth arrest. We tested for the effect of CR and whether or not this would be isolate specific. Data of the natural isolates were analyzed using the COX proportional hazards model and plotted as cumulative survival in time using Kaplan-Meier estimates. The results obtained with the near-isogenic isolates were analyzed in a similar way, except for the use of isolates as a stratifying variable. All data were analyzed using statistics package SPSS© 8.0.0.

Results

Calorie restriction slows down aging in natural isolates of P.anserina

Studies on senescence in *Podospora* usually define life span as growth in cm. Here however, life span is defined in hours until growth arrest. Although mycelial growth rate was affected both by the presence of pAL2-1 homologues (reduced by approximately 15%, under both normal and calorie restricted conditions; two-way ANOVA, $F=18.4$, $df=1$, $P<0.001$) and by CR (increased by approximately 50%, independently of the presence of a plasmid; two-way ANOVA, $F=144.3$, $df=1$, $P<0.001$), this did not influence the outcome of the experiment. In fact, expression in cm would even increase the significance of the findings discussed below. Under conditions with 100-fold reduced glucose content, isolates typically grew faster but

less dense. Compared to isolates grown under normal conditions, they were poorly colored, arrhythmic and reproduced more slowly. Cultures dying under calorie restricted conditions typically showed an increase in microconidial production just before death, observed as a more intensely colored band. Results for all individual isolates have been summarized in Table 4-1. Survival analysis showed a significant life span prolonging effect of calorie restriction (Wald's test, Z=67.2, df=1, P<0.0001, Table 4-2).

Table 4-1. Life spans of natural *P. anserina* isolates under normal (N) and calorie restricted (CR) conditions. Using Southern analysis, *Wa11* and *Wa26* were previously classified as plasmid-free (Van der Gaag *et al.*, 1998).

Isolate		**N**	**CR**	**CR effect**
		hrs ± se (n)	**hrs ± se (n)**	**(CR/N)**
Plasmid free	*Wa03*	283 ± 29 (5)	549 ± 38 (5)	1.94
	Wa07	271 ±17 (5)	1080 ± 106 (5)	3.96
	Wa13	236 ± 12 (5)	696 ± 141 (5)	2.95
	Wa14	243 ± 7 (5)	466 ± 111 (5)	1.92
	Wa16	313 ± 30 (5)	1226 ± 122 (5)	3.92
	Wa17	256 ± 33 (5)	356 ± 33 (5)	1.39
	Wa19	170 ± 17 (5)	223 ± 33 (5)	1.31
	Wa21	243 ± 11 (5)	499 ± 22 (5)	2.05
	Wa23	276 ± 5 (5)	966 ± 92 (5)	3.50
	Wa24	291 ± 13 (5)	842 ± 88 (5)	2.89
	Wa25	147 ± 8 (5)	244 ± 22 (5)	1.66
	Wa29	325 ± 40 (5)	429 ± 66 (5)	1.32
	Wa52	209 ± 5 (5)	1057 ± 120 (5)	5.06
Plasmid containing	*Wa04*	216 ± 51 (5)	354 ± 77 (5)	1.64
	Wa10	152 ± 9 (5)	271 ± 28 (5)	1.78
	Wa11	243 ± 16 (5)	277 ± 56 (5)	1.14
	Wa18	201 ± 29 (5)	284 ± 51 (5)	1.41
	Wa26	229 ± 21 (5)	668 ± 136 (5)	2.92
	Wa30	198 ± 13 (5)	294 ± 23 (5)	1.48
	Wa32	231 ± 28 (5)	210 ± 10 (5)	0.91
	Wa34	174 ± 21 (5)	242 ± 17 (5)	1.39
	Wa45a	287 ± 43 (5)	293 ± 57 (5)	1.02
	Wa119	204 ± 19 (5)	297 ± 78 (5)	1.45
Subtotal plasmid free		251 ± 8 (65)	664 ± 46 (65)	2.65
Subtotal plasmid containing		213 ± 9 (50)	319 ± 25 (50)	1.50
Total		234 ± 6 (115)	514 ± 32 (115)	2.20

Table 4-2. Survival analysis of natural isolates using the COX proportional hazards model (N=230, 0% censored). The joint predictive ability of the specified variables is significant (Goodness of fit χ^2=318, df=3, P<0.0001). Calorie restriction increases life span in an isolate dependent manner.

Variable	B ± se	Wald's test			HR[1]	95% CI
		Z	df	P		
CR	-6.84 ± 0 .83	67.2	1	<0.0001	0.0011	(0.0002 - 0.0055)
Isolate	-	80.9	22	<0.0001	-	-
Isolate * CR	-	79.7	22	<0.0001	-	-

[1.] Hazard Ratio (estimated measure of effect of a given variable on the instantaneous death rate). Values below one indicate a life span prolonging effect, values equal to one indicate no effect and values above one indicate a life span reducing effect.

The response to calorie restriction varies widely among isolates (Wald's test, Z=79,7 df=22, P<0.0001, Table 4-2), ranging from no effect (for example in *Wa45a*, Table 4-1) up to a five-fold life span extending effect (for example in *Wa52*, Table 4-1). Closer inspection of the data shows that the variation in the isolates response corresponds largely to the presence or absence of pAL2-1 homologues (Table 4-1, Figure 4-1). When the same analysis was done, but controlled for plasmid presence, a significant amount of variation remained (Wald's test, Z=54.4, df=22, P=0.0001). Thus most, but not all, variation can be ascribed to the presence or absence of pAL2-1 homologues. Figure 4-1 shows the cumulative survival of the subset of isolates that contain a pAL2-1 homologue or the subset of isolates that do not, under normal and calorie restricted conditions. Plasmid free isolates thus strongly respond to calorie restrion whereas plasmid containing isolates do not.

PAL2-1 homologous plasmids reduce calorie restriction mediated life span extension

The data from the natural isolates alone do not allow us to distinguish a true effect of the plasmid from a potential effect of its associated background. We thus constructed and analyzed near-isogenic combinations of isolates with and without a plasmid, by selecting for plasmid containing and plasmid free sibs among the progeny of self-fertilized isolates. Race tubes of one set of 'isogenic' isolates are shown in figure 4-2. A summary of the results obtained with the sets of 'isogenic' strains is given in table 4-3. Survival analysis showed that the effect of the pAL2-1 homologues did not result from a host background effect. When controlled for host background, the plasmid effect on CR remained (Wald's test, Z=13.5, df=1, P=0.0002).

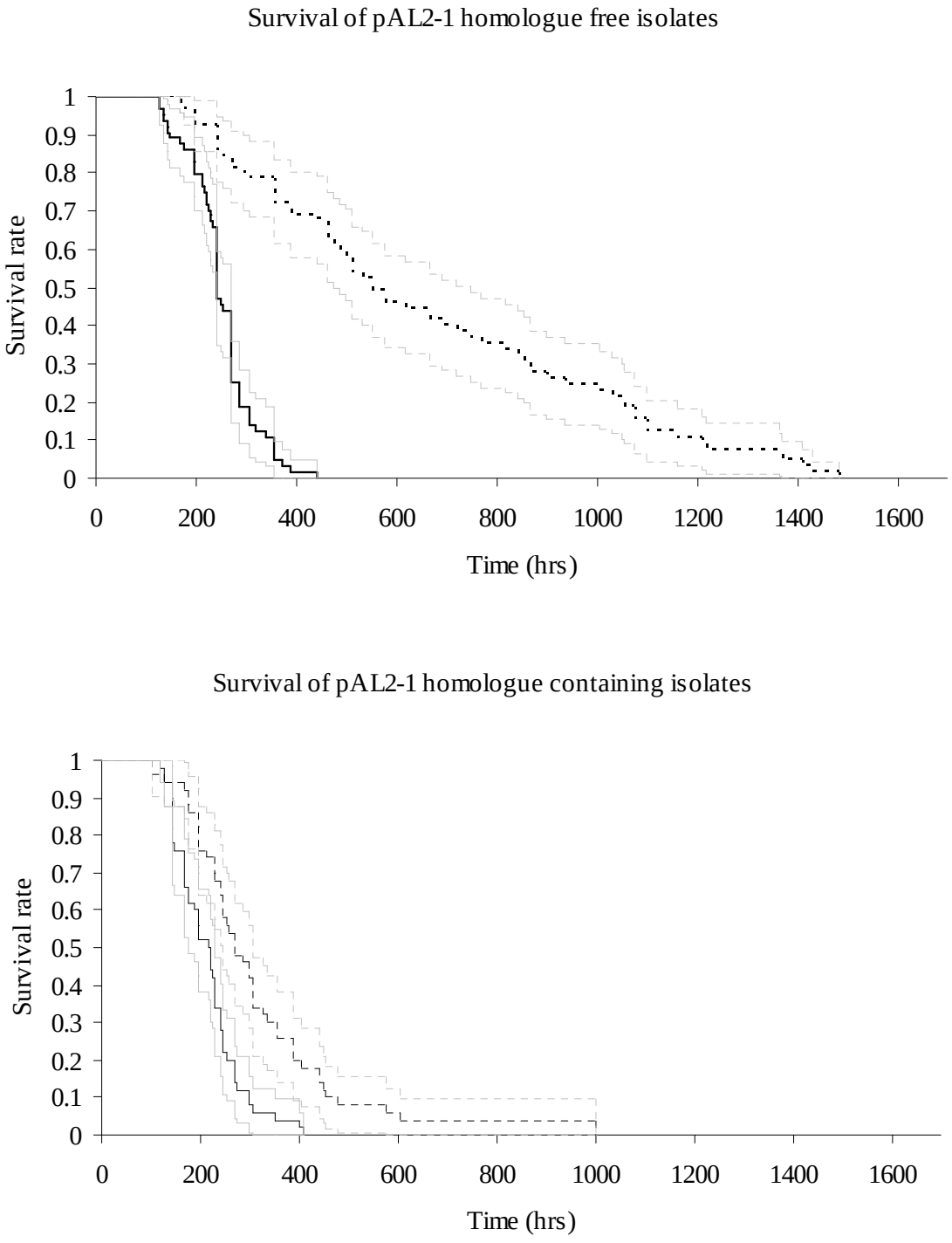

Figure 4-1. Kaplan-Meier survival curves of natural *P. anserina*, plasmid free (top panel) and plasmid containing (lower panel) isolates under normal (solid line) and glucose limited (dotted line) conditions. The presence of pAL2-1 homologous plasmids strongly reduces the life span extending effect of glucose limitation. The 95% confidence intervals are indicated using grey (solid or dotted) lines.

Figure 4-2. Near-isogenic strains derived from wild-type isolate Wa32, with and without a pAL2-1 homologue, grown under normal and calorie restricted conditions. Whereas CR hardly affects life span in Wa32, it extends life span two-fold in its plasmid free siblings. As was also the case in the comparison between natural isolates, pAL2-1 homologues did not have any visible effect on reproduction. From top to bottom duplicate race tubes are shown: plasmid containing and plasmid free strains under standard (top half) and glucose limited (bottom half) conditions. Growth arrested in all isolates except in the plasmid free strain under calorie restricted conditions (lower two tubes).

Table 4-3. Average life spans (LS) in hrs ± se (n) of near-isogenic combinations of strains with or without a pAL2-1 homologue, grown under normal (N) and calorie restricted (CR) conditions. The effect of CR is expressed as the ratio of CR and N.

Isolate	**Plasmid status**	**N**	**CR**	**CR effect**
		LS in hrs ± se	LS in hrs ± se	**(CR/N)**
Wa26	Plasmid containing	214 ± 9 (16)	473 ± 23 (13)	2.21
	Plasmid free	240 ± 9 (30)	672 ± 42 (31)	2.80
Wa32	Plasmid containing	251 ± 21 (13)	286 ± 31 (15)	1.14
	Plasmid free	275 ± 15 (15)	534 ± 14 (15)	1.94
Wa119	Plasmid containing	195 ± 16 (15)	286 ± 34 (15)	1.47
	Plasmid free	160 ± 9 (15)	334 ± 33 (15)	2.09

Discussion

Modular and unitary body plans and calorie restriction

In unitary organisms like mice, rats, spiders, worms and fish, CR has been shown to extend life span (Weindruch and Walford, 1988). Reproductive capacity in unitary organisms generally increases with age, then decreases, followed by senescence and death (Andrews, 1998). Extending life span and postponing reproduction will allow a unitary organism to survive unfavorable conditions. In modular organisms however, the adaptive significance of the response to calorie restriction is more difficult to explain because reproductive value increases with age and systemic senescence is often delayed or absent, although senescence directed at the module can occur. Postponing reproduction does not make sense in the same way as it does in unitary organisms because the reproductive value is potentially infinite, and one would not expect a trade-off between life span and early reproduction as in unitary organisms. The filamentous fungus *Podospora anserina* is a typical modular organism with an iterative, indeterminate growth. Because reproductive output is a function of the size of the soma, the potential reproductive value increases exponentially with age. Yet this coprophilous fungus has a limited life span: In all natural isolates senescence occurs after a strain-specific time (Rizet, 1953, van der Gaag, 1998). Senescence may have evolved in this modular system as a result of its ephemeral growth conditions: Growth is restricted by the temporal availability and limited size of its substrate. This kind of resource limitation causes the genetic and physiological individual to share the same fate, because there is no dispersal via asexual propagules in Podospora, only via sexual spores. Hence, the unit of selection is the entire organism and in this respect Podospora resembles unitary organisms. Therefore, not only aging is expected to evolve in this system, but also the life span extending response to CR. It is clear from the results presented here, that calorie restriction postpones reproduction and senescence in Podospora in an isolate dependent manner.

Calorie restriction and the plasmid pAL2-1

Isolate dependent effects of CR are largely explained by the presence or absence of pAL2-1 homologues. These plasmids apparently reduce life span under calorie restricted conditions and therefore they can be considered senescence plasmids. The plasmid effect is remarkable because pAL2-1 was first described as a longevity plasmid (Hermanns *et al*., 1994). In the latter case, pAL2-1 was integrated into the mitochondrial genome, delaying the senescence process. Recently we obtained another long-lived isolate carrying a pAL2-1 homologue

integrated into the mitochondrial genome (see chapter 5). Because under normal conditions the life span reducing effects of the pAL2-1 homologous plasmids are overshadowed by natural senescence, they are more likely to manifest themselves as longevity inducing plasmids. Considering their effect under calorie restricted conditions though, they are not different in effect from mitochondrial plasmids like pKALILO from *N. intermedia* or pMARANHAR from *N. crassa*. In the case of these latter two plasmids, integration is believed to cause death rather than longevity. Given that *Neurospora* does not senesce, the plasmids can only manifest themselves as life span reducing plasmids. The observed effects of these and similar plasmids obviously depend on whether or not the host senesces. For example, in the slime mold *Physarum polycephalum* that normally senesces, integration of a linear mitochondrial plasmid (Nakagawa *et al*., 1998) has been associated with longevity but not senescence. Despite the fact that plasmid integration has historically been associated with longevity, pAL2-1 may thus be more appropriately called a senescence plasmid.

Life span extension by CR has been found in many organisms. The results presented here demonstrate that CR extends life span also in *P. anserina.* However, the presence of pAL2-1 homologues drastically reduces this effect. We propose a model to explain this. Calorie restriction extends life span in *Saccharomyces cerevisiae* by increasing respiration (Lin *et al*., 2002). If this is a general pathway, plasmids that interfere with mitochondrial function are indeed expected to reduce the effect of CR. For example, death caused by pKALILO from *N. intermedia* is associated with a highly increased copy number of the free plasmid, the appearance of integrated copies and mitochondrial defects like cytochrome deficiencies and abnormal mitochondrial ribosomal profiles (Griffiths, 1992). Notably, CR does not increase life span of pKALILO containing isolates of *Neurospora* (Maas *et al*., unpublished data). If indeed pAL2-1 similarly affects mitochondrial function, this can explain why its presence interferes with the life span extending response to CR.

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Chapter 5

A novel long-lived mutant of *Podospora anserina*

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Abstract

The integration of linear mitochondrial plasmid pAL2-1 into the mitochondrial genome has been associated with life span extension in the filamentous fungus *Podospora anserina*. All of its autonomously replicating homologues on the other hand were, though only under glucose limited conditions, associated with a reduction in life span. We recently obtained a second long-lived isolate containing an integrated pAL2-1 homologue. As expected, the longevity trait was inherited maternally. The plasmid had integrated into the putative promoter region of a messenger containing the *nad2* and *nad3* genes that encode two membrane subunits of respiratory complex I. This had serious repercussions throughout the respiratory chain. The mutation was associated with the induction of alternative respiratory pathways, effectively resulting in a shift towards a relatively less efficient but faster form of respiration. The long-lived mutant showed reduced fertility indicating an energy deficiency, reduced levels of reactive oxygen species (ROS) and increased mitochondrial genome stability.

