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Mutations in Two Zinc-Cluster Proteins Activate Alternative Respiratory and Gluconeogenic Pathways and Restore Senescence in Long-Lived Respiratory Mutants of *Podospora anserina*

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ABSTRACT

In *Podospora anserina*, inactivation of the respiratory chain results in a spectacular life-span extension. This inactivation is accompanied by the induction of the alternative oxidase. Although the functional value of this response is evident, the mechanism behind it is far from understood. By screening suppressors able to reduce the life-span extension of cytochrome-deficient mutants, we identified mutations in two zinc-cluster proteins, RSE2 and RSE3, which are conserved in other ascomycetes. These mutations led to the overexpression of the genes encoding the alternative oxidase and the gluconeogenic enzymes, fructose-1, 6 biphosphatase, and pyruvate carboxykinase. Both RSE2 and RSE3 are required for the expression of these genes. We also show that, even in the absence of a respiratory deficiency, the wild-type RSE2 and RSE3 transcription factors are involved in life-span control and their inactivation retards aging. These data are discussed with respect to aging, the regulation of the alternative oxidase, and carbon metabolism.

THE filamentous fungus *Podospora anserina* is a model organism in which life-span control has been extensively investigated. As in other organisms, it was clear from the beginning that life span is controlled by numerous external and genetic factors. Among these factors, mitochondrial activity seems to play a determinant role (reviewed in Lorin *et al.* 2006). But whereas mutations that compromise mitochondrial function in humans (reviewed in Wallace 2005) and in mice (Kujoth *et al.*, 2005, 2006, 2007; Trifunovic *et al.* 2004, 2005) lead to a variety of pathological life-span-shortening diseases, in *P. anserina* they lead to a spectacular life extension. In this organism, all wild-type cultures exhibit an unavoidable arrest of vegetative growth systematically associated with large rearrangements in the mitochondrial DNA (mtDNA). Inactivation of respiratory complex III (mutant *cyc1-1*) (Sellem *et al.* 2007) or complex IV (mutant *cox5::ble*) (Dufour *et al.* 2000) results in an extreme increase of life span (>30-fold) associated with a reduction in reactive oxygen species (ROS) levels and an increased stability of the mtDNA. In *Caenorhabditis elegans*, a class of mutants (*Mit* mutants) with disruptions (either genetic or mediated by RNA interference) in genes essential for the mitochondrial electron transport chain (ETC) are also long lived (reviewed in Rea 2005 and Rea *et al.* 2007).

How does the loss of genes critical for mitochondrial activity lead to life extension in *P. anserina* and *C. elegans*? One characteristic shared by *C. elegans Mit* mutants and *P. anserina* respiratory mutants is the activation of compensatory metabolic pathways in an attempt to supplement deficits in ETC function. Such pathways could produce less toxicity, *e.g.*, by reducing mitochondrial ROS production or activating antioxidant mechanisms. In *P. anserina*, inactivation of genes essential for complex III or IV activity leads to the induction of an alternative oxidase (AOX) that catalyzes the transfer of electrons directly from the ubiquinol pool to oxygen and does not couple this transfer to proton translocation (Affourtit *et al.* 2002; Moore *et al.* 2002). Some phenotypic traits of the *cox5::ble* and *cyc1-1* mutants can be attributed to the following characteristics: reduced growth rate, loss of fertility, and reduced ROS production. In these mutants, only complex I is conserved as a site of proton gradient formation for ATP synthesis leading to a reduction of the energy yield associated with respiration. Furthermore, the alternative oxidase is thought to have an antioxidant role, preventing over-reduction of the mitochondrial quinone pool known to favor superoxide production (Maxwell *et al.* 1999).

The reasons for the spectacular long-lived phenotype of these mutants are more puzzling. One hypothesis proposed that the reduction of ROS and/or ATP production might be sufficient to account for life-span extension. Another hypothesis invoked a mitochondria-to-nucleus signaling pathway as the reason for this life-span extension (Lorin *et al.* 2001). The AOX is encoded in the nucleus and imported into mitochondria. In fungi, AOX expres-

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sion has been extensively studied in *Neurospora crassa* and in *P. anserina*. The protein is not detectable under standard growth conditions. Its expression is strongly induced by mutations or chemicals that inhibit the ETC, and significant regulation occurs at the level of transcription (Lambowitz *et al.* 1989; Li *et al.* 1996; Lorin *et al.* 2001; Affourtit *et al.* 2002; Tanton *et al.* 2003; Descheneau *et al.* 2005; Chae *et al.* 2007a,b). In higher plants, AOX expression depends on developmental signals, stress conditions, and inhibition of the respiratory chain (reviewed in Clifton *et al.* 2006). The AOX expression therefore implies the existence of one or more pathways for transducing signals from the mitochondria to the nucleus to control the expression of the gene. The mechanisms by which mitochondria communicate with the nucleus have been referred to as retrograde signaling (Butow and Avadhani 2004; Liu and Butow 2006; Rhoads and Subbaiah 2007). In *Saccharomyces cerevisiae*, one retrograde pathway (the RTG pathway) has been extensively studied and shown to be an important determinant of life span (Kirchman *et al.* 1999).

In an attempt to clarify the relationships among AOX expression, retrograde signaling, and life span in *P. anserina*, we isolated fast-growing, short-lived revertants from the long-lived respiratory *cox5::ble*- and *cyc1-1*-deficient mutants. We identified three mutations localized in two zinc-cluster transcription factors that control AOX expression both in *P. anserina* (this work) and in *N. crassa* (Chae *et al.* 2007b). Interestingly, these two mutations activate the expression of the alternative oxidase and also of gluconeogenic genes.

MATERIALS AND METHODS

***P. anserina* strains, growth conditions, transformation, and genetic analysis:** Except strain *TS24* that is used for positional cloning, all the strains used in this study were derived from the *s* wild-type strain (Rizet 1952). The *gpd-aox* strain contains a transgenic copy of the *aox* gene under the control of the strong constitutive *P.anserina gpd* promotor associated with a hygromycin resistance cassette (Lorin *et al.* 2001). The long-lived *cox5::ble* and *cyc1-1* strains have been described in Dufour *et al.* (2000) and in Sellem *et al.* (2007), respectively. The Δ*PaKu70* strain inactivated for the *KU70* mammalian ortholog provides an efficient method for producing deletion mutants (El-Khoury *et al.* 2008). The *TS24* strain used for positional cloning was obtained from the progeny of crosses between the *P. anserina S* (Rizet 1952) and the *P. comata T* (ATCC 36713) strains. *TS24* exhibits the wild-type, fertile *P. anserina* phenotype and retained, on chromosome III, at least 12 simple sequence repeat markers (SSR) characteristic of the *P. comata* strain. The germination medium contains ground corn meal (50 g/liter), agar (12.5 g/liter), and ammonium acetate (4.4 g/liter). Minimal standard medium (M2) contains 1% dextrin as the carbon source (Esser 1974). When necessary, hygromycin, phleomycin, nourseothricin, and antimycin A were added to the medium at 75 μg/ml, 10 μg/ml, 50 μg/ml, and 10 μg/ml, respectively. Transformation experiments were conducted as previously described (Berges and Barreau 1989) on protoplasts obtained by incubation with 40 mg/ml glucanex (Laffort). Genetic methods for *P. anserina* have been described (Esser 1974). For the construction of double-mutant strains, the appropriate single mutants of opposite mating types were crossed. The Δ*rse2* Δ*rse3* strain carrying the two alleles inactivated by the same cassette conferring nourseothricin resistance and the double-mutant strain *rse2-1 rse3-1* carrying the two alleles that both confer the ability to grow without delay on a medium supplemented with antimycin A were identified by analyzing the segregation of the cassette or the resistance to antimycin A in isolated asci.