Introduction

Fungal senescence is typically associated with mitochondrial abnormalities including rearrangements of the mitochondrial genome and concomitant biochemical defects (Griffiths, 1992; Bertrand, 2000). Senescing isolates of the filamentous ascomycete *Podospora anserina* for example accumulate small circular mtDNA derivatives termed senDNAs. Over the course of time these senDNAs are amplified to such an extent that normal mtDNA is no longer observed and this ultimately renders mitochondria dysfunctional. The most prominent one of these mtDNA derivatives is termed senDNA α (also known as pl-DNA, Osiewacz, 2002), a 2.5 Kb circle that corresponds exactly to the mobile group II intron *cox1*-i1, also known as intron α. Recent work however has shown that it is neither just intron α nor any other specific mitochondrial element in itself, but the overall activity of respiratory metabolism and its concomitant production of potentially cytotoxic reactive oxygen species (ROS) that are probably at the core of the senescence process (Dufour and Larsson, 2004; Osiewacz 2002).

The implication of free radicals in the aging process was proposed already half a century ago (Harmann, 1956). They are an inherent by-product of the mitochondrial electron transport chain. Mitochondrial electron transport couples the reduction of oxygen to the establishment of an electrochemical gradient across the inner mitochondrial membrane ($\Delta\psi$), which is used by the mitochondrial ATP synthase to phosphorylate ADP. Not only is this the most important source of cellular energy in aerobic eukaryotes, but it is also a potent source of free radicals that will damage the cell if not appropriately discarded by scavenging systems. Both in animal models and in man, defects of the electron transport chain typically cause cellular energy depletion and increased levels of ROS. The latter is associated with aging and a wide variety of aging-related processes (Shigenaga *et al.,* 1994; Lenaz *et al.,* 2002; Wallace, 2001).

In *P. anserina*, defects of the electron transport chain paradoxically appear to lower the level of intracellular oxidative stress rather than increase it, ultimately resulting in longevity rather than senescence (Dufour *et al.,* 2000). The latter may be explained by the fact that fungi, like plants, can switch to alternative modes of respiration. The respiratory chain of Podospora is organized in a large super-molecular structure called the respirasome (Krause *et al*., 2004). In one particular conformation of this complex structure, electrons do not use the cytochrome c oxidase (respiratory complex IV) dependent pathway. Electrons can be shuttled directly to oxygen by an alternative, nuclear-encoded quinol oxidase (AOXp). The latter mildly uncouples the oxidation of quinol from phosphorylation, thereby potentially lowering both energy and mitochondrial free radical production. Mild uncoupling is known to have this effect (Miwa and Brand, 2003; Merry, 2002). In plants the induction of AOXp and the level of mitochondrial ROS are correlated accordingly (Maxwell *et al.,* 1999), and at first sight this also seems to hold up for Podospora. Whereas actively growing, short-lived wild types of *P. anserina* do not use the alternative pathway, long-lived mutants with defects in complex IV typically all do. The latter all show severely reduced levels of cellular energy as well as reduced levels of oxidative stress (Dufour and Larsson, 2004). Lorin *et al.* (2001) however showed that overexpression of the alternative pathway can antagonize the effect of complex IV dysfunction, remarkably restoring both the original level of cellular energy and the original level of oxidative stress rather than reducing these, ultimately restoring both senescence and fertility. The latter may be explained by an indirect effect of AOXp on the functioning of respiratory complex I. Whatever the exact etiology behind it, there are ultimately at least three factors consistently associated with senescence in *P. anserina*: The level of intracellular oxidative stress, the stability of the mitochondrial chromosome, and the cellular energy level. All long-lived isolates obtained thus far showed reduced levels of oxidative stress, increased mtDNA stability, and suffered from reduced fertility indicating deficient energy production.

Although most previously isolated long-lived mutants were cytochrome c oxidase deficient and completely female sterile, some still showed a moderate degree of fertility, like the long-lived isolate *AL2* (Osiewacz *et al.*, 1989) The latter isolate, a derivative of laboratory strain *A*, contained an 8.4 Kb large linear mitochondrial plasmid with a typical invertron structure (containing long terminal inverted repeats, TIRs; Hermans and Osiewacz, 1992). The plasmid, called pAL2-1, was present as an autonomously replicating element and in addition it had integrated into several sites of the mitochondrial genome of the isolate. One copy was found in the third intron of *cob*, the apocytochrome b gene and another one in a different yet uncharacterized location (Hermanns *et al.*, 1994). In addition to the integrated plasmids, the long-lived isolate contained a 3.6 Kb large homoplasmic deletion that included two mitochondrial *tRNA* genes (Hermanns and Osiewacz, 1996). Integrated and autonomously replicating copies of the plasmid could be transferred along with the longevity trait and this was independent from the latter deletion, hence the plasmid associated sequences rather than the deletion must have been responsible for longevity. Even though it was formally never shown whether the autonomously replicating form of pAL2-1, the integrated form, perhaps both or even an integration associated modification elsewhere in the mitochondrial genome was ultimately responsible, integration was held responsible for longevity and pAL2-1 was thus dubbed a longevity inducing plasmid. The autonomously replicating homologues from the natural population were associated neither with integration nor with longevity and these were therefore deemed neutral (van der Gaag *et al.*, 1998). However, the latter plasmids could be transmitted horizontally with remarkable ease (van der Gaag *et al.*, 1998) which is generally a hallmark of virulence, and thus their status as phenotypically neutral remained questionable. Recently we have shown that none of the naturally occurring pAL2-1 homologues are in fact neutral: Particularly under glucose limited conditions they are all associated with a striking senescence phenotype. Whereas Podospora like all other aging organisms responds to calorie restriction (CR) in a life span extending manner, the isolates that carry a pAL2-1 homologue do not or hardly show this response (Maas *et al.*, 2004). At first sight, pAL2-1 thus appears to induce senescence rather than longevity, though longevity may occasionally arise upon a specific integration event. This situation is highly reminiscent of that found in Neurospora, in which linear mitochondrial plasmids also appear to be responsible for senescence rather than longevity. Whereas plasmid free Neurospora isolates normally do not senesce, those carrying either one of the invertrons pKALILO or pMARANHAR do. The latter allegedly cause senescence by acting as insertional mutagens of the mitochondrial genome (Griffiths 1992).

PAL2-1 containing isolates of *P. anserina* hardly respond to CR (Maas *et al.*, 2004). This probably sets pAL2-1 based senescence apart from the regular aging process of

Podospora, which is responsive to CR. The mutation in isolate *AL2* affected both pAL2-1 based senescence (assuming that the prototypic plasmid from strain *A* was associated with a similar effect on life span as its homologues from the natural population; note that this has formally never been tested) and the regular aging process of Podospora. It had a delaying effect on the formation of senDNA α, but was never characterized with regard to other aspects of mitochondrial function. Recently we obtained another long-lived isolate carrying a pAL2-1 homologue integrated into the mitochondrial chromosome. As expected, the longevity trait was inherited maternally. The plasmid had integrated into the putative promoter region of a messenger containing the *nd2* and *nd3* genes that encode two membrane subunits of respiratory complex I. This did not just affect these two genes, but had serious repercussions throughout the respiratory chain. The mutation was associated with the induction of alternative respiratory pathways, effectively resulting in a shift towards a relatively less efficient but potentially faster form of respiration. The long-lived mutant showed reduced fertility indicating an energy deficiency, reduced levels of reactive oxygen species (ROS) and increased mitochondrial genome stability.

Materials and Methods

Isolates and culturing conditions

Properties of the pseudo-homothallic filamentous ascomycete *P. anserina* have previously been reviewed by Esser (1974). *Wa01, Wa06, Wa30* and *Wa32* all are wild-type *P. anserina* isolates originating from Wageningen, The Netherlands and have been described by van der Gaag *et al.* (1998). Strain *Wa32LL* is a novel, long-lived derivative of *Wa32*. Isolate *s* has been described by Rizet (1953) and the *cox5* deficient strain is a long-lived derivative of *s* (Dufour *et al.*, 2000).

Isolates were grown at 27 ˚C, on Modified Rizet's (MR) medium (Esser, 1974), unless stated otherwise. *Wa32LL* was reciprocally crossed with each of the short-lived isolates *Wa1, Wa6, Wa30* and *Wa32*. Crosses were made by fertilizing single mating type cultures of the female parent with microconidia of the opposite mating type. From each cross, perithecia were collected and dissected in order to obtain complete asci for tetrad analysis. Ascospores were collected onto individual plates which, to induce germination, had been supplemented with 0.06 M ammonium acetate (Esser, 1974). Life span was measured using 50 cm long glass 'race' tubes, and was expressed in distance or in time of growth between the inoculation and growth arrest of a culture. To measure the linear growth rate, the mycelial growth front was marked daily.

Isolation of mitochondria

Mitochondria were obtained by discontinuous density gradient centrifugation. Cultures were grown for one or two days at 27 ˚C in liquid medium. Mycelium was harvested and immediately frozen in liquid nitrogen, ground and suspended in 0.4 M sucrose, 50 mM Tris-HCl and 2 mM EDTA, pH 8.0 containing 15 mM β-mercaptoethanol. The suspensions were centrifuged twice for 10 minutes at 3.000 rpm to pellet nuclei and large cellular debris. The supernatant containing mitochondria and similar-sized particles was subsequently filtered over a 30 μm nylon filter and centrifuged for 25 minutes at 11.000 rpm. The resulting pellet was resuspended in 0.4 M sucrose, 50 mM Tris-HCl and 2 mM EDTA, pH 8.0, loaded onto a discontinuous sucrose gradient and centrifuged for 1 hr at 25.000 rpm. The mitochondrial fraction was collected, centrifuged for 25 minutes at 11.000 rpm and finally resuspended in the appropriate buffers for further analysis.

Preparation of protoplasts

Protoplasts were made from cultures grown for one or two days at 27 ˚C in liquid medium. Mycelium was harvested and resuspended in 0.6 M sucrose, 5 mM Na_2HPO_4 and 45 mM KH_2PO_4, pH 6.0 containing 4% (w/v) β-1,3-glucanase (Glucanex, Novo Nordisk) for cell wall lysis. The suspensions were incubated for one or two hrs at 37 ˚C and gently mixed to free the protoplasts from the partially digested mycelium. They were then filtered over cheese cloth to remove mycelial debris and centrifuged for 10 minutes at approximately 3.000 rpm. The resulting pellet was resuspended and washed twice in 0.6 M sucrose, 5 mM Na_2HPO_4 and 45 mM KH_2PO_4, pH 6.0, and resuspended in appropriate buffers for further analysis.

Nucleic acid analysis

Cultures for DNA extraction were grown for two or three days at 27 ˚C on cellophane disks overlying solid MR medium. Total DNA was extracted from these cultures using standard procedures (Sambrook *et al*., 1989), to be used for either Southern or PCR analysis.

For Southern analysis, DNA was digested using *HaeIII* (Lecelier and Silar, 1994) and separated using gel electrophoresis in 40 mM Tris-HCl, 20 mM acetic acid and 1 mM EDTA (TAE), pH 8.0 containing 1% (w/v) agarose. DNA was visualized using ethidium bromide staining. Electrophoresis was followed by capillary transfer and ultraviolet crosslinking of the DNA to nylon membranes (Hybond^{tm} N, Amersham Biosciences) according to standard procedures (Sambrook *et al.*, 1989). The resulting blots were subsequently hybridized with

[α-^{32}P]-dCTP labeled probes, made using the Megaprime^{tm} DNA labelling system RPN 1606 (Amersham Biosciences). To probe regions α and γ of the mitochondrial genome, we used the complete 2.5 Kb large intron α and the 14.8 Kb large *EcoRI*-1 fragment of isolate *s* mtDNA, respectively (Begel *et al*., 1999). To probe pAL2-1 homologous sequences, we used the 1.9 Kb *XbaI*-3 fragment of pAL2-1 from isolate *A* (Hermanns *et al.*, 1994).

Based on the Southern analysis, a set of PCR primers was designed to amplify the plasmid/mtDNA recombination junctions found in isolate *Wa32LL* and the corresponding wild-type region as found in isolate *Wa32*. These primers are given in Table 5-1. The 5' pAL2-1/mtDNA recombination junction was amplified using primers 01 and 04, the 3' junction using primers 01 and 05, and the wild-type region using primers 04 and 05. In each case, PCR was done using 30 rounds of amplification with an annealing temperature (T_a) of 55 ˚C and an elongation time of one minute. Gel electrophoresis and visualization was done as described above. The DNA fragments were purified using the Qiaquick PCR product Purification Kit (Qiagen) and sequenced directly from these fragments.

RNA was extracted according to standard procedures (Sambrook *et al.,* 1989) from mitochondria obtained as described above, to subsequently be used for either Northern analysis or reverse transcription PCR (RT-PCR).

For Northern analysis, mitochondrial RNA was separated using gel electrophoresis in 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0 and 2.2 M formaldehyde containing 1.2 % (w/v) agarose, followed by capillary transfer and ultraviolet cross-linking of the RNA to nylon membranes (Hybond^{tm} N, Amersham Biosciences), all according to standard procedures (Sambrook *et al.*, 1989). To specifically probe the transcripts of the *nad2* and *nad3* genes, we used PCR products amplified from these respective two genes. A region within *nad2* was amplified using primers nr. 06 and 07 (Table 5-1), and a region within *nad3*-e2 using primers nr. 08 and 09 (Table 5-1). In each case, PCR was done using 30 rounds of amplification with an annealing temperature (T_a) of 60 ˚C and an elongation time of one minute. Products were labeled as described above to be used as a probe for Northern hybridization. The mitochondrial rRNAs served as an internal control.

For RT-PCRs, cDNA was synthesized from 1 μg of DNAseI treated mitochondrial RNA each, using Avian Myeloblastosis Virus (AMV) reverse transcriptase according to the manufacturer's instructions (Promega). Several different primers were used for first strand synthesis: primer nr. 07 located in *nad2*, primer nr. 09 located in *nad3*-e2, or primer nr. 02 located in the TIR region of the pAL2-1 homologue (Table 5-1). Subsequent PCRs were done using sets of primers as indicated in the text. As an internal control, additional RT-PCRs were done using primers nr. 10 and 11 located within the *cytb* gene, or nr. 12 and 13 located within the *atp8* gene (Table 5-1). Electrophoresis and visualization was done as described above.

Table 5-1. Oligonucleotides used. Positions either refer to the sequence of pAL2-1 (numbers 01 and 02, GenBank accession nr. X60707; Hermanns and Osiewacz, 1992) or to that of the mitochondrial genome (numbers 03-13, GenBank accession nr. X55026; Cummings *et al*., 1990).

#	Sequence	Location	Position
01	5' CTC CCC TGC ATC CTC CAA AG 3'	TIR pAL2-1	96-115/8.281-300
02	5' CCC CTC TAT ATA TGG TCC AG 3'	TIR pAL2-1	32-51/8.345-8.364
03	5' GAG AAT TCG ATT TTC TCC CC 3'	*tRNA^{Met}*	11.312-11.331
04	5' GTT GAT TGG TTG GCT GAT TGG 3'	5' of *nd2*	11.338-11.358
05	5' TAT AAG AGC AAT TCG GTT GAA G 3'	*nd2*	11.677-11.698
06	5' CAG TGG AGC AGT CTT TTT AAT G 3'	*nd2*	12.004-12.025
07	5' TAT ACT ATT AAG TAT ATA AAG CCC 3'	*nd2*	12.227-12.250
08	5' CAA GAG AAG TAT AGC ATA TTT GA 3'	*nd3*-e2	14.641-14.663
09	5' CAA TTT TCA ATG CAC TTT TAC CT 3'	*nd3*-e2	14.868-14.890
10	5' TAC GGG CAA ATG TCC TTA TGA G 3'	*cytb*-e3	34.088-34.109
11	5' TAA GGA GCA AAT GTT ATT CTA TCG 3'	*cytb*-e3	34.333-34.356
12	5' ATT TAT ATG CCT CAA TTA GTC CC 3'	*atp8*	89.345-89.367
13	5' GTA CGA GAT AAG AAT AAA CGA AC 3'	*atp8*	89.456-89.478

Protein analysis

Proteins were isolated from mitochondria obtained as described above. They were suspended in 0.7 M sorbitol, 50mM Tris-HCl and 0.2 mM EDTA, pH 7.5, containing protease inhibitor. Concentrations were measured using a Bradford essay (Biorad). For Western analysis, mitochondrial proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes according to standard procedures (Sambrook *et al.*, 1989). AOXp was probed using a monoclonal antibody against AOXp from *Sauromatum gautatum* (Elthon *et al*., 1989). Detection was done with a SuperSignal® West Pico Chemiluminescent Substrate Kit (Pierce).

Analysis of cellular respiration

Oxygen consumption was measured according to the methods described by Dufour *et al.* (2000). Analyses were done using protoplasts, made as described above and suspended in 0.6 M sorbitol, 7.5 mM $MgCl_2$, 10 mM KH_2PO_4, 10 mM imidazole and 0.2 % bovine serum albumin (BSA), pH 7.5, at concentrations of at least 10^8 per ml. Protein content was measured using a Bradford essay (Biorad). Oxygen consumption was measured in an oxygraph (Gilson) equipped with a Clark-type electrode (Hansatech). 50 mM rotenone, 1 mM KCN or 2.5 mM SHAM was used to inhibit respiratory complex I, complex IV or AOXp, respectively.

Analysis of the level of oxidative stress

The level of intracellular oxidative stress was estimated flow-cytometrically according to the methods described by Dufour *et al*. (2000). Protoplasts were made as described above and suspended in 0.6 M sorbitol and 10 mM Tris-HCl, pH 7.5 containing 80 μM 2', 7'-dihydrodichlorofluorescein diacetate (H_2DCF-DA). H_2DCF-DA is a cell-permeant substrate that after intracellular hydrolysis yields the oxidation sensitive fluorogenic compound H_2DCF. The intracellular oxidation of the latter compound, yielding fluorescent DCF, was measured in 15 minute time intervals using a XL3C flow-cytometer (Coulter, France). As a control, a *cox5* deficient, long-lived isolate (*Cox5::ble*) was used that is known to have a reduced level of intracellular oxidative stress (Dufour *et al.*, 2000).

In addition to the flow-cytometric approach, we used a superoxide specific staining procedure based on the methods described by Munkres (1990). Young, equally sized mycelial colonies of *Wa32* and *Wa32LL* were flooded with 5 mM MOPS, pH 7.5 containing 2.5 mM nitro blue tetrazolium chloride (NBT, Merck), and incubated for one hour. NBT is a colourless chromogenic substrate which is reduced by superoxide to give a dark colored, water-insoluble formazan deposit (Halliwell and Gutteridge, 1985).

Results

A maternally inherited longevity trait in strain Wa32LL

Wa32LL is a spontaneous longevity mutant of a single mating type derivative (mat -) of short-lived wild type *Wa32* previously described by van der Gaag *et al.* (1998). Compared to the *Wa32*, *Wa32LL* has a more than ten-fold increased life span. We were able to culture it for at least 200 days, whereas its ancestor Wa32 had an average life span of only two weeks (see chapter 4). The long-lived isolate shows a slightly reduced growth rate (4.3 ± 0.2 mm/day versus, 4.9 ± 0.3 in the wild type), a reduction in the amount of aerial hyphae, less intense pigmentation (Figure 5-1), and impaired sexual development. When used as a protoperithecial (female) parent, fruiting bodies of *Wa32LL* were as numerous as in the wild type, though they appeared days later and most contained no or very few asci with ripe spores. Nonetheless, viable offspring could be obtained from these crosses. In confrontational crosses (using mycelial confrontations between different single mating-type isolates rather than fertilization with microconidia), the long-lived isolate initially appeared to be male sterile, but we have not been able to reproduce this result. The male sterility effect may thus have been unstable. Otherwise it may be conditional. In any case, it was not strictly associated with longevity.

Tetrad analysis of the longevity trait showed that it is inherited maternally. Reciprocal crosses were made between *Wa32LL* and several different wild types. By dissecting perithecia, fifteen complete asci were collected from each of these crosses. When *Wa32LL* was used as the protoperithecial parent, all spores from each ascus gave rise to long-lived colonies (For practical reasons the experiment was stopped after 50 days and longevity is here therefore defined as having a life span of more than 50 days). Given a few exceptions, all isolates resulting from self-fertilization of these were also long-lived. In contrast, progeny obtained from crosses using *Wa32LL* as a microconidial (male) parent was short-lived (<25 days) and showed wild-type phenotypic characteristics. We therefore concluded that the longevity trait found in isolate *Wa32LL* is cytoplasmic.

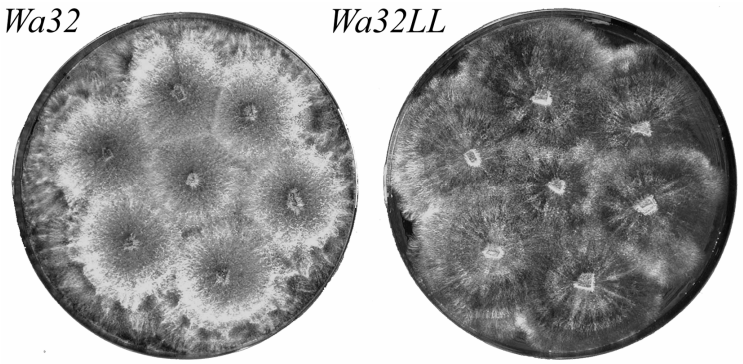

Figure 5-1. Cultures of natural isolate *Wa32* (left) and its long-lived derivative *Wa32LL* (right). Compared to its wild type ancestor, isolate *Wa32LL* shows a slight reduction in the amount of aerial hyphae and is slightly less intensely colored.

Longevity is associated with the stable integration of a pAL2-1 homologue into the mitochondrial chromosome

Most spontaneous extra-chromosomal longevity mutants of *P. anserina* found thus far, showed complete or partial deletions of intron α and its flanking sequences (reviewed by Griffiths, 1992 and Bertrand, 2000). Southern analysis of this mtDNA region, here referred to as region α, did however not reveal any differences between *Wa32LL* and its ancestor. Instead, an 8.4 Kb large insertion was detected within the region containing the mitochondrial *nad2* and *nad3* genes, here also referred to as the γ region because it is part of the common core sequence from which the γ family of senDNAs is derived. This insertion appeared to be present in a homoplasmic or near-homoplasmic state and was not detected in the mitochondrial genome of the wild type, neither by Southern nor by PCR (see further on). A closer comparison of the mtDNA restriction profiles of isolates *Wa32* and *Wa32LL* revealed no major differences other than this insertion. Previously a similar-sized, but autonomously replicating pAL2-1 homologue was found in isolate *Wa32* (van der Gaag *et al,* 1998) and thus we tested whether the insertion corresponded to this plasmid. Southern analysis showed that

this was the case (Figure 5-2). The plasmid/mtDNA recombination junctions were amplified using PCR and the products were sequenced. This showed that in *Wa32LL* the pAL2-1 homologue had inserted in the putative promoter region of *nad2*. It was found 102 bp away from the initiation codon of this latter gene (corresponding to position 11555/11556 of the mtDNA sequence of *P. anserina*, in the original sequence only 49 bp upstream of the initiation codon of the *nad2* gene, GenBank accession nr. X55026, Cummings *et al*., 1990).

When compared to the corresponding region from the wild-type isolate, the inserted plasmid copy was flanked by short target site duplications. The duplicated region 3' of the integrated plasmid was a perfect inversion of that 5' of the integrated plasmid, hence in effect forming an extension of the terminal inverted repeat (TIR) sequence of the plasmid. These duplications were not found in the mtDNA of the wild type, indicating that they came about *de novo* during the integration process. Similar duplications are typically found next to integrated invertron copies and may originate from intermolecular recombination events between different integrated copies (Bertrand, 1986), or recombination events involving a single-stranded, pan-handle shaped replication intermediate (Griffiths, 1992). A similar structure is also found at integration sites in senescent subcultures of *P. anserina*, suggesting that it is unrelated to the long-lived phenotype of *Wa32LL*.

PAL2-1 was initially discovered in isolate *AL2*, a long-lived derivative of *P. anserina* laboratory strain *A*, and it has therefore historically been associated with the induction of longevity (Hermanns *et al.*, 1994, Hermanns and Osiewacz, 1996). More recently however, we have shown that particularly under glucose limiting conditions, the pAL2-1 homologues are actually associated with a senescence syndrome. *P. anserina* isolates that contain a pAL2-1 homologue have a phenotype strikingly similar to that induced by the invertrons pKALILO and pMARANHAR from Neurospora (Maas *et al.*, 2004, see also chapter 4), a senescence phenotype allegedly caused by the integration of these plasmids into the mitochondrial genome (Griffiths, 1992; Bertrand, 2000). Analogous to the situation in Neurospora, integration of pAL2-1 is not just found in long-lived isolates (*AL2* and *Wa32LL*), but also in senescent subcultures of short-lived isolates (Figure 5-2).

In the wild type we found two different sites of integration. Both of these were sequenced: One was located in the *ORF P'* gene (corresponding to position 28.805/28.806 in the mtDNA sequence of strain *A*, GenBank accession nr. X55026; Cummings *et al*, 1990), which presumably is a non-functional remnant of the DNA dependent RNA polymerase (*DdRp*) gene encoded by an integrated predecessor of the pAL2-1 homologues. The other site of integration was located in the same region as that found in *Wa32LL*, namely in the intergenic region located upstream of *nad2* (corresponding to position 11.338/11.339 in the mtDNA sequence of strain *A*, GenBank accession nr. X55026, Cummings *et al*, 1990).

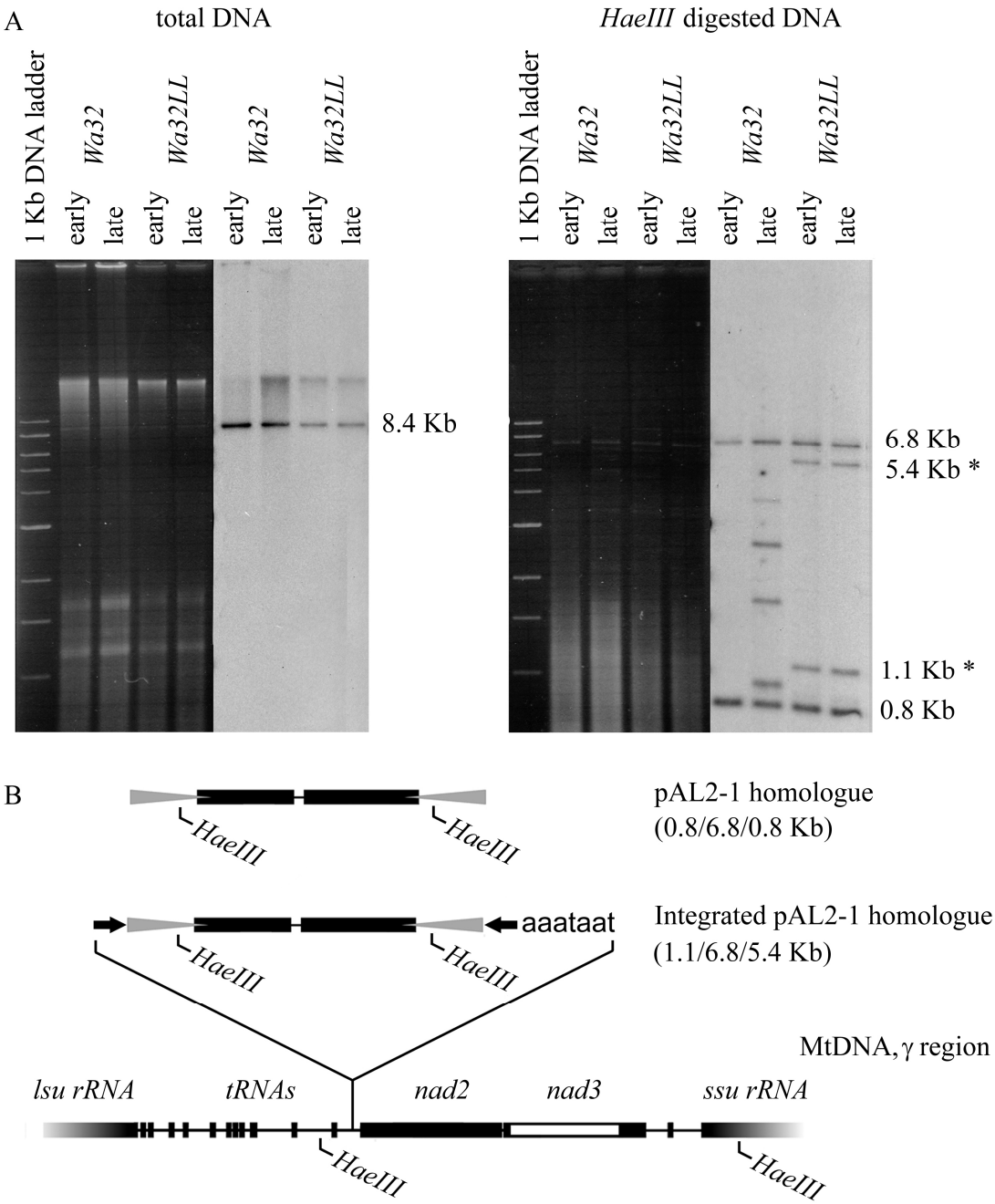

Figure 5-2. Long-lived isolate *Wa32LL* contains an integrated pAL2-1 homologue upstream of *nad2*. **A.** Ethidium bromide stained undigested (left) and *HaeIII* digested (right) total DNA, including corresponding blots probed with the *XbaI*-3 fragment of pAL2-1. The 8.4 KB fragment and the 6.8 and (two) 0.8 Kb fragments correspond to the autonomously replicating form of the plasmid; the 1.1 and 5.4 Kb fragments respectively correspond to the 5' and 3' sides of the integrated copy including its flanking region. The latter fragments co-hybridized with a γ region specific probe, the *EcoRI*-1 fragment of the mtDNA of strain *s* (Southern data not shown). **B.** Physical map of the mtDNA region γ and pAL2-1, indicating *HaeIII* restriction sites and the site of plasmid integration in *Wa32LL*.

The latter insertion was located much further away from the *nad2* gene than the one in *Wa32LL*, only a few base pairs downstream from one of the mitochondrial *tRNA*^{Met} genes. Integration is thus not necessarily linked to longevity as suggested in earlier work (Hermanns *et al.*, 1994; Hermanns and Osiewacz, 1996). It may have a conditional effect, depending for example on the timing or exact location of the initial integration event, or even a combination of these and other factors. Formally it may even be an epi-phenomenon of mitochondrial malfunction.

The integrated pAL2-1 homologue appears to alter the expression of nd2 and nd3 in Wa32LL

Since the integrated sequence in *Wa32LL* was found close to the startcodon of *nad2*, within the putative promoter region of this gene, we tested whether it would perhaps negatively affect the expression of this latter gene. Using Northern analysis (data not shown) with the mitochondrial rRNAs as loading control, transcripts containing *nad2* could indeed not be detected in *Wa32LL*. Neither could transcripts containing *nad3*, which is co-transcribed, be detected. In the wild type, *nad2* and *nad3* nonetheless appeared to be present on a single RNA species of approximately 2.6 Kb. The latter RNA presumably reflected a mature transcript, processed at the flanking tRNAs and with the intron of *nad3* spliced out (as predicted from the mtDNA sequence according to Cummings *et al.*, 1990). It thus appeared that the plasmid insertion in *Wa32LL* interfered with the expression of its downstream two genes *nad2* and *nad3*.

Using RT-PCR we were able to detect a processed messenger both in *Wa32* and in *Wa32LL* (Figure 5-3), showing that it was not just absent but merely less abundant in the long-lived isolate. CDNAs were made using a primer for first strand synthesis located within the second exon of *nad3* (Nr. 09, Table 5-1). From these cDNAs, a fragment was amplified spanning *nad2* and *nad3*, but lacking *nad3*-i1 thus confirming that these two genes are cotranscribed as may be expected from a mitochondrial RNA species. Using a semi-quantitative approach (by using various combinations of the amount of template and the number of reaction cycles in the RT-PCRs and a densitometric evaluation of the corresponding Southern blots that were made), we roughly estimated that the amount of RNA containing *nad2* and *nad3* was 10 to 20-fold reduced in *Wa32LL*. The expression levels of *atp8* and *cytb* on the other hand appeared not or hardly affected.

We also tested whether or not the integrated plasmid was perhaps removed at the RNA level. This was not the case. CDNAs were made using primers for first strand synthesis located either within *nad2* or within *nad3* (Nrs. 07 and 09 respectively, Table 5-1), and in each case the 3' plasmid/mtDNA recombination junction could be amplified (using

combinations of primers nr. 02 and 07 or nr. 02 and 09 respectively, Table 5-1). CDNAs were additionally made using a primer located within the TIR of the plasmid (Nr. 02, Table 5-1), and from these also the 5' plasmid/mtDNA junction could be amplified (using primers nr. 02 and 03, Table 5-1). The latter product spans the entire region upstream of the integrated plasmid copy including the *tRNA*^{Met} gene, thus in addition to its effect on *nad2* and *nad3* the integrated copy potentially affects the correct processing of tRNA^{Met}.

In short, the integrated plasmid found in *Wa32LL* thus negatively affects the expression of *nad2* and *nad3*. It is not removed from the RNA precursor and may, in addition to its effect on these two genes, interfere with the processing of the tRNA^{Met} situated upstream of the integration site. The latter has however not yet been tested.