Life-span measurements: Life spans were measured on M2 medium on three to five subcultures derived from two to five independent spores exhibiting a given genotype. Cultures were grown in 30 ml/30 cm race tubes at 27° in the dark. The life span of a strain was defined in centimeters as the mean length (given with standard errors) of growth of parallel cultures between the point of the incubation of freshly germinated spores and the arrested edge of the dead culture. Survival curves, plotted as the percentage of surviving cultures in the course of time, also defined the life span (in days) as the time at which 50% of the cultures are still alive.

SSR markers and localization of gene *rse2*: Twelve SSR markers overlapping 2 Mb on the long arm of chromosome III were found polymorphic between the *P. anserina* and *P. comata* isolates. Their characteristic markers are presented in supporting information, Table S1. To position gene *rse2*, crosses between (*TS24*) *rse2*$^+$ (*comata* origin) and (*s*) *rse2-1* (*anserina* origin) parental strains provided us with a collection of monocaryotic spores for which linkage analysis was performed. The nature of the *rse2* allele was determined by growth on antimycin A, and the nature of the 12 SSR markers was identified by PCR analysis. PCR amplifications were performed on rapid mini-preparations of DNA extracted from mycelium grown 24 hr after the germination of each spore.

Nucleic acid and protein manipulation: Southern blots were done using total DNA extracted by the mini-preparation method (Lecellier and Silar 1994). Western blot analysis of the AOX protein was performed on isolated mitochondria as previously described (Sellem *et al.* 2007). Immunochemistry was performed with an anti-AOX mouse monoclonal antibody generated against the AOX of *Sauromatum guttatum* (Elthon *et al.* 1989). Additionally, blots were reprobed with an anti-βATPase rabbit antibody (a gift from J. Velours) as a standardization control. The bound antibodies were detected using an enhanced chemiluminescence detection system (Pierce Supersignal West picochemiluminescent substrate). Quantifications of the signal intensity that reflects the amount of protein were performed using the ImageQuant program on at least three independent blots (Molecular Dynamics, Amersham Bioscience, Piscataway, NJ).

Quantitative RT–PCR: Total RNA from various strains grown for 48 hr on standard medium (1% dextrin) covered with cellophane disc was extracted using the RNeasy plant kit (Qiagen) with RLT buffer and DNAase I according to the manufacturer's instructions except that mycelium was broken with glass beads in a Fastprep apparatus (40 sec, intensity 6.5). For quantitative RT–PCR (qRT–PCR) analysis, 2 μg of RNA was reverse transcribed and random primed with oligo$(dT)_{20}$ using the Supercript II reverse transcriptase (Invitrogen) according to the instructions of the supplier. Pairs of primers for PCR were developed for the *aox* (5′-GATGTCTGTTCCCCATCGAC-3′/5′-GAGGAAATGTTGGCAGTGGT-3′), *gpd* (5′-CACCGAGGACGAGATTGTCT-3′/5′-TCAGGGAGATACCAGCCTTG-3′), *fbp* (5′-CACCGGTGACTTTACGCTCC-3′/5′-GGAGAATTGGAGGGCGTGGC-3′), and *pck* (5′-ACCAAACCATCCGACATGC-3′/5′-GGTCTTGTTTACTGTGTTGA-3′) genes to give products of ∼40–50 bp in length. One primer of each set was designed across an exon/intron boundary to avoid amplification of any contaminating genomic DNA. The product of the first-strand cDNA

reaction was diluted 10-fold before real-time PCR analysis. Amplifications were performed in duplicate in a LightCycler (Roche) using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche) with no reverse transcriptase controls to estimate the contribution of contaminating DNA. Amplification efficiencies were measured for each primer pair and every set of amplification reactions. For each strain, the levels of *aox*, *fbp*, and *pck* transcripts were normalized using the *gpd* transcript level, which was used as a standard because its expression remained stable in all the strains and conditions examined. At least three independent experiments were performed from one to three different RNA preparations. For a given strain and a given gene, results are expressed as the level of expression of this gene in this strain relative to the level of expression of this gene in the wild-type strain.

Cloning of *rse2* and *rse3* genes: PCR amplifications of the *rse2* and *rse3* mutated genes were performed using the primer pairs 5′-GGCTCGAGGACGGGAACCGGGAAG-3′/5′-GGGG ACTAGTCGAAGGGGCGGCATTGTG-3′and 5′-CCCCCATG GCCGAGTAAA TACTGGATTTTG-3′/5′-CCCAGATCTGCCG CGTGACCAGGACC-3′. They were cloned in the *Xho*I/*Spe*I sites of the PBCHygro vector (Silar 1995) or in the *Nco*I/*Bgl*II sites of the pAPI508 vector (El-Khoury *et al.* 2008), respectively. Transformation of wild-type protoplasts resulted in hygromycin- or nourseothricin-resistant strains purified through genetic crosses with the wild type.

Inactivation of *rse2* and *rse3* genes: Seven hundred nucleotides of the 5′ and 200 nucleotides of the 3′ region of the *rse3* wild-type gene were amplified with the primers 5′-GAAAGC GGCCGCGTGACCAGGACCAAG-3′/5′-GGGCCATGGCTCT ATCTGGACGG GACGGC-3′and 5′-GGGAGATCTGGAGTGC AGTTATACTTGG-3′/5′-CACGCGGCCGC TTTCGCCTCTTC TTTAAAC-3′, respectively, as described in El-Khoury *et al.* (2008) and cloned in the *Bgl*II/*Nco*I sites of the pAPI508 vector containing the nourseothricin resistance cassette. Protoplasts of the Δ*KU70* strain were transformed and nourseothricin-resistant transformants were isolated and purified through a genetic cross with the wild-type. Nourseothricin resistance cosegregated with antimycin sensitivity. Following the same strategy, *rse2* was inactivated using the primers 5′-AGGAAAAAAGCG GCCGCTGGGAAAGGGGAAGGAAG-3′/5′-GAAGATCGCAGT CGTTCGGCTTTGT-3′ for the 5′ region and 5′-AGGAAGC TTGGTGGGAGCATCGACAAA-3′/5′-AGGAAAAAAGCGGCC GCAAT CCGCCTCTCGGTCTT-3′ for the 3′ region and cloned in the *Hin*dIII/*Bgl*II sites of the pAPI508 vector.

RESULTS

Mutations in genes *rse2* and *rse3* restore senescence in *cox5::ble* and *cyc1-1* contexts: Inactivation of complex III (*cyc1-1*) or complex IV (*cox5::ble*) leads to a spectacular increase in life span associated with several phenotypic defects: alteration in germinating mycelium; poorly colored, thin growing mycelium; reduction of the growth rate; and female sterility (Dufour *et al.* 2000, Sellem *et al.* 2007). To shed light on the parameters responsible for these different characteristics, we isolated suppressor mutations able to improve the phenotype of these mutants. Spontaneous revertants were obtained independently as sectors of aerial fast-growing mycelium from *cox5::ble* and *cyc1-1* cultures. Most cultures grown in race tubes led to such sectors. Two of them, sectors 2 and 3, were isolated from *cox5::ble* and *cyc1-1* cultures, respectively. They were crossed with wild type to test the genetic basis of the reversion and to obtain pure revertant strains (the sectors probably contain a mixture of mutant and revertant nuclei). The presence of extragenic suppressors was revealed by recovery in the progeny of the crosses of three types of ascospores: ascospores that germinate to give sparse mycelium like the original mutant, ascospores that germinate normally, and ascospores that display an intermediate germinating mycelium giving rise to a growing mycelium similar to that of the initial sectors. Genetic analysis of the two pure revertant strains revealed that the two suppressor mutations named *rse2-1* and *rse3-1* were unlinked to the original *cox5::ble* and *cyc1-1* mutations. The characteristics of the revertants and of the strains carrying the suppressor mutations dissociated from the initial respiratory mutation are shown in Figure 1 and Table 1.

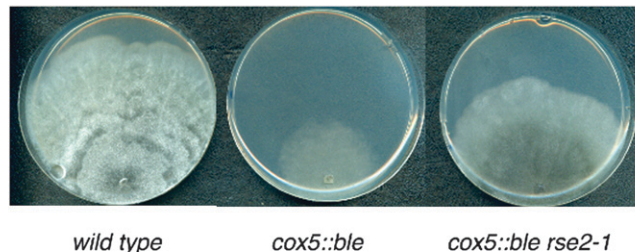

Figure 1.—Mycelium aspect of the wild-type, *cox5::ble*, and *cox5::ble rse2-1* strains. Petri plates of M2 medium were inoculated with an explant of each strain and incubated for 10 days at 27°. The wild-type strain exhibits a dense, aerial, colored mycelium and is fast growing whereas the *cox5::ble* mutant exhibits a thin, poorly colored mycelium and is slow growing. The *cox5::ble rse2-1* revertant exhibits an intermediate phenotype.

As shown in Table 1, the *rse2-1* or *rse3-1* mutations do not restore a wild-type phenotype to the *cox5::ble* and *cyc1-1* mutants. However, they considerably improve the germination of the ascospores (germinating thalli of *cox5::ble rse2-1* and *cyc1-1 rse3-1* appeared more dense and grew better than germinating thalli of *cox5::ble* and *cyc1-1*), they improve the aspect of the growing mycelium that appears more aerial and colored, and they restore a growth rate of 0.48 ± 0.1 cm/day compared with 0.21 ± 0.01 cm/day for the mutants. In contrast, they do not restore female fertility to the *cox5::ble* and *cyc1-1* mutants or normal ascospore pigmentation to *cyc1-1*. As *P. anserina* crosses yield dicaryotic ascospores, the recovery of heterocaryotic *cox5::ble rse2-1*/*cox5::ble rse2*$^{+}$ and *cyc1-1 rse3-1*/*cyc1-1 rse3*$^{+}$ ascospores allowed us to test the dominance/recessivity of the suppressors and to conclude that they are dominant with respect to the improved phenotypes.

Interestingly, analysis of longevity of the *cox5::ble rse2-1* and *cyc1-1 rse3-1* strains revealed that the two suppressors also restored the senescence phenomenon (hence the name *rse* for *r*estorator of *se*nescence). Longevity of

TABLE 1

Phenotypic properties of the *cox5::ble* and *cyc1-1* respiratory-deficient mutants and of the *rse2-1* and *rse3-1* suppressors

	Wild type	*cox5::ble*	*cyc1-1*	*cox5::ble rse2-1*	*cyc1-1 rse3-1*	*rse2-1*	*rse3-1*
Mycelium aspect	Aerial	Thin	Thin	Aerial	Aerial	Aerial	Aerial
Ascospore coloration	Black	Black	Colorless[a]	Black	Colorless[a]	Black	Black
Germination rate[b]	+++	+	+	++	++	+++	+++
Growth rate[c] (cm/day)	0.60 ± 0.03	0.21 ± 0.01	0.21 ± 0.01	0.48 ± 0.10	0.48 ± 0.11	0.50 ± 0.04	0.51 ± 0.04
Female fertility	Fertile	Sterile	Sterile	Sterile	Sterile	Fertile	Fertile

[a] This phenotype exhibits a variable penetrance (Sellem *et al.* 2007).
[b] Germination rate is given as +++ for wild-type thalli, + for sparse and slow-growing thalli, and ++ for thalli of intermediate phenotype on germination medium.
[c] Growth rates are mean values ± standard deviation.

the revertants was ~90 days (60 ± 10 cm) compared to ~17 days (11.3 ± 1.6 cm) for the wild-type strain and >2 years (>300 cm) for the *cox5::ble* and *cyc1-1* mutants. An analysis of the mtDNA content of the senescent revertant cultures revealed the presence of mtDNA rearrangements called senDNAs as in senescent wild-type cultures (Belcour *et al.* 1999; Albert and Sellem 2002). However, in contrast to the wild-type strain in which senDNAα is systematically observed in a large amount, the senDNAs in the revertants originated mainly from the γ region as previously shown in other mutants (Lorin *et al.* 2001; Figure S3).

Recombination between *cox5::ble* and *rse3-1*, on the one hand, and between *cyc1-1* and *rse2-1*, on the other hand, revealed that either mutation, *rse2-1* or *rse3-1*, is able to suppress complex III and complex IV loss-of-function mutations.