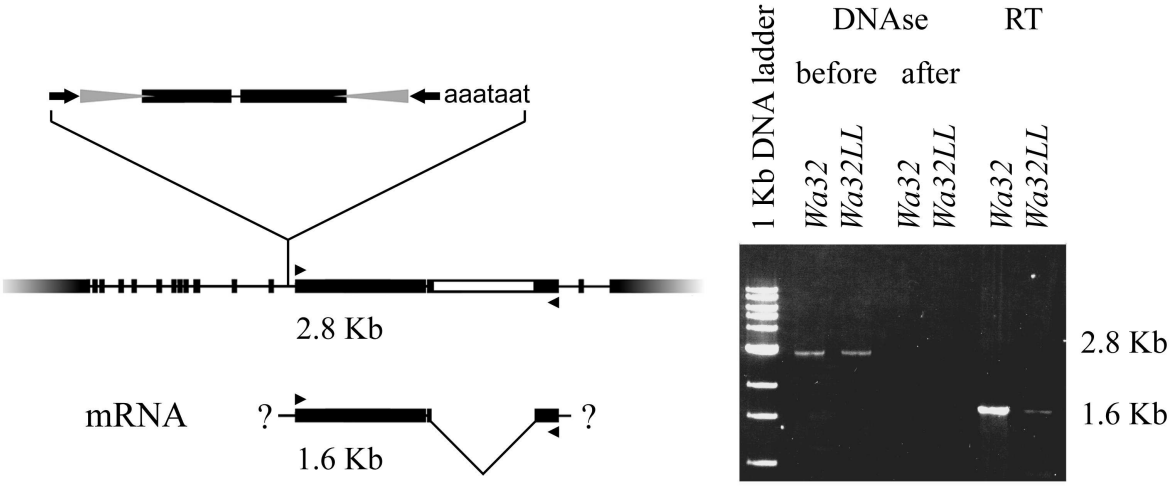

Figure 5-3. Despite the fact that mature transcripts carrying *nad2* and *nad3* initially appeared to be present only in Wa32, using RT-PCR they were found both in *Wa32* and in *Wa32LL*. A physical map of the region surrounding these genes is given (corresponding to that in 5-2b), indicating the location of the primers and the expected products (left). An example of the RT-PCR analysis is given (right). The expected 1.6 Kb product is present both in Wa32 and in Wa32LL (faint band).

Longevity is associated with the induction of alternative respiratory pathways

We measured the rate of oxygen consumption in isolates *Wa32* and *Wa32LL* according to the methods described by Dufour *et al. (*2000). To be able to evaluate the relative contributions of the different respiratory chain components in the two isolates, we tested the effects of several specific inhibitors: We used rotenone as an inhibitor of the proton-translocating NADH dehydrogenase (Complex I), cyanide (CN) as an inhibitor of cytochrome c oxidase (Complex IV), and salicylhydroxamic acid (SHAM) as an inhibitor of the alternative oxidase (AOXp). Inhibitors of the alternative NADH dehydrogenases have not yet been described.

Compared to *Wa32* protoplasts, *Wa32LL* protoplasts showed an increased level of oxygen consumption (Figure 5-4). Oxygen consumption in Wa32LL was relatively less sensitive to rotenone than in the wild-type, suggesting a malfunction in Complex I, consistent with the RNA analysis. However, in absolute terms the amount of rotenone sensitive respiration was not at all significantly reduced, and Complex I may thus be fully intact in the long-lived isolate and present at a level comparable or possibly even higher than that in *Wa32*.

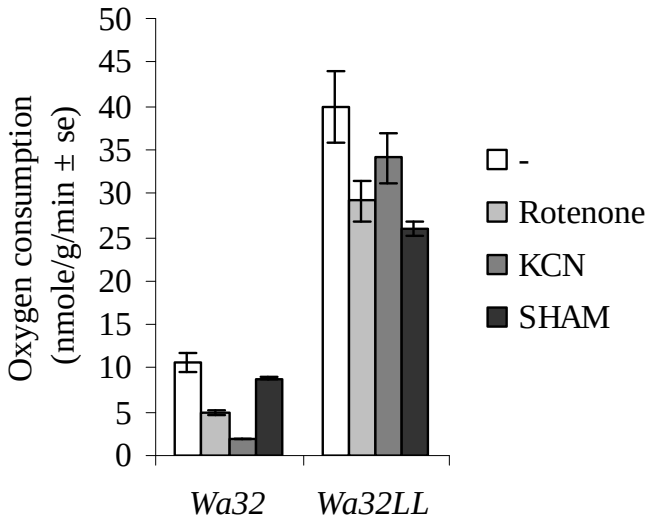

Figure 5-4. Oxygen consumption in *Wa32* and *Wa32LL* protoplast suspensions. Specific inhibitors were used to assess the contributions of the different respiratory chain components: Rotenone specifically inhibits the mitochondrial NADH dehydrogenase (Complex I), KCN specifically inhibits cytochrome c oxidase (Complex IV), and SHAM specifically inhibits AOXp.

Preliminary results obtained with blue-native polyacrylamide gel electrophoresis (BN-PAGE) and subsequent colorimetric staining seemed to confirm that there was no difference between the NADH dehydrogenase activity of Complex I in the two isolates (data not shown). Thus, despite its potential effect on the *nad2* and *nad3* subunits, the insertion of the pAL2-1 homologue did not appear to affect NADH dehydrogenase activity in *Wa32LL*. Nonetheless, the composition of the respirasome was affected. The relative and absolute capacity of the alternative rotenone-insensitive pathway appeared to be increased, indicating that *Wa32LL* relies more heavily either on the alternative mitochondrial NAD(P)H dehydrogenases than *Wa32* does, or that it relies more heavily on succinate:ubiquinone oxidoreductase or Complex II than *Wa32* to feed electrons into the transport chain.

Other modifications were observed downstream of coenzyme Q. In *Wa32*, oxygen consumption was highly sensitive to CN, showing that this isolate heavily depended on Complex IV as the terminal oxidase. In *Wa32LL* however, oxygen consumption was not as sensitive to CN, showing that in this isolate the alternative oxidase was induced. This was confirmed using Western analysis, which showed that AOXp was indeed present at a high level in *Wa32LL* (Western not shown). Oxygen consumption was not just relatively insensitive to CN in *Wa32LL* but also to SHAM (Figure 5-4), showing that in addition to the

alternative oxidase, a functional Complex IV was present in the isolate. This was consistent with preliminary experiments using blue-native polyacrylamide gel electrophoresis (BN-PAGE) and subsequent colorimetric staining (data not shown). Thus, unlike *Wa32*, *Wa32LL* possesses both terminal oxidase pathways. To what relative extent electron transport proceeds through either of these can not be concluded at this point. In any case, the combined capacity of the two terminal oxidase pathways tested individually was much larger than expected merely on the basis of total oxygen consumption, which indicates that the respiratory chain has an overcapacity in *Wa32LL* compared to that in *Wa32*

In short, both the relative and absolute contributions of complexes I and IV appear to be different in *Wa32LL* from these in *Wa32*. Unexpectedly, the mutation in *Wa32LL* appears to have effects throughout the respiratory chain, including the induction of alternative pathways that do not contribute to proton translocation. The latter may be explained by the fact that the different components of the respirasome are not assembled independently as discussed further on.

Longevity is associated with a reduced level of intracellular oxidative stress

The alternative respiratory pathway was previously suggested to lower mitochondrial ROS. Under at least some conditions it presumably causes a slight loss of respiratory control by mildly uncoupling electron transport from proton translocation across the inner mitochondrial membrane. Although its exact role in the senescence process is still unclear as also mentioned in the introduction section, all long-lived mutants nonetheless show the induction of this pathway. We thus tested whether the mitochondrial modifications observed in *Wa32LL* would be associated with a reduction in the cellular level of oxidative stress. This was done using a flow-cytometric approach according to Dufour *et al.* (2000). Protoplasts were incubated with the cell-permeant, non-fluorescent substrate 2’7’-dihydrodichlorofluorescein diacetate (H_2DCFDA), which is hydrolyzed intra-cellularly to form H_2DCF. The latter is a fluorogenic compound that reacts with intracellular hydrogen peroxide and other ROS yielding the intensely fluorescent compound 2’7’-dichlorofluorescein (DCF). The intensity of fluorescence in the incubated protoplasts was measured in intervals of 15 minutes. DCF accumulated significantly less fast in protoplasts of *Wa32LL* than in protoplasts of the wild type *Wa32* (Figure 5-5). The estimated level of intracellular oxidative stress found in *Wa32LL* was approximately a third of the original level, which is in the order of magnitude of that found in a previously described *cox5* deficient mutant of isolate *s* (Dufour *et al.* 2000, see figure 5-5).

We used an additional measure of the amount of oxidative stress employing nitro-blue tetrazolium (NBT), according to the methods described by Munkres (1990). The latter assay

gave similar results. Equally-sized mycelia of *Wa32* and *Wa32LL* were flooded with a solution containing NBT, a chromogenic probe that specifically reacts with superoxide, yielding an intensely dark-blue colored formazan deposit. Significantly less formazan was formed in mycelia of isolate *Wa32LL* than in mycelia of *Wa32* (Figure 5-5). Together these observations indicate that the level of oxidative stress is significantly reduced in *Wa32LL*.

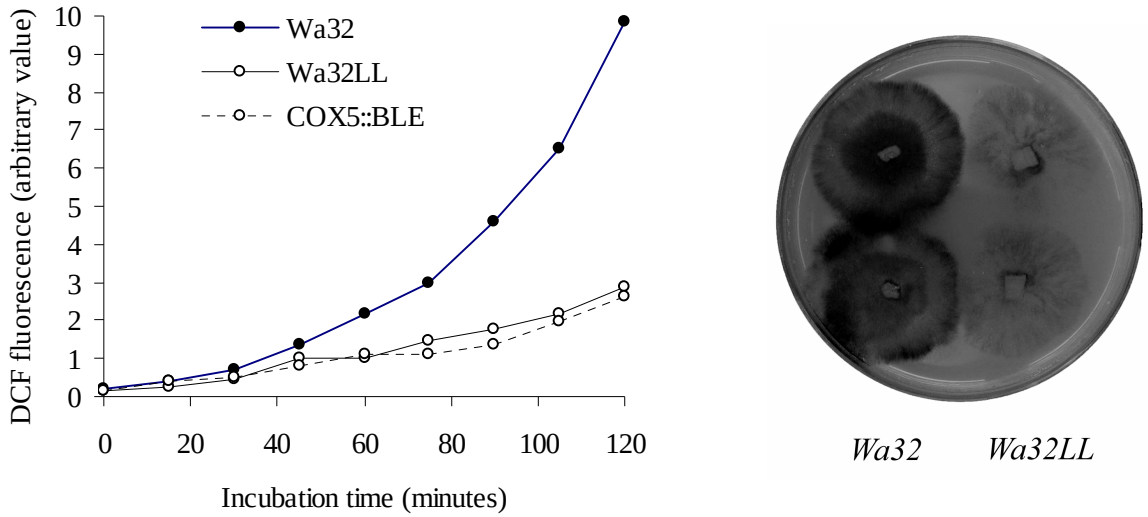

Figure 5-5. Estimates of the level of oxidative stress in isolates *Wa32* and *Wa32LL*. Flow-cytometric analysis using H_2DCF as a fluorogenic probe (left) and example of colorimetric staining using NBT (right). Protoplasts of isolates *Wa32* (● and solid line), *Wa32LL* (○ and solid line) or *cox5::BLE* (○ and dotted line) were incubated with H_2DCF-DA, and fluorescence was measured every 15 minutes. Values are averages of measurements of at least 10^4 cells and expressed in arbitrary units.

An overall stabilization of the mitochondrial chromosome in isolate Wa32LL

As shown in Figure 5-2, the pAL2-1 integration found in Wa32LL appears to prevent additional integration events of the free plasmid. Perhaps integrated copies confer some kind of immunity to the autonomously replicating element, as for example commonly known from prophages. It could be that pAL2-1 integration even exerts a more general effect on the overall stability of the entire mitochondrial chromosome, for example via a ROS or energy-level mediated signalling pathway. Therefore, we tested whether the integrated pAL2-1 homologue would attenuate also intron α, for example by preventing the formation of dsDNA circles. Intron α is present and expressed in Wa32LL (Northern data not shown), thus clearly this is a relevant question. As observed using Southern analysis, the senescing wild type readily accumulated senDNA α. In several cases region α was even entirely deleted and these

cases corresponded to the accumulation of other types of senDNA, including senDNA γ. Over a period of 100 days of growth, this was never observed in the long-lived isolate (Figure 5-6). Thus, the integrated plasmid copy found in *Wa32LL* appears to be associated with an overall stabilization of the mitochondrial genome, preventing both additional integration events of the free pAL2-1 homologue and the accumulation of different types of senDNA.

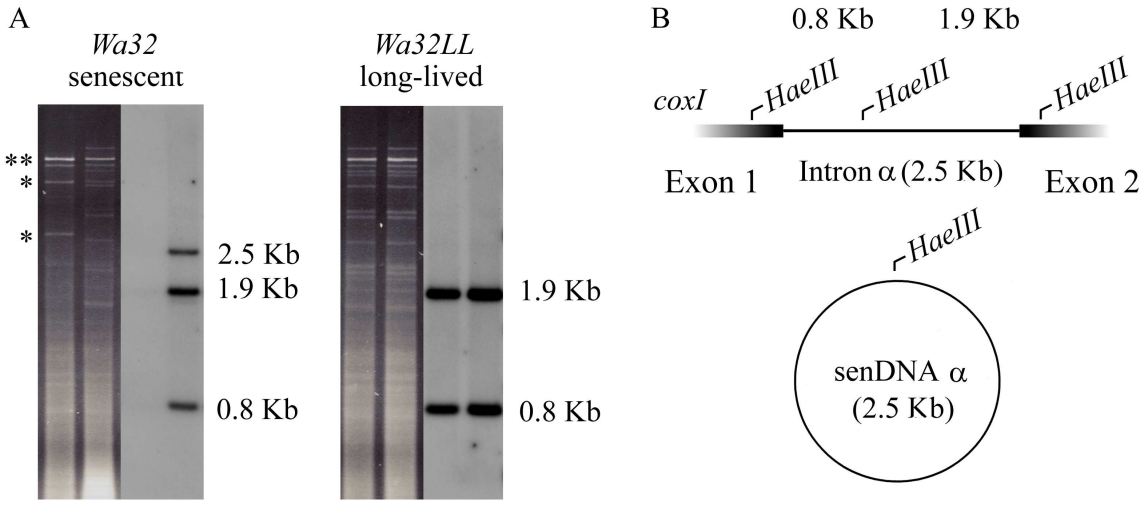

Figure 5-6. MtDNA stability in *Wa32* and *Wa32LL*. Ethidium bromide stained, *HaeIII* digested total DNA of senescent or late subcultures of *Wa32* and *Wa32LL* including the corresponding Southern blots probed with intron α (Left), and restriction map of mtDNA region α indicating *HaeIII* restriction sites (right). The 1.9 and 0.8 Kb fragments correspond to intron α, including parts of the flanking exons. The 2.5 Kb fragment corresponds to senDNA α. Asterisks indicate senDNA γ, effectively corresponding to a deletion of region α: The fragments in the utmost left lane cohybridize with a γ region specific probe, the *EcoRI*-1 fragment of the mitochondrial genome of strain *s* (not shown).

Discussion

Integration of pAL2-1 or its homologues is not necessarily a causal agent of longevity

The integration of pAL2-1 into the mitochondrial genome was also previously associated with longevity (Hermanns *et al*., 1994). This appears to be exceptional though, because none of the naturally occurring pAL2-1 homologues were previously associated either with integration or with longevity (van der Gaag *et al*., 1998). Under CR conditions the latter homologues, including the one found in isolate *Wa32*, even appear to shorten rather than increase life span (Maas *et al.*, 2004). Because in contrast to the prototypic plasmid its naturally occurring homologues initially all appeared to be present as an autonomously replicating element only

(Van der Gaag *et al.*, 1998), it was tempting to speculate on a causal relationship between integration and the induction of longevity. Here however, we have demonstrated that integrated copies can be found not only in long-lived but also in short-lived subcultures of Podospora. Clearly, integration does thus not lead to longevity per sé. It may be a conditional cause or otherwise perhaps an epi-phenomenon of mitochondrial malfunction. The negative effect of pAL2-1 on life span is normally overshadowed by the 'regular' aging process of Podospora and comes to light only under calorie restricted conditions. Under normal conditions thus only those cases will come to light, in which integration is associated with longevity, like in *AL2* or in *Wa32LL*.