The mutations *rse2-1* and *rse3-1* are responsible for the constitutive expression of the alternative oxidase: We previously showed that constitutive overexpression of the alternative oxidase in the *cox5::ble* and *cyc1-1* mutants improved mycelium aspect and growth rate and also restored the senescence process. This was demonstrated by expressing a fusion (*gpd-aox*) between the *gpd* promoter and the *aox* coding sequence in these mutants (Lorin *et al.* 2001; Sellem *et al.* 2007). Because of the similarities between the effects of the *gpd-aox* transgene and the *rse2-1/rse3-1* mutations, the level of expression of the alternative oxidase was examined in strains carrying these mutations. The *gpd-aox* strain was used as a control. The Western blot analysis (Figure 2) corroborated previous results (Lorin *et al.* 2001) showing that AOX is undetectable in the wild type grown under standard conditions whereas it is induced in long-lived respiratory mutants (*cyc1-1*, for example, in Figure 2) and very strongly expressed in the *gpd-aox* strain. Our results show that it is also expressed in the strains carrying the *rse2-1* or *rse3-1* mutations. However, the AOX level is 2- to 3-fold lower in *rse2-1* and *rse3-1* strains than in strains carrying the *gpd-aox* transgene. These results were confirmed by qRT-PCR experiments shown in Figure 3. Expression levels of the *aox* gene were normalized to the *gpd* gene, and the *aox* mRNA copy number was given a value of 1 in the wild-type strain. *Aox* mRNA copy number increased ~20-fold in *rse3-1*, 40-fold in *rse2-1*, and 60- to 80-fold in *gpd-aox* strains. In the *cox5::ble* (and *cyc1-1*) strain, *aox* transcript levels were increased ~20-fold compared to wild type. These levels were increased ~3-fold in the presence of *rse2-1* or *rse3-1* mutations and ~5-fold in the presence of the *gpd-aox* transgene. Altogether, these results are in accordance with data obtained by Western blot. The expression of the *aox* gene in *rse2-1* and *rse3-1* strains was also confirmed by testing the ability of these strains to grow on a medium containing antimycin A. Antimycin A is an inhibitor of complex III, leading to induction of the alternative oxidase in wild-type cells and allowing them to grow in the presence of the drug after a delay necessary for the induction whereas the *gpd-aox*, *rse2-1*, and *rse3-1* strains grow without delay on this medium because of the constitutive expression of AOX. Heterocaryotic strains *rse2-1/rse2*$^+$ and *rse3-1/rse3*$^+$ also grow without a delay on medium containing antimycin A, confirming the dominance of these mutations. The double mutant *rse2-1 rse3-1* was constructed by genetic cross, and it exhibited a phenotype very similar to that of each simple mutant, indicating the absence of a synergistic effect between the two mutations.

The *rse2* and *rse3* genes encode two zinc-cluster transcription factors: Since the strains carrying the wild-type *rse2*$^+$ and *rse3*$^+$ or the mutated *rse2-1* and *rse3-1* alleles differ by their growth with or without delay on a medium containing antimycin A, the segregation of these alleles can be easily analyzed through crosses. Genetic analysis showed that *rse2* and *rse3* were localized on chromosomes III and IV, respectively, near *ura5* for *rse2* and near *sir2* for *rse3*.

Taking advantage of the genome sequence of *P. anserina* (Espagne *et al.* 2008) and of the characterization on each of the seven chromosomes of marker polymorphisms between the geographic strains *P. anserina* and *P. comata*, segregation analysis of gene *rse2* with the markers linked to gene *ura5* was undertaken. A total of 198 monocaryotic spores derived from the cross *rse2-1* (*anserina*) × *rse2*$^+$ (*comata*) were generated. The *rse2-1/rse2*$^+$ segregation was determined by growth on anti-

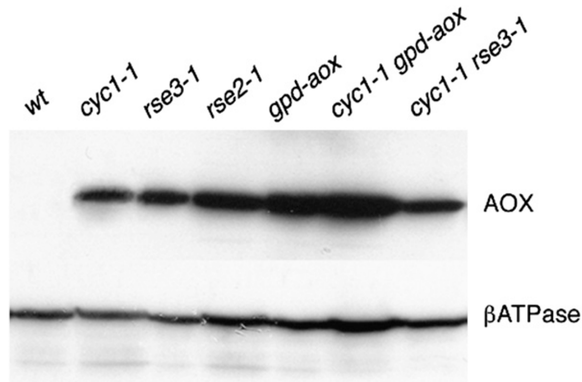

FIGURE 2.—Western blot analysis of the AOX protein. Mitochondria (10 μg of mitochondrial protein) were extracted from the wild-type (wt), *cyc1-1*, *rse3-1*, *rse2-1*, *gpd-aox*, *cyc1-1 gpd-aox*, and *cyc1-1 rse3-1* strains and loaded onto a 12% SDS–PAGE acrylamide gel. The AOX was revealed with a mouse antiserum against *S. guttatum* AOX provided by T. Elthon. As an internal control, the blot was reprobed with a rabbit anti βATPase provided by J. Velours.

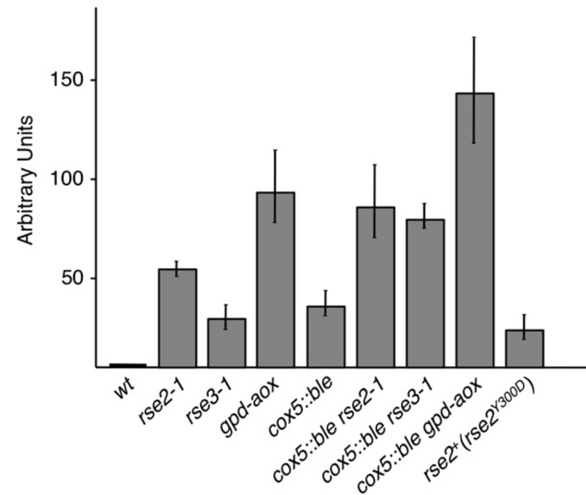

FIGURE 3.—Relative abundance of *aox* transcripts. For each strain, the levels of *aox* and *gpd* transcripts were determined by quantitative RT–PCR performed on one to three different RNA preparations (one to three replicates). For each experiment, the level of *aox* transcripts was normalized using the level of *gpd* transcripts as a reference. The graph shows the level of *aox* transcripts relative to the level of *aox* transcripts in the wild type for each strain. The error bars correspond to standard error. The $rse2^{+}(rse2^{Y300D})$ strain corresponds to a strain in which an ectopic copy of the mutated $rse2^{Y300D}$ has been integrated.

mycin A, and each spore was genotyped for 12 simple sequence repeat markers covering 2 Mb. All the polymorphic markers and the *rse2* gene segregated in a 1:1 ratio. Of the 12 molecular markers, 2 always remained in parental association with the *rse2* alleles. They covered ~250 kb containing 67 predicted open reading frames (ORFs) and three encoding putative transcription factors of which one (Pa_3_6340) is a zinc-cluster transcription factor containing the canonical motif CX2CX6CX5-12CX2CX6-8C. Genomic DNA was prepared from *rse2-1* and wild-type strains. PCR products that contained the ORF Pa_3_6340 were generated and sequenced. The *rse2-1* gene was shown to contain a single T-to-G substitution changing a Tyr into an Asp codon at position 300. Sequence comparisons revealed that this protein is homologous to the recently reported AOD2 protein from *N. crassa*, which acts synergistically with another transcription factor of the zinc-cluster family, AOD5 (CHAE *et al.* 2007b). This prompted us to search by BLAST the homolog of this protein in the *P. anserina* genome. The protein most related to AOD5 was Pa_4_8760 (54.7% identity) located on chromosome IV to which the *rse3-1* mutation was genetically assigned. Sequencing of the corresponding gene in wild-type and *rse3-1* strains revealed a G-to-T substitution changing a Gly into a Val codon at position 642 in the *rse3-1* strain. The structure of the two genes and the position of the mutations are shown in Figure 4.