In the related genus Neurospora, linear mitochondrial plasmids of the invertron-type also exist: pKALILO and pMARANHAR. Whereas Neurospora isolates normally do not senesce, those that carry either one of these plasmids do, and allegedly so because they integrate into the mitochondrial genome (Griffiths 1992, Bertrand, 2000), which at first sight appeared to be the exact opposite of the situation found in Podospora. All of the Neurospora cultures that carry one of the invertrons and senesce indeed show integrated plasmid copies, and because of this it was very tempting to speculate on a causal relationship. It must be said though, that this causal relationship was never formally proven: In addition to integrated plasmid copies, senescent cultures of Neurospora typically harbor vast quantities of autonomously replicating plasmid and the latter may equally well be the cause of death. Notably, we recently obtained natural isolates of Neurospora that carry pKALILO but do not senesce and some of these contain stably integrated or variant copies of pKALILO in addition to the autonomously replicating element (see chapters 2 and 3).

The effect of integration is ultimately thus unclear. Integration may have conditional effects depending for example on its timing, exact location, or even a combination of these and other factors. Integration could formally even be a secondary event, an indirect consequence of mitochondrial malfunction.

Respiratory modifications associated with plasmid integration and longevity in Wa32LL

The plasmid found in long-lived isolate *Wa32LL* had integrated adjacent to the *nad2* gene, a subunit of the membrane embedded subcomplex of the mitochondrial NADH dehydrogenase or respiratory complex I. Since the integrated plasmid copy was the only major genetic difference detected, a logical step was to investigate whether or not it would affect this complex. At the RNA level the integration did appear to interfere with Complex I. The NADH dehydrogenase activity of complex I however did not appear to be affected in preliminary BN-PAGE experiments. The NADH dehydrogenase activity however is a

function of the subcomplex extending into the mitochondrial matrix rather than of the membrane embedded subcomplex, and these two subcomplexes may function independently from one another as found in Neurospora (Alves and Videira, 1998). Hence, this does thus not necessarily exclude the possibility that *Wa32LL* suffers from a complex I malfunction. Remarkably, more pronounced effects were found elsewhere in the respiratory chain: Complex IV was negatively affected and AOXp strongly induced. This could be explained by an effect of the integrated copy on one or more of the tRNA genes situated upstream of the integration site. The latter would affect mitochondrial translation in general, with potential effects throughout the respiratory chain. Alternatively though, and perhaps more likely, the unexpected switch to alternative respiration may be explained by the fact that the different components of the respiratory chain are not assembled independently. They are organized in a respirasome. Krause *et al.* (2004) showed that although AOXp accepts electrons directly from quinol thus bypassing Complex III, the alternative pathway involves distinct super-complexes of I and III. They concluded that Complex III is necessary for the assembly and/or stability of Complex I. A single alteration, for example in Complex I, could thus have pleiotropic effects throughout the respiratory chain. *Wa32LL* could thus be a Complex I mutant with serious repercussions elsewhere in the respirasome. Formally, the unexpected complex IV deficiency could also be the consequence of another mutation in the mitochondrial genome. At this point, the latter can not be ruled out. As discussed earlier, integration itself is not strictly associated with the induction of longevity and is thus perhaps not the direct cause of longevity. Thus, although *Wa32LL* may effectively be a complex I mutant, complex interactions within the respiratory chain appear to obscure the effect.

Longevity is associated with a shift towards a faster but less efficient form of respiration

Interestingly, Wa32LL shows increased respiration. Although little emphasis has previously been placed on this effect, the same has actually been observed in other long-lived mutants, also there in combination with the induction of alternative respiratory pathways (for example in the mutants g*risea* and *ex*; Borghouts *et al.*, 2001, or *cox17::ble;* Stumpferl *et al*. 2004). Increased respiration may be an effect of partial respiratory uncoupling mediated by the alternative pathway, but the discrepancy between our results from the protein analysis, that was done using isolated mitochondria, and the respiratory analysis, that was done using whole cells, suggests that there may be more mitochondria or perhaps a larger surface of mitochondrial membrane in the long-lived mutant. Respiratory defects may be compensated for by up-regulating the amount or size of mitochondria and it would be interesting to do also cytological analyses of the various long-lived mutants. Notably, it is known also from the

facultatively aerobic yeast *Saccharomyces cerevisiae*, that life span extension via calorie restriction (CR) is correlated with a shift towards respiration (Lin *et al.*, 2002). CR requires the conservative use of resources. The long-lived mutants may mimic the effects of CR.

Conclusively, our data show that pAL2-1 associated longevity corresponds to a metabolic switch towards a potentially faster but less efficient form of cellular energy production. Presumably, the fungal invertrons are thus best seen as a form of potentially 'oncogenic' mitochondrial plasmids. Although our data indicate there may be no direct causal connection with integration into the mitochondrial genome, this remains to be elucidated.

Acknowledgements

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Chapter 6

Integration sites of the invertrons pKALILO and pAL2-1

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Abstract

Linear mitochondrial plasmids of the invertron type are known to integrate into the mitochondrial genomes of their hosts. Although this has been known for several decades, the exact mechanism by which they do so remains highly elusive. To gain more insight into the mechanism of integration, we characterized novel plasmid/mtDNA recombination junctions using semi-random two-step PCR. In many cases, deletions and/or additional insertions had occurred at the recombination junctions that were found. Plasmids were either completely integrated or missing several base pairs from their termini. In some cases, recombination junctions coincided exactly with the ends of known mitochondrial RNA species, indicating the involvement of a reverse transcription step. The mechanism of integration is therefore probably heterogeneous. Furthermore, integration sites were distributed in a non-random fashion. They were often found within or near one of the mitochondrial rRNA genes. A model is proposed to explain the structure and distribution of invertron integration sites.

Introduction

Mitochondrial plasmids abound in fungi (Griffiths, 1995). Though most of them appear to be relatively innocuous, in the laboratory several of them have severe effects on their fungal hosts. Two of these plasmids are the invertrons pKALILO and pMARANHAR that are found in species of the genus Neurospora. They are small linear molecules with long terminal inverted repeats (TIRs) that appear to encode only the basic molecular machinery that is needed for their own replication. Both pKALILO and pMARANHAR are associated with a senescence syndrome that is characterized by a progressive deterioration of the mycelium, followed by permanent growth arrest and death (Rieck *et al.*, 1982, Bertrand *et al*., 1985, Bertrand *et al.* 1986, Court and Bertrand, 1992). Over time, the plasmids often rise to extremely high titers and integrate into the mitochondrial genomes of their hosts. The invertrons thus act as insertional mutagens. For yet unknown reasons, the mtDNA molecules that carry an integrated plasmid copy gradually replace the wild-type molecules until the latter are hardly or no longer detected (Myers *et al*., 1989). Isolates that carry one of the invertrons

concomitantly show a plethora of mitochondrial aberrations (Rieck *et al.*, 1982). Features of senescent cells include extensive vacuolization, swelling and even lysis. Typical features of apoptosis though, such as cell shrinkage, chromatin condensation and nucleosomal DNA fragmentation have apparently not been observed (Bok *et al.*, 2003). This, along with the fact that it spreads infectiously (Debets *et al.*, 1994), indicates that 'invertron-based' senescence is not part of the normal developmental program but perhaps best seen as a systemic disease. The pAL2-1 homologous mitochondrial plasmids from *P. anserina* are associated with a similar senescence syndrome. This is however only observed under a dietary regimen of reduced food intake otherwise known as calorie restriction (CR) (Maas *et al.*, 2004). Under CR conditions Podospora isolates live several-fold longer than under regular dietary conditions, except for the isolates that carry one of the pAL2-1 homologous plasmids. These do not or hardly so. Like pKALILO and pMARANHAR, also pAL2-1 integrates into the mitochondrial genome. This was first observed in a long-lived strain (Hermanns *et al.*, 1994), but also occurs in senescent isolates of Podospora (see previous chapter).

The mode by which the plasmids integrate is unknown. Integration of pKALILO allegedly involves short sequence homology with the mtDNA target site: Sequences of mtDNA target sites matched that of a short region within the terminus of pKALILO (Bertrand, 1986; Bertrand and Griffiths, 1989), and pKALILO/mtDNA recombination junctions concomitantly involved short deletions. Integration of pAL2-1 on the other hand did not appear to involve sequence homology. At the pAL2-1/mtDNA recombination junction described by Hermanns *et al.* (1995), a short insertion was found rather than a deletion, suggesting a different mode of integration. There could be a plasmid- or host-specific mode of integration, or else it could be that secondary recombination events occasionally obscure the primary structure of an integration site. A typical feature of integration sites that is found both in Neurospora and in Podospora, is the presence of large inverted mtDNA repeats (Bertrand, 1986; Hermanns *et al.*, 1995). These were found using restriction fragment length polymorphism (RFLP) analysis and sequencing. Previous analyses indicated they may be extremely long. The way in which they are generated has however not been elucidated. They could for example result from intermolecular recombination events between integrated plasmid copies (Bertrand, 1986), or from events involving a single stranded (ss-)DNA replication intermediate (Griffiths, 1992).

The distribution of plasmid integration sites has never systematically been analyzed. Although pKALILO carrying isolates have been subject to extensive RFLP analysis (Bertrand, 1986; Myers *et al.*, 1989), exact sequence data of integrated plasmid copies is scant. In the first report that documented integrated copies of pKALILO, copies were found in the *HindIII*-15 and *HindIII*-20 fragments of the intron DNA of *rnl*, the gene encoding the

mitochondrial large subunit rRNA (Bertrand *et al.,* 1985). Sequencing showed that the copy found in the *HindIII*-15 fragment was located just upstream of *s-5,* the gene encoding the S5 mitochondrial ribosomal protein (Bertrand, 1986). The precise location of that in the *HindIII*-20 fragment was never determined, but this must have been somewhere within or downstream of *s-5*. Bertrand *et al.* (1985) therefore initially suggested that integration sites are preferentially located within the intron of the *rnl* gene. Subsequent RFLP analyses though indicated that they are probably not restricted to that location (Bertrand, 1986, Myers *et al*., 1989). Integrated copies of pAL2-1 were first reported from a long-lived derivative of standard laboratory strain *A* of *P. anserina* (Hermanns *et al.*, 1994). This long-lived strain carried at least two different copies of pAL2-1 that were stably integrated into the mitochondrial genome. One of these was located within the third intron of the mitochondrial *cob* gene. The second one was never characterized. Recently we found integrated copies of pAL2-1 homologue also in senescent Podospora isolates (see chapter 5). Senescent subcultures of *Wa32* for example contained integrated copies within *ORF P* and within the intergenic region between the $tRNA^{Met}$ and *nd2* genes. Subcultures of *Wa32LL,* (a long-lived derivative of *Wa32*) contained an integrated copy also within the latter intergenic region (also see previous chapter). As is probably also the case with pKALILO, the integration of pAL2-1 homologues is thus not site-specific. Whether or not it is completely random cannot be concluded from the limited amount of data thus far available.

To gain more insight into the mode of invertron integration, we used semi-random two-step PCR (ST-PCR; Chun *et al.,* 1997) and sequencing to characterize novel plasmid/mtDNA recombination junctions. In many cases, deletions and/or additional insertions had occurred at the junctions that were found. Plasmids were either completely integrated or missing several base pairs from their termini. In some cases, recombination junctions coincided exactly with the ends of known mitochondrial RNA species, indicating the involvement of a reverse transcription step. Notably, the latter cases coincided with suppression of the senescence phenotype (see chapter 3). Thus, the mode of integration is probably heterogeneous and perhaps even connected to the expression of senescence. Furthermore, integration sites were distributed in a non-random fashion. Both in Neurospora and in Podospora, most were located within or near one of the mitochondrial rRNA genes. A model is proposed to explain the structure and distribution of invertron integration sites.

Materials and methods

Strains and culturing conditions

Neurospora isolates used in the ST-PCR analysis were all pKALILO carrying isolates from a recent collection of Kauaian *N. intermedia* wild types (Maas *et al*., 2005, see chapter 2). Cultures were grown at 25 ˚C, on Vogel's Minimal Medium (VMM; Davis and DeSerres, 1970). *NI21.05* and *NI23.18* are short-lived wild types; *NI05.01* and *NI28.01* are long-lived wild types. Other Neurospora strains, including *NT39.03, P561* and *1766*, are mentioned in the text but were not used in the ST-PCR analysis.

P. anserina isolate *Wa30* was used in the ST-PCR analysis. The latter is a pAL2-1 homologue carrying isolate from a collection of Dutch wild types (van der Gaag *et al*., 1998). Cultures were grown at 27 ˚C, either on regular cornmeal medium or on medium with 100-fold reduced glucose content (Maas *et al*., 2004, see chapter 4) as indicated in the text. Other *P. anserina* strains, including *Wa32*, *Wa32LL* and *AL2*, are mentioned in the text but were not used in the ST-PCR analysis.

ST-PCR based analysis of integration sites

Cultures were, in the case of Neurospora, grown for approximately one day at 25 °C in liquid VMM or, in the case of Podospora, grown for two or three days at 27 °C on cellophane disks overlying solid cornmeal medium. Mycelium was harvested and ground using liquid nitrogen, followed by a phenol/chloroform based DNA extraction using standard procedures according to Sambrook *et al*. (1989).

The identification of plasmid integration sites was based on the semi-random two-step PCR (ST-PCR) procedure given by Chun *et al.* (1997), which is a way of amplifying unknown flanking sequences. First, a reaction was done using a PCR primer pointing outwards from within the TIR region of either pKALILO (5' GAA ATG ATA AAA AGA TCA CAA AGG G 3') or pAL2-1 (5' CCA AAG AAT TGA AAA TCG TCT CCC 3'), each in combination with the partially degenerate primer given in the original ST-PCR protocol (5' GGC CAC GCG TCG ACT AGT AC $N_{(10)}$ GAT AT 3'). The latter primer anneals at sites throughout the genome that contain the pentanucleotide sequence 5' ATATC 3'. This sequence is found approximately once every 300 bp, both in the mitochondrial genome of *N. crassa* and in that of *P. anserina*. It is distributed uniformly and the sampling of integration sites using this method was therefore probably unbiased (see results section). Next, a nested reaction was done using another primer pointing outwards from within the TIR region of

either pKALILO (5’ GGG TTA AAA TAG GAA CAA AAG GGG 3’) or pAL2-1 (5’ GGA GTG AGG GGG TGG GGG G 3’), each in combination with the other primer given in the original ST-PCR protocol, which comprises the specific part of the partially degenerate primer (5’ GGC CAC GCG TCG ACT AGT AC 3’). This second reaction allows a specific amplification of the plasmid/mtDNA recombination junction. Reaction conditions were exactly as described in the original protocol. Electrophoresis was done in 40 mM Tris-HCl, 20 mM acetic acid and 1 mM EDTA (TAE) pH 8.0 containing 1% (w/v) agarose, followed by visualization of the products using ethidium bromide (Sambrook *et al.*, 1989).

ST-PCR products were (gel-) purified using a High Pure PCR Product Purification Kit (Roche) and cloned in *E. coli* DH5-α using the pGEM®-T Easy Vector system (Promega). Plasmid DNA was recovered using a High Pure Plasmid Isolation Kit (Roche) and sequencing was done according to the methods described by Sanger (1977), using Big Dye™ terminators (Applied Biosystems). Sequences thus obtained were compared to the mitochondrial genome sequence of *N. crassa* (Corresponding to the mtDNA sequence of standard laboratory strain 74-OR23-1A as determined by the Neurospora Sequencing Project; Whitehead Institute/MIT Center for Genome Research) or that of *P. anserina* (Cummings *et al.*, 1990; Genbank database accession nr. X55026), using BLASTn version 2.2.9. (Altschul *et al.*, 1997).

Statistical analysis

Circular distribution data were analyzed using Rayleigh’s uniformity test and/or Rao’s spacing test with the software package Oriana version 2.00 (Kovach). Frequency data were analyzed using a Likelihood ratio test with the software package SPSS version 11.0 (SPSS inc.).