To confirm that the Y300D mutation in the *rse2-1* strain and the G642V mutation in the *rse3-1* strain are responsible for the constitutive expression of the AOX and the restoration of senescence in strains deficient for the III/IV respiratory complex, we took advantage of the dominance of the two suppressor mutations. The mutated genes were cloned in plasmids pBCHygro (SILAR *et al.* 1995) and pAPI508 (EL-KHOURY *et al.* 2008) and introduced into a wild-type strain by transformation. Hygromycin- and nourseothricin-resistant transformants resulting from a nonhomologous integration were selected for each transformation. These $rse2^{+}(rse2^{Y300D})$ and $rse3^{+}(rse3^{G642V})$ transformants, which carried an endogenous wild-type allele and an ectopic mutant allele, showed constitutive overexpression of the alternative oxidase [see $rse2^{+}(rse2^{Y300D})$ in Figure 3], revealing that the introduction of the Y300D mutation in the *rse2* gene, or of the G642V mutation in the *rse3* gene, is sufficient to cause this phenotype. Furthermore, strains with the *cox5::ble* $rse2^{+}(rse^{Y300D})$ genotype obtained by genetic cross exhibited the same growth phenotype and longevity as *cox5::ble rse2-1* (90 days compared to >2 years for *cox5::ble* $rse2^{+}$).

The *rse2* and *rse3* genes of eight other revertants derived from *cox5::ble* or *cyc1-1* cultures were sequenced. One revertant had a single base-pair substitution changing a glycine to a serine at position 303 in the *rse2* gene, only three amino acids from the $rse2^{Y300D}$ mutation (Figure 4). The seven other revertants carried wild-type alleles for *rse2* and *rse3*.

The *rse2* and *rse3* gene products also activate the expression of gluconeogenic genes: The *rse2* and *rse3* gene products are conserved in several ascomycetes (see Figure S1 and Figure S2). RSE2 is homologous to AOD2 from *N. crassa* (53% identity), AcuM from *Aspergillus nidulans* (34% identity), and RDS2 from *S. cerevisiae* (28.8% identity). Recently, it was shown that RDS2 in *S. cerevisiae* (SOONTORNGUN *et al.* 2007) and AcuM and

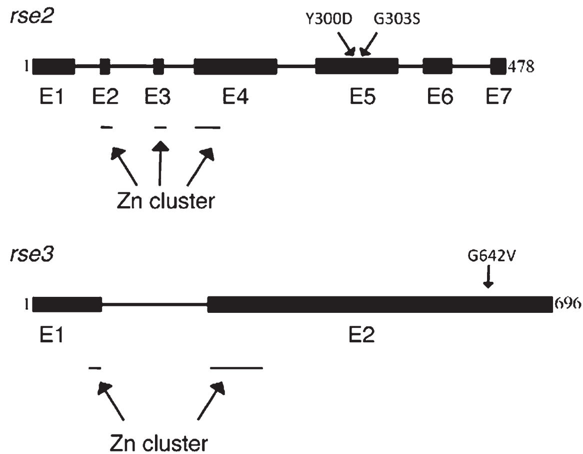

Figure 4.—Structure of the *rse2* and *rse3* genes. The amino acids mutated in the *rse2* gene (top) and the *rse3* gene (bottom) are indicated. Exons (E) are shown as solid boxes and introns as solid lines. The first and the last amino acids of each protein are indicated. Sites within exons that contain a motif identified as a possible zinc cluster are indicated by thin lines below the genes.

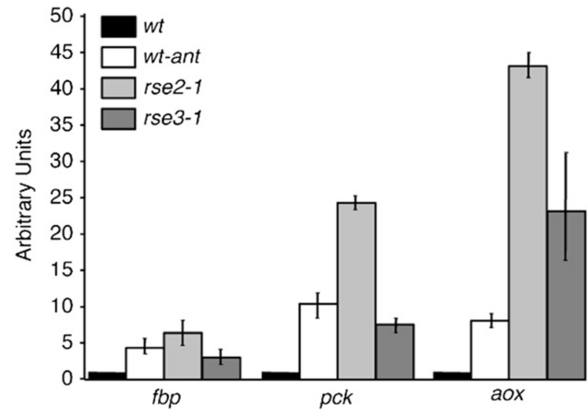

Figure 5.—Quantification of *fbp*, *pck*, and *aox* expression. Total RNA was extracted from cultures of the wild-type strain grown under normal conditions (solid) or in the presence of antimycin A (10 μg/ml) (open) and from cultures of the $rse2^{Y300D}$ (light shading) and $rse3^{G642V}$ (dark shading) mutants grown under normal conditions. qPCR reactions were performed on cDNA to quantify the level of *fbp*, *pck*, *aox*, and *gpd* transcripts in each strain. Experiments were performed at least three times. As in Figure 3, in each experiment, the level of *fbp*, *pck*, and *aox* transcripts was normalized using the level of *gpd* transcripts as a reference. The diagram shows the level of *fbp*, *pck*, and *aox* transcripts in the different strains and culture conditions relative to the level of these transcripts in the wild type. The error bars correspond to standard error.

AcuK in *A. nidulans* (Hynes *et al.* 2007) act as activators of the expression of genes encoding central enzymes in the gluconeogenic pathway, in particular phosphoenolpyruvate carboxykinase (PCK) and fructose-1,6-biphosphatase (FBPase). PCK catalyzes an early step in gluconeogenesis and converts oxaloacetate to phosphoenolpyruvate, and FBPase catalyzes the final step in hexose monophosphate formation by dephosphorylating fructose-1,6-biphosphate to yield fructose-6-phosphate. This prompted us to examine the expression of the genes encoding these two enzymes in $rse2^{Y300D}$, $rse3^{G642V}$, and wild-type strains by quantitative RT–PCR. Two coding sequences potentially encoding PCK (Pa_4_3160) and FBPase (Pa_4_ 9360) were identified by homology searches. RNA was isolated from the $rse2^{Y300D}$, $rse3^{G642V}$, and wild-type strains grown on standard medium (containing 1% dextrin), and the abundance of transcript levels of the genes encoding Pa_4_ 9360 (*Papck*) and Pa_4_ 9360 (*Pafbp*) were examined in the three strains. The expression of the *aox* gene was determined in parallel and the level of transcripts expressed from the constitutive *gpd* gene was used as a reference control. Figure 5 shows the expression level of each gene in a given strain compared to the expression level of the *gpd* gene in the wild-type strain. These experiments corroborate the results shown in Figure 3: an increase of *aox* mRNAs levels of ~40-fold in $rse2^{Y300D}$ and of ~20-fold in $rse3^{G642V}$. They also reveal that the expression of *Papck* and *Pafbp* is significantly increased in the mutant stains: for *Papck* ~20-fold in $rse2^{Y300D}$ and ~5-fold in $rse3^{G642V}$ and for *Pafbp* ~5-fold in $rse2^{Y300D}$ and ~2-fold in $rse3^{G642V}$.

To be sure that the activation of the *Papck* and *Pafbp* genes in the mutant strains does not result from a qualitative change of the properties of the mutated transcription factors, the expression level of the two genes and of the *aox* gene was also tested in a wild-type strain grown on antimycin A. As shown in Figure 5, the expression of the *aox*, *Papck*, and *Pafbp* genes is increased 5-fold, 10-fold, and 3-fold, respectively, in the wild-type strain grown on antimycin A, indicating that *Papck* and *Pafbp* are indeed coregulated with the *aox* gene.

Genes *rse2* and *rse3* are nonessential in respiratory-competent strains grown in standard conditions but both are essential for induction of the alternative oxidase: To determine the function of these transcription factors more clearly, strains deleted for the *rse2* and *rse3* genes were constructed by replacement with a cassette conferring resistance to nourseothricin (El-Khoury *et al.* 2008). The correct replacement was verified by Southern blot analysis (data not shown). Using *rse2::nat* (Δ*rse2*) and *rse3::nat* (Δ*rse3*) as parents in genetic crosses, we subsequently isolated the double-deleted strain Δ*rse2* Δ*rse3*. All these strains were viable and displayed no impairment of growth, pigmentation, or fertility on standard synthetic M2 medium (the same mycelium aspect as the wild-type strain; *cf.* Figure 1). These results demonstrate that *rse2* and *rse3* are nonessential genes in respiratory-competent strains grown in standard conditions. However, none of these strains, including the simple Δ*rse2* and Δ*rse3* deletions, are able to induce the *aox* gene. This was demonstrated in two ways. First, none of the deleted strains is able to grow on a medium containing antimycin A even after the lag necessary for the wild type to begin growth. Second, the association by genetic cross of mutations *cox5::ble* and

Δ*rse2* or *cox5::ble* and Δ*rse3* led to lethal spores that were unable to germinate. These results indicate that the inactivation of either of the two genes, *rse2* and *rse3*, prevents the expression of the *aox* gene under inducing conditions and therefore that both proteins RSE2 and RSE3 are required for *aox* induction. Finally, we constructed the Δ*rse2 rse3*^{G642V} and *rse2*^{Y300D} Δ*rse3* strains in which the deleted allele of one gene is associated with the mutated allele of the other. None of these strains was able to grow on a medium containing antimycin A, demonstrating that even when one of the two proteins is present in its mutant form, the other protein is still required to induce the alternative oxidase.

The RSE2 and RSE3 transcription factors are involved in life-span control even in the absence of mitochondrial dysfunction: As stated above, the modification of the transcriptional pattern in the *rse2*^{Y300D} and *rse3*^{G642V} strains revealed that the suppressor mutations modify the expression or the activity of the RSE2 and RSE3 products. We examined the abundance of *rse2* and *rse3* transcript levels in the wild-type, *rse2*^{Y300D}, and *rse3*^{G642V} strains by quantitative RT–PCR experiments. Transcripts of both genes were virtually unchanged in the three strains (data not shown). This strongly supports the hypothesis of a modification of the activity and not of the abundance of the RSE2 and RSE3 products in the *rse2*^{Y300D} and *rse3*^{G642V} strains. In a respiratory-deficient context, *cox5::ble* or *cyc1-1*, the *rse2*^{Y300D} and *rse3*^{G642V} mutations are responsible for severe life-span reduction. We investigated whether, in respiratory-competent strains, the *rse2*^{Y300D} and *rse3*^{G642V} mutations and the resulting gene expression modifications also lead to a modified life span. As shown in Figure 6, the *rse2*^{Y300D} and *rse3*^{G642V} strains displayed a decreased life span compared to wild type (∼12 *vs.* 17 days) whereas deletion of either of the *rse* genes results in an increased life span (∼30 days for Δ*rse2* and 35 days for Δ*rse3*). These results suggest that the RSE products contribute to shortening life span and that the gene expression modifications due to the *rse2*^{Y300D} and *rse3*^{G642V} mutations accentuate this effect. It is very unlikely that the decreased life span of the *rse2*^{Y300D} and *rse3*^{G642V} strains results from the overexpression of the AOX, since as previously shown (Lorin *et al.* 2001 and Figure 6), we confirmed that the *gpd-aox* strain carrying the *gpd-aox* transgene displays the same longevity as the wild type.

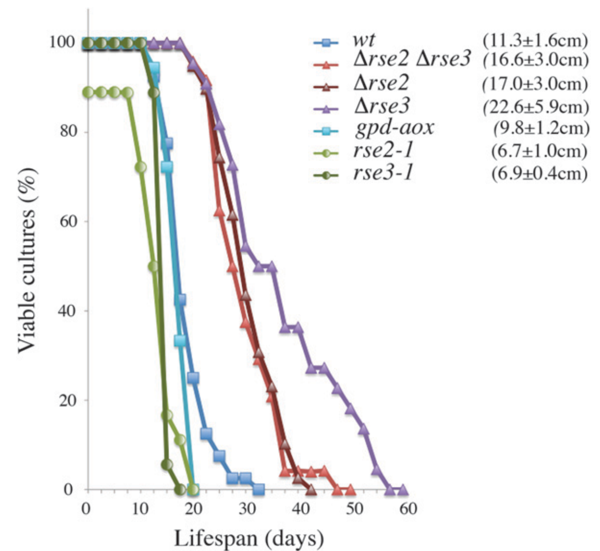

Figure 6.—Life-span analyses. For each genotype, at least 18 subcultures (representing the two mating types) were grown on M2 medium at 27° in race tubes. Data were plotted as the cumulative survival in time using Kaplan–Meier estimates. The mean longevity in centimeters ± standard error is in parentheses.

DISCUSSION

Characterization of mutations in two conserved zinc-cluster proteins that control the expression of the alternative oxidase and gluconeogenic genes: We report in this study the characterization of mutations in two transcription factor genes, *rse2* and *rse3*, each encoding a zinc-cluster protein controlling the induction of the alternative oxidase and the expression of gluconeogenic genes. These mutations were selected in a screen for suppressors of the long-lived respiratory mutants *cox5::ble* and *cyc1-1*. They partially suppress the detrimental effects and restore the senescence process in these mutants. The three mutations reported here are located in two different genes; several other suppressors nonallelic to *rse2* and *rse3* were obtained but have not yet been studied in detail. Thus, the screen was not exhaustive. The two gene products contain Zn(2) Cys(6) binuclear cluster DNA-binding domains. Database searches and recent published data reveal that these genes are present in other ascomycetes. RSE2 corresponds to AOD2 from *N. crassa*, AcuM from *A. nidulans*, and RDS2 from *S. cerevisiae*. RSE3 corresponds to AOD5 from *N. crassa* and AcuK from *A. nidulans*.

We show here that the mutations *rse2*^{Y300D} and *rse3*^{G642V} confer higher levels of mRNA of the alternative oxidase and gluconeogenic genes compared to the wild-type strain and are dominant, strongly suggesting that they correspond to gain-of-function mutations. The mutations *rse2*^{Y300D} and *rse2*^{G303S} are not located in a conserved predicted functional domain (Schjerling and Holmberg 1996); however, their clustering pinpoints one region in RSE2 with potential significance for the function of this transcription factor and indicates that the integrity of this region is necessary to keep the transcription factor in a less-active form. Both the affected residues Y300 and G303 are conserved in *aod2* (*N. crassa*), *RDS2* (*S. cerevisiae*), and *acuK* (*A. nidulans*) and lie in a region of the protein highly conserved between the four organisms. In the same way, the G642 of *rse3* is conserved in *aod5* (*N. crassa*) and *acuK* (*A. nidulans*) and belongs to a short region that is conserved among the three organ-

isms (see Figure S1 and Figure S2). The question how these single amino acid substitutions modulate the activity of the RSE2 and RSE3 transcription factors is unresolved. Although the levels of *rse2* and *rse3* mRNA in the mutant strains are unchanged, hypotheses such as an increased protein stability conferred by the mutations cannot be excluded. However, some other interesting possibilities can be proposed. For example, the *rse3^{G642V}* mutation in the C-ter of RSE3 could make the transactivation domain more accessible or increase its intrinsic activation properties (Schjerling and Holmberg 1996). The mutations *rse2^{Y300D}* and *rse2^{G303S}* in RSE2 could reveal a latent activation domain or could change an interaction with an inhibitory protein. The hypothesis in which conserved motifs in zinc-cluster proteins could have an inhibitory role is based on several studies in *S. cerevisiae* showing that deletion of these motifs renders these proteins constitutively active (MacPherson *et al.* 2006).