Results

Integration sites of pKALILO

PKALILO integrates into the mitochondrial genome of Neurospora. Several integration sites have previously been described. The first ones were found only within the intron of the *rnl* gene (Bertrand *et al.,* 1985), but subsequent RFLP analyses showed that they are probably not restricted to that location (Bertrand, 1986, Myers *et al.,* 1989). Using an adapted ST-PCR procedure (Chun *et al.,* 1997), we identified several novel integration sites. The plasmid flanking sequences were amplified, cloned, sequenced and compared with the known mitochondrial genome sequence of *N. crassa* (Corresponding to the mtDNA sequence of

standard laboratory strain 74-OR23-1A as determined by the Neurospora Sequencing Project; Whitehead Institute/MIT Center for Genome Research). A summary of the sites thus and previously found is given in Table 6-1a. Integrated copies of pKALILO were found in a range of locations throughout the mitochondrial genome. Several though not all were located within or close to the *rnl* and *rns* genes that encode the mitochondrial rRNAs. Notably the ones from isolates *NI05.01* and *NI28.01*, that did not show any signs of senescence (Maas *et al.*, 2005), were located elsewhere. Due to the low number of insertion sites characterized no calculations were made, but together with the previously collected RFLP data, the data are highly suggestive of a bias in the distribution of integration sites (see also Figure 6-1). As discussed further on, a similar bias was found in Podospora. ST-PCR relies on the use of a semi-random primer that could have influenced the results. We thus screened the mitochondrial genome for potential annealing sites of the latter primer (*i.e.* the sequence 5' ATATC 3'). Annealing sites are found approximately once every 300 bps and are distributed uniformly over the mitochondrial genome (Rayleigh's test for circular uniformity, Z=0.644, df=1, P=0.525 when taking all possible priming sites into consideration; Z=1.546, df=1, P=0.213 when taking only forward priming sites into consideration; Z= 1.501, df=1, P=0.223 when taking only reverse priming sites into consideration. Similar results were obtained using Rao's spacing test). Our analysis was therefore unbiased with regard to the method of sampling. Forward priming sites were nonetheless slightly more abundant (117 forward versus 83 reverse priming sites, a marginally significant difference from the expected 1:1 ratio, Likelihood ratio test, G=5.81, df=1, P=0.016) and our methods could have positively biased the relative number of 5' plasmid/mtDNA recombination junctions found. All (six out of six) plasmid/mtDNA recombination junctions that were found using ST-PCR however corresponded to 3' plasmid/mtDNA junctions (Table 6-1a).

There were several cases in which the plasmids appeared to have recombined with the mitochondrial genome via a form of homologous recombination: Some integrated copies of pKALILO were incomplete and in two cases these showed short sequence homology with the mitochondrial genome (Table 6-1a, numbers 1 and 2). One of the junctions found in strain *NI21.05* coincided exactly with a so-called *PstI* palindrome (Table 6-1a, number 1). The *PstI* palindromes are members of the GC-rich family of repeats and are found at recombination junctions also in mtDNA deletion mutants (Almasan and Mishra, 1991). They typically consist of two closely spaced *PstI* sites (5' C TGC AGT ACT GCA G 3') and are flanked by clusters of C's or G's on each side (Yin *et al.*, 1981).

Not all integrated plasmid copies were incomplete. The ones in *NI05.01* and *NI28.01* were complete. This can probably be ascribed to the template switching activity of a reverse transcriptase (RT) involved in their formation. This more elaborately discussed in chapter 3.

Table 6-1. Known and novel integration sites of the mitochondrial invertrons pKALILO and pAL2-1. Sequences are given in the left column, with parts derived from the plasmids indicated in grey, and regions of homology between the plasmids and the mitochondrial genomes of their respective hosts in dark grey. References to earlier work are indicated in the table. The phenotype of each strain is indicated, which is either short-lived (SL) or long-lived (LL) (*i.e.* signs of senescence have been observed or not). (**A**) Integration sites of pKALILO and homologues in the mitochondrial genome of Neurospora. The first five are novel; the other twelve were already known from previous analyses. The first seven were found using ST-PCR and sequencing; the other sites were all deduced from RFLP patterns and hybridization experiments. Number nine was sequenced, though the exact sequence data has not been made publicly available (Bertrand, 1986). Where possible, the exact locations of the plasmid/mtDNA recombination junctions are given; numbers correspond to that of nucleotides within the known ~ 65 Kb mitochondrial genome sequence of standard laboratory strain 74-OR23-1A of *N. crassa* (As determined by the Neurospora Sequencing Project; Whitehead Institute/MIT Center for Genome Research). It must be noted that all sequences were from isolates of either *N. intermedia* (*NI21.05, NI23.18, NI05.01, NI28.01, P561* and $f_1^{P561 \cdot 1766}$) or *N. tetrasperma* (*NT39.03*) and not from isolates of *N. crassa*. The mitochondrial genome sequence of *N. crassa* was used though, because the full mitochondrial genome sequences of the other two species have not yet been determined. All Neurospora strains were wild-type isolates from the Kauaian population except the ones analyzed by Myers *et al.* (1989). The latter (indicated as $f_1^{P561 \cdot 1766}$) were derived from a cross between the Kauaian *N. intermedia* wild-type isolate *P561* as a female and the plasmid free Taiwanese *N. intermedia* wild-type isolate *1766* as a male. (**B)** Integration sites of pAL2-1 and homologues in the mitochondrial genome of Podospora. The first eleven are novel; the other four were already known from previous analyses (Hermanns et al., 1992; also see previous chapter). The first eleven were found using ST-PCR and sequencing; the other sites were found using RFLP and sequencing. Where possible, again the exact locations of the plasmid/mtDNA recombination junctions are given; numbers correspond to that of nucleotides within the known ~100 Kb mitochondrial genome sequence of standard laboratory strain *A* of *P. anserina* (Cummings et al., 1990; GenBank database accession nr. X55026). All Podospora strains are wild-type isolates from the Dutch population of *P. anserina*, except *Wa32LL and AL2.* The latter two are long-lived derivatives of *Wa32* (see previous chapter) and *A* (Osiewacz *et al.*, 1989) respectively.

6-1a. Integration sites of pKALILO and homologues in the mitochondrial genome of Neurospora

#	Sequence (5'→ 3')	5'/3'	Location		Strain	Phenotype	Reference
	TGCCCCTTAC AC		pKALILO terminus				
01	TGCCCCTGCA GTACTGCAGG	3'	between *cox2* and *tRNA*^{Met}	61,282-89	*NI21.05*	SL	this work
02	TGCCCCCGGT TAATGGCGGC	3'	in *rnl*-e1	13,250-55	*NI23.18*	SL	this work
03	TGCCCCTTAC ACACTTACCG	3'	between *nad5* and *cob*	32,735/36	*NI05.01*	LL	this work
04	TGCCCCTTAC ACTTCTCATT	3'	exact 5' border of *tRNA*^{Arg}	43,360/61	*NI28.01*	LL	this work
05	TGCCCCTTAC ATAAATATTT	3'	in novel retroelement	-	*NI28.01*	LL	this work
06	TGCCCCTTAC CGGGGGATCG	3'	in *rnl*-e1	12,244/45	*NI21.05*	SL	Maas *et al.*, 2005
07	TGCCCCTTAC TTAACGGATT	3'	in *rnl*-e1	12,501/02	*NT39.03*	SL	Maas *et al.*, 2005
08	(deduced from RFLP data)	-	in *rnl*-i1	-	*P561*	SL	Bertrand *et al*, 1985
09	sequence data unavailable	3'	in *rnl*-i1, 5' of *S-5*	14,427/28	*P561*	SL	Bertrand, 1986
10	(deduced from RFLP data)	-	in *rnl*-e1	-	*P561*	SL	Bertrand, 1986
11	(deduced from RFLP data)	-	in *urf5* (*nad5)*	-	*P561*	SL	Bertrand, 1986
12	(deduced from RFLP data)	-	between *cox2* and *rns* (two copies)	-	*unknown*	SL	Bertrand, 1986
13	(deduced from RFLP data)	-	in *rnl*-e2	-	$f_1^{P561 \cdot 1766}$	SL	Myers *et al.*, 1989
14	(deduced from RFLP data)	-	in *rnl*-i1 (two copies)	-	$f_1^{P561 \cdot 1766}$	SL	Myers *et al.*, 1989
15	(deduced from RFLP data)	-	between *cox3* and *rnl* (three copies)	-	$f_1^{P561 \cdot 1766}$	SL	Myers *et al.*, 1989
16	(deduced from RFLP data)	-	between *cox2* and *rns* (two copies)	-	$f_1^{P561 \cdot 1766}$	SL	Myers *et al.*, 1989
17	(deduced from RFLP data)	-	upstream of *oli2* (*atp6)* (two copies)	-	$f_1^{P561 \cdot 1766}$	SL	Myers *et al.*, 1989

6-1b. Integration sites of pAL2-1 and homologues in the mitochondrial genome of Podospora

#	Sequence (5'→ 3')	5'/3'	Location		Strain	Phenotype	Reference
	TACGTCTCTC TC		pAL2-1 terminus				
01	TACGTATCGG GCCAACCATA	3	in *LrRNA-i1* (within maturase)	3,919-21	*Wa30*	SL	this work
02	TACGTATCAC AGTACAAAGA	5	in *ORF P*	28,808-12	*Wa30*	SL	this work
03	TACGTATCTC GACTATGTTG	5	in *ND1-i4* (within maturase)	98,017-25	*Wa30*	SL	this work
04	TACGTATCTC AACAAAAAAA	3	between $tRNA^{Cys}$ and *COI*	38,434-42	*Wa30*	SL	this work
05	TACGTATCTC TAGGATCTAA	5	in *ND3-i1* (within maturase)	14,362-67	*Wa30*	SL	this work
06	TACGTATCTC TCATTTTTTC	5	in *Cytb-i1* (within maturase)	32,164-70	*Wa30*	SL	this work
07	TACGTATCTC TCATTCCTTT	5	between *ORF C* and *ATP6*	22,656-60	*Wa30*	SL	this work
08	TACGTATCTC TAGTGCGTTA	5	between *ORF C* and *ATP6*	22,979/80	*Wa30*	SL	this work
09	TACGTATCTC TAAGCTCCAC	3	between *ORF C* and *ATP6*	23,009/10	*Wa30*	SL	this work
10	TACGTATCTC TCAAGTACTA	5	in pAL2-1, in *DdRP*	3,021/22	*Wa30*	SL	this work
11	TACGTATCTC TCGGTAAATA	3	in pAL2-1, in *DdRp*	2,599/00	*Wa30*	SL	this work
12	TACGTATCTC TGGCACAAAG	5	in *ORF P*	28805/6	*Wa32*	SL	see chapter 6
13	TACGTATCTC TGTTGATTGG	3	between $tRNA^{Met}$ and *ND2*	11,337-9	*Wa32*	SL	see chapter 6
14	TACGTATCTC TGGCTAATAA	5	between $tRNA^{Met}$ and *ND2*	11,555-7	*Wa32LL*	LL	see chapter 6
15	TACGTCTCTt tatatatttt	5	in *Cytb-i3* (5'of maturase)	34674/5	*AL2*	LL	Hermans *et al.* (1992)

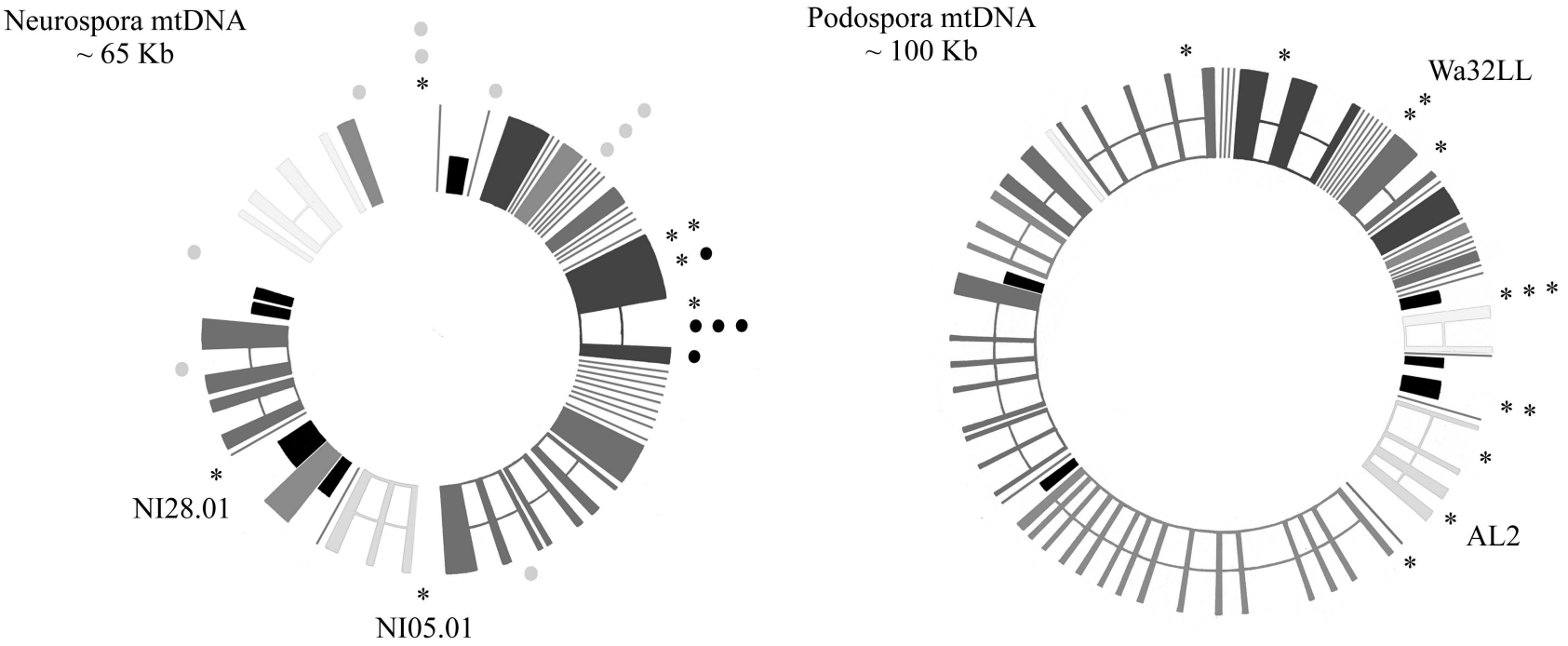

Figure 6-1. Physical maps of the mitochondrial genomes of Neurospora (left) and Podospora (right), indicating the distribution of known and newly characterized invertron integration sites (Table 6-1). Their respective topologies were based on the mtDNA sequences of *N. crassa* standard laboratory strain 74-OR23-1A (As determined by the Neurospora Sequencing Project; Whitehead Institute/MIT Center for Genome Research) and *P. anserina* strain *s/A* (Cummings *et al.*, 1990). The twelve o' clock positions of the maps correspond to the start of the traditional circular map of these sequences (Kennel *et al.*, 2005; Cummings *et al.*, 1990). Asterisks indicate sites that were found using ST-PCR or RFLP and sequencing. Closed circles indicate approximate locations that were deduced from RFLP patterns (see table 6-1). Sites associated with a long-lived phenotype are indicated with the names of the corresponding strains. Large filled boxes indicate exons and small open boxes indicate introns. The mitochondrial rRNAs and most of the mitochondrial tRNAs are encoded in the upper right quadrants.

PAL2-1 integration sites

Like pKALILO, also pAL2-1 integrates into the mitochondrial genome. The plasmid was first discovered in a long-lived derivative of *P. anserina* strain *A* (Osiewacz *et al.,* 1989). The latter strain carried two integrated copies of pAL2-1, one of which was located in the third intron of the mitochondrial *cob* gene (Hermanns *et al*., 1995; Hermanns *et al*., 1994; Osiewacz *et al.,* 1989). Hermanns and Osiewacz (1996) surmised that the longevity trait resulted from the integration of pAL2-1, but integrated pAL2-1 homologues can also be found in senescent isolates of *P. anserina* (see previous chapter) and are therefore not necessarily causal agents of longevity. As was done for pKALILO, we used an adapted ST-PCR procedure to identify novel sites of integration. The plasmid flanking sequences were amplified, cloned, sequenced and compared with the known mitochondrial genome sequence of *P. anserina.* This way, eleven novel sites were identified: Nine of these were located within the mitochondrial genome (Cummings *et al.,* 1990, GenBank accession number X55026); two were located within the gene that encodes the DNA dependent RNA polymerase (*DdRp*) of the plasmid itself (GenBank database accession nr. X60707; Hermanns and Osiewacz, 1992). A summary is given in Table 6-1b. Integrated copies of pAL2-1 were found in various locations throughout the mitochondrial genome, again frequently within or close to the genes that encode the mitochondrial rRNAs. Their distribution appeared to be biased (Rayleigh's test of circular uniformity; $Z=4.832$, df=1, $P=0.005$ when taking ST-PCR data only into account; $Z=7.699$, df=1, $P<0.001$ when taking all data into account. Similar results were obtained using Rao's spacing test.). Again we tested for a bias that could have resulted from the use of ST-PCR. We screened the mitochondrial genome of *P. anserina* (according to Cummings *et al.*, 1990, GenBank accession number X55026) for potential annealing sites of the semi-random primer (*i.e.* the pentanucleotide sequence 5' ATATC 3'). Like in the Neurospora mitochondrial genome, also in the *P. anserina* mitochondrial genome these occur approximately once every 300 base pairs and are distributed uniformly (Rayleigh's test for circular uniformity, $Z=1.283$, df=1, $P=0.277$ when taking all possible sites into consideration; $Z=4.458$, df=1, $P=0.012$ when taking only the forward priming sites into consideration; $Z=0.706$, df=1, $P=0.494$ when taking only the reverse priming sites into consideration. Similar results were obtained using Rao's spacing test). Again forward priming sites were more abundant (210 versus 147 reverse priming sites, a significant deviation from the expected 1:1 ratio, likelihood ratio $G=11.18$, df=1, $P=0.001$), which could have resulted in a bias towards 5' recombination junctions. This may have been the case, though again a larger sample size would be required to address this matter. Most of the integration sites corresponded to sites within introns or intergenic regions. This also appeared to be the case

for integration sites of pKALILO (see Table 6-1a) but does not necessarily reflect a biologically significant bias, given that in both species most of the mitochondrial genome consists of introns and intergenic regions. A larger sample size would be required to address this.