Regarding the relationships between RSE2 and RSE3, the observation that the Δ*rse2 rse3*$^+$, Δ*rse3 rse2*$^+$, Δ*rse2 rse3^{G642}*, and *rse2^{Y300D}* Δ*rse3* strains are unable to induce the alternative oxidase unambiguously demonstrate that both proteins are required for this induction even when one of them is present in a mutated form. This result agrees with observations reported for *N. crassa* in which neither the *aod2* nor the *aod5* mutants are able to induce AOX (Descheneau *et al.* 2005) and in *A. nidulans* in which neither the *acuK* nor the *acuM* mutants are able to induce PCK (Hynes *et al.* 2007). In *N. crassa*, electrophoretic mobility shift assays showed that AOD2 and AOD5 act synergistically to bind an alternative oxidase induction motif (AIM) present in the promoter of the *aod-1* gene, which encodes the alternative oxidase. These data support the hypothesis that the two proteins interact with each other (Chae *et al.* 2007b). The AIM motif consists of one pair of CGG repeats separated by 7 bp and is essential for the inducible expression of the *aod-1* gene. It is present in the upstream sequence of the *aox* gene of *P. anserina* and other Sordariales (Chae *et al.* 2007a); however, it is absent from the 900-bp upstream coding sequence of the *Papck* and *Pafbp* genes whose expression is also controlled by RSE2 and RSE3. Several explanations can be proposed for this observation. One possibility is that there is a cryptic motif that we have not spotted in the promoter of these genes. Another one is that activation of these genes requires other factors interacting with RSE2 and RSE3 and determining DNA-binding specificity. A third hypothesis is that RSE2 and RSE3 are indirect activators of the gluconeogenic genes by regulating the production of an inducing molecule.

RSE2, RSE3, and the control of longevity in *P. anserina*: We have previously shown that in *P. anserina*, inactivation of genes encoding components of the cytochrome pathway leads to the induction of the alternative oxidase and to a spectacular increase of life span (Dufour *et al.* 2000; Sellem *et al.* 2007). We have also shown that the introduction of the *gpd-aox* transgene in the long-lived *cox5::ble* and *cyc1-1* leads to increased expression of the alternative oxidase in comparison with induced expression of this enzyme in the nontransgenic *cox5::ble* mutant and to the restoration of senescence associated with an improvement of the phenotype (Lorin *et al.* 2001; Sellem *et al.* 2007). We show here that, in response to a block of the cytochrome pathway, there is induction of the *aox* gene but also of the *pck* and *fbp* gluconeogenic genes and that this induction is under the control of the two zinc-cluster proteins RSE2 and RSE3. These results therefore question the reasons for the very great life span of the respiratory-deficient mutants of *P. anserina* and the role of the different pathways that are induced in the control of longevity. The observation that gain-of-function mutations of genes *rse2* and *rse3* lead to a decreased life span whereas deletion of these genes, in particular of *rse3*, results in an increased life span, strongly suggests that some (direct or indirect) targets of the RSE2 and RSE3 proteins contribute to shortening life span. Although the *aox* gene is greatly induced in the *rse2^{Y300D}* and *rse3^{G642V}* mutants, it seems unlikely that the reduction of the life span of these mutants results from this induction because the *gpd-aox* transgenic strains show no reduction of life span. The *aox* gene is therefore probably not involved in the control of life span in a respiratory-competent context. In contrast, in a *cox5::ble* or *cyc1-1* context, we found a correlation among the mycelium phenotype, longevity, and AOX levels. Increased *aox* gene expression leads to a reduction in life extension and counters the detrimental phenotypic effects due to the *cox5::ble* or *cyc1-1* mutations. This positive correlation between the amount of AOX and the improvement of the phenotype of *cox5::ble* and *cyc1-1* mutants supports our proposed mechanism that increasing the electron flow through the alternative pathway is accompanied by increased oxygen consumption and increased ATP formation at the first coupling site (Lorin *et al.* 2001, 2006).

Many studies have demonstrated the central role of metabolic regulation in the aging process. While it is impossible to highlight all such studies, it is worth noting that a simple reduction of available glucose in the media results in life extension in *P. anserina* (Maas *et al.* 2004) and in yeast (Lin *et al.* 2000). More relevant to our discussion is that, in *S. cerevisiae*, a metabolic shift from glucose metabolism and fermentation toward respiration plays a central part in this life extension (Lin *et al.* 2002). In the same way, caloric restriction induces a metabolic reprogramming characterized by a transcriptional shift toward energy metabolism and upregulation of gluconeogenesis in mouse skeletal muscle (Lee *et al.* 1999). Recently, transcript profiling data from *C. elegans* dauer larvae and long-lived *daf-2* mutant adults revealed increased expression of genes encoding gluconeogenic enzymes (McElwee *et al.* 2006). It is therefore possible that gluconeogenesis is a

conserved pathway in the control of longevity in a wide spectrum of organisms.

A block of the respiratory cytochrome pathway is expected to lead to a wide spectrum of transcriptional changes in the cell. Our study shows that the two zinc-cluster proteins RSE2 and RSE3 are involved in this transcriptional response by activating the expression of the genes encoding the alternative oxidase and major enzymes of gluconeogenis. To identify the other genes whose expression is regulated by RSE2 and RSE3, transcriptome profiling of the strains carrying the different alleles (wild type, gain of function, deleted) of genes *rse2* and *rse3* will be conducted. This should allow us to gain insights into the physiological role of these zinc-cluster proteins and especially their role in the cellular response to a defect in respiratory function. We are convinced that these data will clarify the parameters involved in the control of the life span in *P. anserina*.