As was the case in Neurospora, the termini of integrated copies were often incomplete and/or showed homology with the mitochondrial genome. Formally, the sequence length variation may reflect natural variation of the autonomously replicating element. However, since the first eleven sequences (Table 6-1b) were all derived from one and the same isolate (Wa30) yet show heterogeneous termini, this is quite unlikely. As was the case in Neurospora, termini of some integrated plasmid copies were complete. There was no clear indication that this resulted from a process involving RT activity as in Neurospora. However, the latter cannot be excluded.

In short, integrated plasmid copies appear to be distributed in a region-specific manner. Both in Neurospora and in Podospora they are frequently found in the region encoding the mitochondrial rRNAs (Table 6-1, Figure 6-1). Often they show short deletions of their termini and short homology with the mitochondrial sequence. Formally, this may have resulted from secondary recombination events. Some plasmid/mtDNA recombination junctions found in Neurospora appeared to have resulted from recombination via an RNA intermediate and the mode of integration is thus probably heterogeneous.

Structural organization of integration sites

RFLP analyses previously indicated that integrated invertron copies are flanked by inverted repeats of mtDNA (Bertrand, 1986; Hermanns *et al.,* 1995). In our analysis, ST-PCR often gave only one product and this may well reflect the presence of such inverted repeats. We analyzed the mtDNA sequences on either side of one of the integrated copies found in the mitochondrial genome of *P. anserina* strain *Wa30* (Table 6-1b, number 3) and compared these to the wild-type mtDNA sequence from a young culture of the same strain. Short duplications were found. These occurred in an inverted position as well as in the regular orientation (see Figure 6-2). The duplications were not already present in young cultures, indicating that they came about *de novo*. In most other cases the corresponding 5' or 3' recombination junctions could not be found, which could mean that the majority of the integrated plasmid copies are flanked by longer inverted repeats. From RFLP data Bertrand *et al.* (1986) concluded that the inverted repeats often exceed several Kbs in size, which is consistent with this observation. The organization of the integration site of the plasmid found in *Wa30* was similar to that in *Wa32LL* and thus does probably not relate to the phenotype (*i.e.* short- or long-lived).

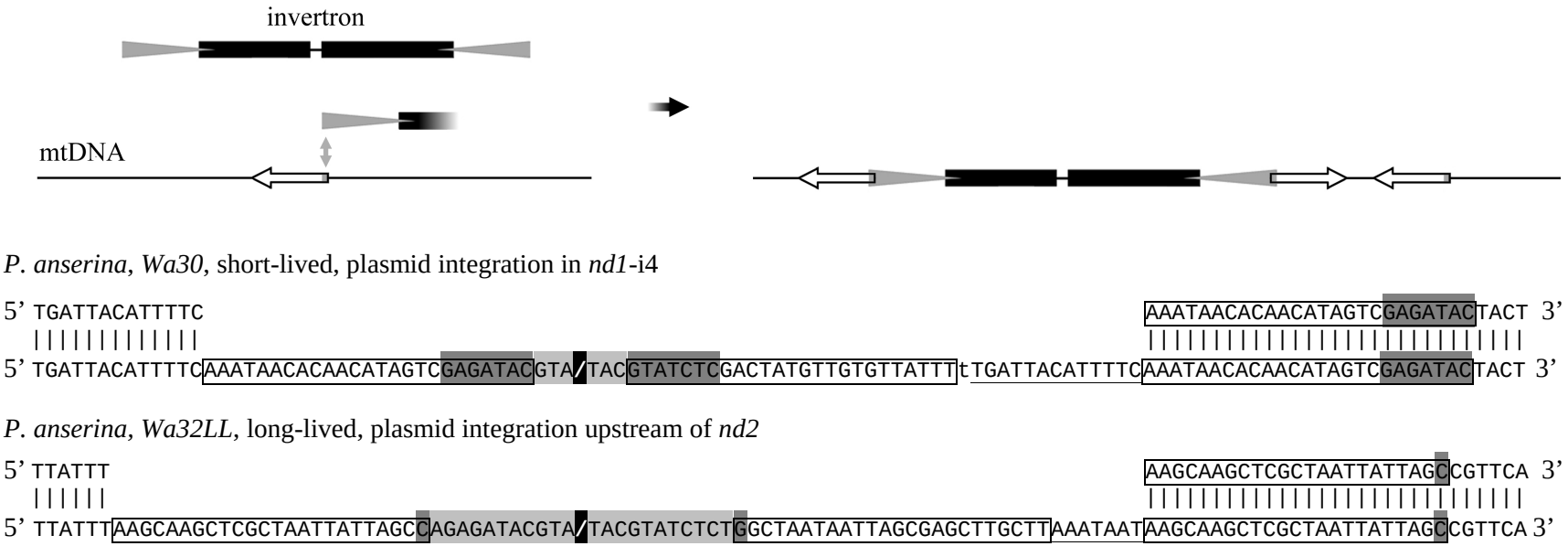

Figure 6-2. Plasmid integration results in mtDNA duplications. A schematic representation (top) is given of the structural organization before and after integration. In addition, two sequence alignments (bottom) are given of plasmid flanking sequences from *P. anserina* strains *Wa30* and *Wa32LL* with the wild-type mtDNA regions from juvenile cultures of the corresponding strains (in the case of *Wa32LL* with that from a juvenile culture of its ancestor *Wa32*). The 5' (left) and 3' (right) plasmid and plasmid flanking mtDNA sequences are separated by a forward slash (black). Note that multiple sequence alignments are possible and this is one interpretation. As in table 6-1, plasmid sequences are indicated in grey, with homology to the mitochondrial sequences in dark grey. Duplications are boxed. The inverted duplication downstream of the integrated plasmid in Wa30 was linked to the rest of the sequence by an additional base pair (lowercase lettering). In both cases, either the direct or inverted downstream duplication is actually slightly larger than indicated using boxed text (underlined).

Discussion

To gain more insight into the mode of invertron integration, we used ST-PCR (Chun *et al.*, 1997) and sequencing to characterize novel plasmid/mtDNA recombination junctions from Neurospora and Podospora isolates, respectively carrying pKALILO or pAL2-1. As discussed below, both in the case of pKALILO and pAL2-1, the integration process was heterogeneous in nature and seemingly region-specific. Integration is probably best seen as a mistake, perhaps resulting from a form of erroneous mtDNA repair.

The mode of integration of mitochondrial invertrons

Bertrand and Griffiths (1989) proposed that the integration of pKALILO involves short sequence homology with the mtDNA target site. This was based on an unpublished observation that the sequences of the target sites matched that of a short region within the terminus of the plasmid. Integrated copies of pKALILO concomitantly lacked several base pairs from their termini. In contrast however, the integration of pAL2-1 did not appear to involve sequence homology with the mtDNA target site: At the pAL2-1/mtDNA recombination junction described by Hermanns *et al.* (1992) a short insertion was found, suggesting a different mode of integration. The question thus remained whether the short sequence homology model of integration could be generalized to Podospora. There could be several different modes of integration. It could also be that the structure of integration sites is occasionally obscured by secondary recombination events.

In our analysis, most recombination junctions could be explained with the short sequence homology model of integration. Both in Neurospora and in Podospora, there were cases in which the sequence of the target site matched that of the corresponding plasmid. Integrated plasmid copies often lacked several base pairs from their termini. This was however not always the case. In some cases integrated copies were complete, which shows that integration does not necessarily have to involve deletions. In the *N. intermedia* strains *NI28.01* and *NI05.01* integration appeared to have involved a RNA intermediate. This is more elaborately discussed in chapter 3. Notably in these two isolates the senescence phenotype of pKALILO was suppressed. Thus, the mode of integration is heterogeneous and perhaps even connected to the expression of senescence. Though the incomplete termini could formally also be explained by existing variation in the autonomously replicating elements (note that only the prototypic plasmids have thus far entirely been sequenced), this is highly unlikely given that the termini of integrated copies varied even within single isolates. The incomplete termini must therefore have resulted from *de novo* deletion events. They may have resulted directly

from the integration process, or from a secondary event. Notably one of the recombination junctions in Neurospora coincided with a so-called *PstI*-palindrome, an element that was associated with recombination junctions also in mtDNA deletion mutants (Almasan and Mishra, 1991). Cahan and Kennel (2005) recently showed that the presence of these *PstI*-palindromes is inversely correlated with that of plasmid-related sequences in the mitochondrial genome, suggesting that they serve a role in eliminating these and similar foreign DNA sequences. These observations together thus raise the question whether the incomplete termini result directly from the integration process or from a secondary recombination event. It would be interesting to study all the potential changes that occur in recombination junctions during the senescence process rather than just the outcome of the entire process.

The distribution of invertron integration sites

The distribution of insertion sites was neither site-specific nor completely random, but appeared to be region-specific as initially suggested by Bertrand *et al.* (1985) for pKALILO. Both in Neurospora and in Podospora, most of the insertion sites were located within the region containing important components of the mitochondrial translational apparatus including the mitochondrial rRNAs and most of the mitochondrial tRNAs. This bias could be explained by a bias in the integration process itself. The mtDNA region affected could for example be more susceptible to recombination in general. It may be more heavily transcribed and thus more accessible to integration. Viral integration is sensitive to the transcriptional state of the targeted locus (reviewed by Sandmeyer et al., 1990) and this is also the case for linear plasmid DNA (Srikantha *et al.*, 1995). The biased distribution could formally also be explained by intracellular selection. MtDNA molecules carrying an insert in the affected region could have a replicative advantage over molecules with an insert elsewhere. It may reflect the progressive deletion of the mitochondrial genome around an origin of replication situated near the rRNA loci.

In short, the mode of integration is heterogeneous and insertion sites appear to be distributed in a region-specific way. Also because there is no specific integration function known to be associated with the plasmids, integration is probably best viewed as an error. This was also suggested by Griffiths (1992). It could be that the termini of the plasmids are erroneously recognized as dsDNA breaks. This would occur with a higher chance in senescent tissue because plasmid titers typically increase during the senescence process. Although for a long time it was considered to be non-existent, mtDNA repair is well established nowadays. MtDNA repair mechanisms include base excision, mismatch as well as recombinational repair

mechanisms (reviewed by Croteau *et al.*, 1999). Plasmid integration could thus result from erroneous recombinational mtDNA repair. A similar process is known for example from hepaDNAviruses (Bill and Summers, 2004): Integration of hepaDNAviruses depends on the production of linear viral dsDNA and occurs at dsDNA breaks, presumably via non homologous end-joining (NHEJ). Notably, in the latter system, as was the case at the plasmid/mtDNA recombination junctions we found, short deletions or insertions were observed at the viral/host DNA recombination junctions. It would be interesting to test more directly whether the induction of dsDNA breaks affects the mode of action of the mitochondrial invertrons.

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Chapter 7
Summarizing discussion and conclusion

Fungal senescence is a heterogenous phenomenon

The findings presented in this thesis allow us to make a distinction between several fungal senescence phenomena that have by some authors been classified as one. The senescence phenomenon in Podospora as originally described by Rizet is clearly different from that in Neurospora. The latter, pKALILO based senescence, in fact proved to be analogous to pAL2-1 based senescence, a phenomenon previously overlooked because it is in the shadow of the regular senescence syndrome of Podospora as shown in chapter 4. The senescence phenomenon in Podospora is multicausal, whereas invertron based senescence in Neurospora obviously is not, which in fact would have been a very good argument *a priori* for it not to be called senescence. According to its evolutionary definition that was stated in the introduction, aging is expected to be multifactorial because it results from the accumulation of traits with deleterious effect late in life. Perhaps even more important, the invertrons are not intrinsic causes of death. They are highly infectious even among incompatible natural isolates, and thus probably best seen as an infectious disease.

In **Chapter 2**, the frequency and effect of pKALILO in the heterothallic, outcrossing species *N. intermedia* were compared to those of the pseudohomothallic, selffertilizing species *N. tetrasperma*. Selffertilization would generally cause the interests of host and intracellular parasite to align. Results were in line with this: The frequency and virulence of pKALILO were lower in *N. tetrasperma* than in *N. intermedia*. However, the frequency of the allegedly phenotypically neutral plasmid pHANALEI-2 was also lower in *N. tetrasperma*, thus drawing into doubt either the phenotypic neutrality of pHANALEI-2 or the fact that the frequency of pKALILO has anything to do with its virulence.

In **Chapter 3**, a novel mitochondrial retro-element was described that was found in long-lived isolates of *N. intermedia*. One intruiging possibility is that the elements interfere with each other in a similar way as DI viral particles, thus cancelling out each others phenotypic effects. In fact, thus far no two senescence plasmids have been found in one and the same strain despite their relatively high frequencies in the population. This could point for example at the existence of incompatibility groups in mitochondrial plasmids. It would be interesting to try and combine different senescence plasmids in one cytoplasm to test this hypothesis.

In **Chapter 4,** the effects of calorie restriction on senescence in Podospora were investigated. Calorie restriction could in some strains extend life span by a factor five, which is more than is usually observed in unitary organisms. The main cause of variation in the response to calorie restriction was the mitochondrial plasmid pAL2-1. This plasmid was originally believed to induce longevity, but is in fact associated with a senescence syndrome under calorie restricted conditions similar to that associated with the structurally similar element pKALILO from Neurospora.

In **Chapter 5**, a novel spontaneous longevity mutant of *P. anserina* was characterized. Unlike most previously described longevity mutants, the strain was not completely female sterile, though together with the other existing mutants building a strong case for antagonistic pleiotropy theory of aging. The mutant had severe alterations throughout the respiratory chain, potentially mildly uncoupling oxidative phosphorylation from electron transport, and resulting in increased but less efficient metabolism. The longevity mutation corresponded to the insertion of one of the plasmids that in Chapter 4 were shown to negatively affect life span. Integration is thus, unlike previously thought, neither per sé associated with senescence nor with longevity. It may be that the cells carrying mtDNA with inserts of pAL2-1 and pKALILO are normally eliminated by the host before sectors get a chance to escape. In other words: Longevity induction by integration of pAL2-1 or pKALILO may be timing dependent. Otherwise it could be site specific or it could be a secondary effect.

In **Chapter 6,** the structure and distribution of plasmid/mtDNA recombination junctions involving the two invertrons pAL2-1 and pKALILO were investigated. The recombination junctions corresponded predominantly to sites within the mitochondrial rDNA regions of the two respective hosts. They often but not invariably contained additional insertions and/or deletions, as is known from hepaDNAviruses that insert at dsDNA breaks. It could be that the rDNA regions are more heavily transcribed and consequently more susceptible to recombination. If the plasmids indeed insert at DNA breaks this suggests that the age dependent rearrangements could be due to errors in mtDNA repair.

Invertron based senescence, evidence for the mutation accumulation theory of aging?

Given all the above, it seems as if invertron based senescence should indeed be classified as a disease. Invertrons have a structure that is also typical of certain DNA viruses, and they posses viral-like polymerases, so the link with phages and viruses is not far-fetched. Be it as it may, the first signs of invertron based senescence typically set in, both in Neurospora and in

Podospora, not earlier than a week after ascospore germination. This is approximately the amount of time that is necessary for the sexual cycle to be completed. It could thus be that the elements assert their effects in the shadow of selection. The plasmids appear to integrate at dsDNA breaks as proposed in chapter 7, so perhaps the senescence syndrome results from the progressive decline in mtDNA repair with age, in combination or not with a progressive loss of control over plasmid replication. It would be interesting to test if the plasmids also integrate at specific mtDNA breaks or recombination junctions, and whether they interact with other senescence or senescence related phenomena like for example with the *premature death* phenomenon. It would also be interesting to test whether other elements like some of the optional introns have an effect on CR. Also these elements may have accumulated in the shadow of natural selection. The fact that pAL2-1 based senescence is normally overshadowed by the regular, calorie restriction sensitive senesce phenomenon could indicate that its presence should be considered a form of mutation accumulation.

Senescence a laboratory artefact?

What also requires testing is whether the invertrons assert an effect in nature, for example by collecting vegetative material directly from the field. It could be that invertron based senescence is largely a laboratory artefact. The culturing methods usually employed in Neurospora for example, are highly unnatural in the sense that bulk inocula are used in serial subculturing. The natural mode of vegetative propagation probably involves only one or just few but related macroconidia to start a new colony. In nature the invertrons may have no or barely an effect because somatic selection may purge the detrimental effects of the plasmids.

Antagonistic pleiotropy of fungal senescence

All the longevity mutants thus far show severely reduced fertility. Mitochondrial instability, oxidative stress, and the cellular level of energy appear to be intimately connected. It could be that according to the free radical theory of aging, mitochondrial oxidative stress is the key parameter in the aging process. However, this is always associated with the level of energy and the latter could be an equally important parameter in the process. Thus far there is no evidence that these parameters can be dissociated. The most direct way of testing this possibility would be to use artifical selection for increased reproduction and increased life span simultaneously.

Samenvatting

Veroudering evolueert in de schaduw van natuurlijke selectie: Omdat de cumulatieve kans om te sterven aan externe doodsoorzaken toeneemt naarmate een organisme ouder wordt, zal er doorgaans slechts geringe selectie bestaan tegen intrinsieke, genetische factoren die relatief laat in het leven een negatief effect hebben. Bijgevolg zullen dergelijke factoren over meerdere generaties accumuleren. Dit staat bekend als de “mutatie accumulatie” theorie van veroudering. Als deze factoren bovendien een pleiotroop, positief effect vroeg in het leven zouden hebben, bijvoorbeeld op de fertiliteit, zou er zelfs selectie vóór dergelijke factoren kunnen zijn. Dit staat bekend als de “antagonistische pleiotropie” theorie van veroudering. Veroudering is naar verwachting dus een multicausaal proces dat het resultaat is van intrinsieke factoren met een negatief effect laat in het leven en een mogelijk positief effect vroeg in het leven. Het kan gezien worden als het resultaat van een gebrek aan investering in lichamelijk onderhoud laat in het leven, een erfenis van het evolutionaire verleden van een organisme.

In tegenstelling tot unitaire organismen zoals veel dieren, kunnen modulaire organismen zoals planten, schimmels en kolonievormende invertebraten zich klonaal of vegetatief vermeerderen. Bij dergelijke organismen is er geen duidelijk onderscheid tussen de kiembaan, de cel lijn bestemt voor de voortplanting, en het soma, het lichaam. Daar de kiembaan niet mag verouderen, zouden deze modulaire organismen dus geen veroudering moeten kennen. Hoewel modules of delen van modulaire organismen aan veroudering onderhevig kunnen zijn, verouderen dergelijke organismen dan ook inderdaad zelden als geheel. Desondanks zijn er voorbeelden bekend van organismale veroudering bij schimmels en planten, waarbij alle vegetatieve delen van een enkel individu gelijktijdig sterven.

Dit proefschrift gaat over veroudering in twee genera filamenteuze schimmels: Neurospora en Podospora. De vraag is of er overeenkomsten bestaan, zowel op het proximate of mechanistische, alswel op het ultimate of evolutionaire niveau, tussen veroudering zoals dat plaatsvindt bij schimmels en veroudering zoals dat in de regel plaatsvindt bij dieren. Het werk in dit proefschrift laat zien dat veroudering bij sommige schimmels, zoals in ieder geval bij de pseudohomothallische ascomyceet *P. anserina*, inderdaad een intrinsiek en multicausaal proces is, zoals verwacht verwacht mag worden. Mitochondriën blijken hierin een cruciale rol te spelen. Een analyse van de bestaande natuurlijke variatie voor levensduur, laat zien dat de belangrijkste bron van variatie samenhangt met de af of aanwezigheid van mitochondriële plasmiden, moleculaire parasieten die interfereren met de ademhaling. Variatie die spontaan in het lab ontstaat, hangt echter vaak samen met de mutaties in de electron transport keten. Deze zijn geassocieerd met de inductie van alternatieve ademhalings

routes en dit leidt via een nog onbekende weg tot een stabilizering van het mitochondrieel genoom, een verlaagd niveau van de hoeveelheid vrije radicalen die aanwezig zijn in de cel en minder energie. Derhalve zijn de stammen die een dergelijke mutatie dragen langlevend en minder fertiel tot zelfs compleet steriel. Ook blijkt een reductie in de hoeveelheid glucose in het dieet net als bij dieren te leiden tot levensduurverlenging. Dit kan teniet gedaan worden door de aanwezigheid van een mitochondrieel plasmide, hetgeen aangeeft dat deze manier van levensduurverlenging waarschijnlijk afhangt van het correct functioneren van de mitochondriën.

Dankwoord

Ter afsluiting wil ik hier graag iedereen bedanken die op welke manier dan ook een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift. Veel mensen waren hierbij betrokken, hetzij direct, door een bijdrage aan de ideeënvorming, de praktische uitvoering of het schrijven, hetzij indirect, door een bijdrage aan de plezierige omstandigheden waaronder ik de afgelopen vier en een half jaar heb gewerkt. In een dankwoord loop je altijd het risico om onverhoopt een aantal namen te vergeten, en daarom zeg ik alvast: Iedereen bedankt! Een aantal mensen wil ik hier echter iets nadrukkelijker bedanken.

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Dit proefschrift heeft wellicht een meer moleculair genetisch karakter gekregen dan oorspronkelijk de bedoeling was. Ik bracht tijdens mijn tijd als A.I.O. twee periodes van enkele maanden door in het Centre de Génétique Moléculaire (C.G.M.), onderdeel van het Centre National de la Recherche Scientifique (C.N.R.S.) te Gif sur Yvette, Frankrijk. Gedurende die tijd heb ik in het laboratorium van Annie Sainsard-Chanet veel geleerd over moleculaire genetica. Je voudrais remercier tous les gens du laboratoire de Gif. Vous êtes tous très sympas et c’est bien de travailler avec vous. Annie, Carole, Sandrine, Antoine, Riyad, Severine, Sophie, Odile, Patricia, Delphine, et aussi Marie-Claire et Erwin, merci beaucoup.

Gedurende mijn tijd in het Laboratorium voor Erfelijkheidsleer heb ik veel Bsc. en Msc. studenten, mede- A.I.O.s en O.I.O.s, postdocs en ander volk mee mogen maken. Het was mij een waar genoegen om jullie allemaal te mogen leren kennen. Annelies, Jarco, Pieter van ’t H., Hugo, Anneleen, Bart, Eric, Arnaud, Bram en Margriet, de resultaten van jullie experimenten hebben hetzij direct, dan wel indirect bijgedragen aan dit proefschrift. Ik ben jullie zeer dankbaar. Ik heb veel van jullie geleerd en hopelijk was dat wederzijds. Bas en Jannie, bedankt voor het verzamelen van de bodemmonsters. Zoals jullie kunnen zien, zijn ze goed terecht gekomen. Anne, Marijn, Duur, Peter, Henk, Judith, Ronny, Siemen, Merijn,

Karoly, Stefan, Danny, Michelle, Pieter W., Dominika, Elaine, Andrés, Tamás, Mark, Edu, Bertha, Marijke, Marjan, Corrie, Aafke, Klaas, Arjan, en iedereen die ik bij deze nog niet bij naam genoemd heb, bedankt voor de leuke tijd die ik heb gehad bij Erfelijkheidsleer. Merijn en Margriet, ik ben vereerd dat jullie mijn paranimfen willen zijn.

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Gif sur Yvette, 7 September 2005

Curriculum vitae

Op 11 april 1977 werd ik, Marc François Paul Marie Maas geboren te ‘s Hertogenbosch. Na in 1995 mijn VWO diploma te hebben behaald aan het Jacob Roelands Lyceum te Boxtel, ben ik dat jaar aan de opleiding Biologie begonnen van Wageningen Universiteit. Na mijn propedeuse in 1996 te hebben behaald, koos ik voor de specialisatie Populatie biologie. Tijdens mijn doctoraalperiode deed ik twee afstudeervakken en een stage. Mijn eerste afstudeervak deed ik bij het Laboratorium voor Entomologie in Wageningen, onder begeleiding van Richard Stouthamer. Tijdens dat afstudeervak keek ik naar de phenotypische effecten van de PSR (Paternal Sex Ratio) factor, een geslachtsmanipulerend b-chromosoom dat ondermeer voorkomt bij parasitoide wespen en ervoor zorgt dat vrouwtjes die paren met dragers van dat chromosoom alleen nog maar zonen kunnen krijgen. Hierna heb ik in het kader van een stage samen met Ties Huigens, Raymond Klaassen en Martijn Timmermans veldonderzoek gedaan in de Californische Mojave woestijn naar een andere geslachtsmanipulerende factor die voorkomt bij deze wespen: De parthenogenese inducerende Wolbachia, een intracellulaire bacterie die er voor zorgt dat vrouwtjes enkel nog maar dochters kunnen krijgen. Dit onderzoek was onderdeel van het promotie onderzoek van T. H., en werd mede begeleid door Robert F. Luck van het Laboratorium voor Entomologie in Riverside, Californië (V.S.). Terug in Wageningen heb ik een afstudeervak gedaan bij het Laboratorium voor Erfelijkheidsleer, onder begeleiding van Fons Debets. Tijdens dit afstudeervak heb ik mij bezig gehouden met veroudering bij schimmels, een onderwerp waarop ik uiteindelijk aangesteld zou worden als A.I.O. (Assistent in Opleiding). In 2000 studeerde ik af en vanaf oktober dat jaar was ik werkzaam als A.I.O. bij het laboratorium voor Erfelijkheidsleer in Wageningen. Dit werd de eerste vier jaar gefinancierd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (N.W.O.) en gedurende het laatste half jaar door Wageningen Universiteit. In deze periode heb ik onder begeleiding van Fons Debets en Rolf Hoekstra diverse aspecten van veroudering bij schimmels bestudeerd waarvan u de resultaten hier heeft kunnen lezen. Daarnaast heb ik enkele cursussen begeleid en/of verzorgd waaronder Moleculaire en Evolutionaire Ecologie, en studenten in hun afstudeerfase begeleid. Tijdens deze periode heb ik ook twee keer enkele maanden werkervaring opgedaan bij het Centre de Génétique Moleculaire (C.G.M.), onderdeel van het Centre National de la Recherche Scientifique (C.N.R.S.) te Gif-sur-Yvette, Frankrijk, in het laboratorium van Annie Sainsard-Chanet waar ik sinds juli dit jaar werkzaam ben.

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Appendix

Ad Chapter 2. Neurospora isolates collected from Hawaiian soil (isolate codes refer to species, sample numbers and isolate numbers). Indicated are their origins (numbers refer to sites), mating types, and the presence of plasmids and that of senescence (expressed in number of subcultures. One subculture corresponds to two or three days. An isolate was classified as nonsenescent (-) when it grew more than 50 subcultures).

Isolate	**Origin**	**Mating type**	**Plasmid**		**Life span**
			pKALILO	**pHANALEI-2**	
NI05.01	Kauai, 06	a	+		-
NI06.01	Kauai, 06	a	+		35
NI06.02	Kauai, 06	a		+	-
NI07.01	Kauai, 07	a			-
NI07.02	Kauai, 07	a			-
NI08.04	Kauai, 07	A	+	+	21
NI11.02	Kauai, 08	A	+	+	9
NI11.03	Kauai, 08	a	+	+	4
NI13.01	Kauai, 10	A			-
NI13.02	Kauai, 10	a	+	+	19
NI13.03	Kauai, 10	A	+	+	19
NI14.01	Kauai, 10	a			-
NI15.01	Kauai, 10	a			-
NI15.02	Kauai, 10	a	+		-
NI15.03	Kauai, 10	A			-
NI16.01	Kauai, 10	a	+		10
NI16.03	Kauai, 10	a	+		8
NI16.04	Kauai, 10	a	+		14
NI16.05	Kauai, 10	a	+		8
NI16.06	Kauai, 10	a		+	-
NI16.07	Kauai, 10	A	+		21
NI16.08	Kauai, 10	a		+	-
NI16.09	Kauai, 10	A		+	-
NI18.02	Oahu	a	+		20
NI20.01	Kauai, 01	a		+	-
NI20.03	Kauai, 01	a		+	-
NI21.02	Kauai, 04	A			-
NI21.03	Kauai, 04	a	+		20
NI21.04	Kauai, 04	A		+	-

NI21.05	Kauai, 04	A	+		9
NI22.01	Kauai, 02	a	+		7
NI23.01	Kauai, 02	A		+	-
NI23.02	Kauai, 02	A		+	-
NI23.03	Kauai, 02	a			-
NI23.07	Kauai, 02	a	+		25
NI23.10	Kauai, 02	a			-
NI23.11	Kauai, 02	A			-
NI23.12	Kauai, 02	A			-
NI23.13	Kauai, 02	a		+	-
NI23.18	Kauai, 02	a	+		14
NI25.01	Kauai, 09	a		+	-
NI26.03	Kauai, 05	A			-
NI28.01	Kauai, 01	a	+		-
NI29.02	Kauai, 01	a	+		10
NI29.03	Kauai, 01	a			-
NI29.06	Kauai, 01	A	+		9
NI29.10	Kauai, 01	a	+		13
NI30.04	Kauai, 01	A	+		-
NI31.01	Kauai, 03	a			-
NI31.02	Kauai, 03	A	+		-
NI31.03	Kauai, 03	a	+		9
NI32.01	Kauai, 03	A			-
NI35.01	Kauai, 05	A	+		26
NI39.01	Kauai, 11	a			-
NI39.02	Kauai, 11	a		+	-
NI40.01	Kauai, 11	a	+	+	18
NI40.02	Kauai, 11	A			-
NI40.03	Kauai, 11	a			-
NI40.04	Kauai, 11	a			-
NI40.05	Kauai, 11	A			-
NI40.06	Kauai, 11	a			-
NI40.07	Kauai, 11	A	+	+	5
NI42.01	Kauai, 11	a			-
NI42.02	Kauai, 11	A	+		12
NT08.01	Kauai, 07	A/a			-
NT08.02	Kauai, 07	A/a			-
NT08.03	Kauai, 07	A/a			-
NT09.02	Kauai, 08	A/a			-

NT09.03	Kauai, 08	A/a	+		14
NT09.04	Kauai, 08	A/a			-
NT09.05	Kauai, 08	A/a			-
NT09.06	Kauai, 08	A/a			-
NT09.07	Kauai, 08	A/a	+		16
NT09.08	Kauai, 08	A/a			-
NT11.01	Kauai, 08	A/a			-
NT11.04	Kauai, 08	A/a			-
NT16.02	Kauai, 10	A/a			-
NT17.01	Kauai, 10	A/a		+	-
NT18.01	Oahu	A/a	+	+	21
NT19.01	Kauai, 01	A/a			-
NT19.02	Kauai, 01	A/a			-
NT19.03	Kauai, 01	A/a	+		23
NT19.04	Kauai, 01	A/a			-
NT19.05	Kauai, 01	A/a	+		28
NT20.02	Kauai, 01	A/a	+		30
NT20.04	Kauai, 01	A/a			-
NT21.01	Kauai, 04	A/a			-
NT22.02	Kauai, 02	A/a	+		24
NT23.04	Kauai, 02	A/a			-
NT23.05	Kauai, 02	A/a			-
NT23.06	Kauai, 02	A/a	+		-
NT23.08	Kauai, 02	A/a	+		22
NT23.09	Kauai, 02	A/a			-
NT23.14	Kauai, 02	A/a			-
NT23.15	Kauai, 02	A/a			-
NT23.16	Kauai, 02	A/a			-
NT23.17	Kauai, 02	A/a			-
NT23.19	Kauai, 02	A/a			-
NT23.20	Kauai, 02	A/a			-
NT23.21	Kauai, 02	A/a			-
NT23.22	Kauai, 02	A/a			-
NT23.23	Kauai, 02	A/a			-
NT23.24	Kauai, 02	A/a			-
NT23.25	Kauai, 02	A/a			-
NT26.01	Kauai, 05	A/a			-
NT26.02	Kauai, 05	A/a			-
NT27.01	Kauai, 01	A/a			-

NT28.02	Kauai, 01	A/a	+	15
NT28.03	Kauai, 01	A/a	+	22
NT28.04	Kauai, 01	A/a		-
NT28.05	Kauai, 01	A/a		-
NT28.06	Kauai, 01	A/a		-
NT28.07	Kauai, 01	A/a		-
NT29.01	Kauai, 01	A/a		-
NT29.04	Kauai, 01	A/a		-
NT29.05	Kauai, 01	A/a		-
NT29.07	Kauai, 01	A/a		-
NT29.08	Kauai, 01	A/a		-
NT29.09	Kauai, 01	A/a		-
NT29.11	Kauai, 01	A/a		-
NT30.01	Kauai, 01	A/a		-
NT30.02	Kauai, 01	A/a		-
NT30.03	Kauai, 01	A/a	+	29
NT30.05	Kauai, 01	A/a		-
NT32.02	Kauai, 03	A/a		-
NT33.01	Kauai, 03	A/a		-
NT35.02	Kauai, 05	A/a	+	-
NT39.03	Kauai, 11	A/a	+	18

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Cover: Photo of *Podospora anserina*