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Mutations in Two Zinc-Cluster Proteins Activate Alternative Respiratory and Gluconeogenic Pathways and Restore Senescence in Long-Lived Respiratory Mutants of *Podospora anserina*

Carole H. Sellem, Elodie Bovier, Séverine Lorin and Annie Sainsard-Chanet

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```
PaRSE2     1 MTATEAME------RTAGNET------------KDIK-SESNTKPKDHHAPSTDDQQQHN
NcAOD2     1 MTGTEATE------KPNGKEAGT----------KDITKSGSDTKPKDHHPTPADDVQ---
AnACUM     1 MTEKNTTQPGSAPDQYAGNGTAENTGPSKTLDGNKVGRVDSNAKENAQKSPASKTESTPA
ScRDS2     1 -------------------------------------MSANSG-----------------

                                  •  •      •     •  •        •
PaRSE2    42 QHNHHTTHHQTPKKRRKVSHACLYCRRSHMTCDLARPCTRCVKRNIGHLCHDEPRDQGSN
NcAOD2    42 ---------KAPKKRRKVNHACLYCRRSHMTCDLERPCTRCIKRNIGHLCHDEPRDTESR
AnACUM    61 G------TRTSPKKRRKVNHACVYCRRSHMTCDSERPCTRCIKRNIGHLCHDEPREP-SK
ScRDS2     7 -----------VKRASKAFKTCLFCKRSHVVCDKQRPCSRCVKRDIAHLCRED--DIAVP

PaRSE2   102 KSKSVVAPSTTHGSASQSDLGRGNMNQTAADALRLASFDGSLSSGTGSAASAAFDAAAAL
NcAOD2    93 KAKSVLGTSTLHDSESQPDIGRN----ATDKAMRPPGFDSGMGNG-----SVQVAGAAAV
AnACUM   114 RSRSEHEQSAADEEGSSNNEYSK-----VHAMPRKVDIQDAAGQQ------ILADGSLGL
ScRDS2    54 NEMPSQHESSPNDNNIQGKYANKAHTGIPSDYQNEPVNKSGSTYG--EELSPKLDSSLVN

PaRSE2   162 GQSNQLQLVQPTAVSGLQGSTLGSSMNQFP---------------------------HEV
NcAOD2   144 GRGAPLQLVQPGSVAGIQASALGGSMNQFAGLPDSWLTTQNHYHDMHNFHPNYMV-APEV
AnACUM   163 TPSS-MNAVQPGPMSSSTSQNMSVTSQQQLLGYNDWVGGQNQFQDMHTFHPSYMFNAPEV
ScRDS2   112 DTTSLLLPQQPVFVSENVGSEFSSLNEFLSMLEN------------------------PL

PaRSE2   195 SSEFDVLNNYLRANIFEDPVAAPDGQ-----NQGRPIPGFP------------------S
NcAOD2   203 TNEFNLLNEFLSAGLLEESAFMSDDHGLILGANQSAVSGLPNANNNGNNASSNNKGNSSS
AnACUM   222 TNEYNLLGDFLSNSLLDDGSIFQNEDMQRMYSDPTLINSMAVLG--------------GP
ScRDS2   148 LTQTSLSSSSASNVHLENGSQTTQSPLEYQNDNRRDEIGVARQENR------------SP

PaRSE2   232 SSSMPPPATAPGASLPTANSEQSAAVSK------DKTR-EYYLQAADPSGNADADDRMLQ
NcAOD2   263 TSGMLPPSATQGTSMLPPSSDQTTAIGKPASTNLDNARDAYYLQAADPSGNDTPEERMQR
AnACUM   268 STSLLQQSQTLQPQQSQQNQGDTASGATIGN---DKARETYYMTAADPAGSDPPEERMNK
ScRDS2   196 TIMSGSSNSISKGDKQDQEKEESRILANANENSAPTPKEQFFLTAADPSTEMTPEHRLKL

                            *  *
PaRSE2   285 VLTAKVEAGLLQPFNYIKGYQSLQTYLNEHVSPGSRQKILRQLDRFRPKFREKMQGRDHM
NcAOD2   323 LLRAKYEAGLLKPFNYILGYKRLSDYLDGHVSPTSKQKILKQIDRFRPKFREKIQLLTDM
AnACUM   325 LLKAKYDAGLLRPFNYVKGYARLSAYMEKNLQVSSRQKIARQLDKFRPKFRDRMQKLTDI
ScRDS2   256 VINAKLEAGLLKPYNYAKGYARLQDYMDKYMNQSSKQRILKPLSTIRPAFRTIARSLKDV

PaRSE2   345 FLTINEMQIESSLMEYDRVFASMAVPACCWRRTGEIFRGNKEMAELIDVPVEDLRNGKTK
NcAOD2   383 DLLMVEMWFERTLLEYDRVFASMAVPACCWRRTGQIFRGNKEMAELIGVPVESLRGGQIA
AnACUM   385 ELILVEMWFERSLMEYDRVFASMAIPACCWRRTGEIFRGNKEMAELIGVPIELLRDGKLA
ScRDS2   316 DLVLVEESFERMLLSYDRVFTSMSMPACLCRRTGEIYRANKEFASLVDCTVDDLRDGKLA

PaRSE2   405 LHEILTEESVVRYWEEFGTIAFDPLHDTLLTACTLKSPNKS-SKKVVNCCFSFRIKKDNA
NcAOD2   443 LHEILTEESNVRYWEEFGTIAFDPAHDTLITACSLKNPNDDKGTKVVNCCFSFRIRRDDH
AnACUM   445 IHEIIVEDQLVSYWEKFGAIAFDNTQKAMLTSCTLKNPNATSPTEGIPCCFSFTIRRDNH
ScRDS2   376 IYELMTEESAVNFWEKYGSIAFDKGQKAVLTSCSLR---TKDGIRKRPCCFSFTIRRDRY

PaRSE2   464 QVPG-----LIIG---------------NFIPHDP-------------------------
NcAOD2   503 KIPS-----LIVG---------------NFLPHDP-------------------------
AnACUM   505 NIFLT---PLILVRLLLLEISYQRNGIPNRKKHSSRFQDQTQSLSVCRGALKQLTRFAIQ
ScRDS2   433 NIPICIVG--------------------NFIPLS--------------------------

PaRSE2       ----
NcAOD2       ----
AnACUM   562 KLLF
ScRDS2       ----
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FIGURE S1.—Alignment of RSE2 with homologs from *Neurospora crassa* (Nc), *Aspergillus nidulans* (An) and *Saccharomyces cerevisiae* (Sc). Black and grey highlights indicate identical and similar residues, respectively. Black circles indicate the zinc cluster domain. Stars indicate the position of the mutations Y300D and G303S.

```
PaRSE3    1 MPEDGGPFGSEAAEASGAMSETENEYDDHEPHHKDEDD-RMSEQNTTPDGVDAGGEVKKK
NcAOD5    1 MPDDVGP---AEAEVSGAVSESDNEYDETEVTTKDDDDEKMAERSVASEGVETNGDQKKK
AnACUK    1 METETGK----GATAPPAES-SGVEQDTAAVGAPADQPPKTNANATSNANGEDQPANGQK

                             •  •      •     •  •        •
PaRSE3   60 YDPKDPLRPRRKKARRACYACQRAHLTCGDERPCQRCIKRGLQDSCQDGVRKKAKYLHDA
NcAOD5   58 YDPKDPLRPRRKKARRACYACQRAHLTCGDERPCQRCIKRGLAEACQDGVRKKAKYLHDA
AnACUK   56 ANPKDPSRPRRKKARRACFACQRAHLTCGDERPCQRCIKRGLQDACHDGVRKKAKYLHDA

PaRSE3  120 PPEALRPVLGPN--YNPNAPSSRHGGQRHHS-VSTDA-STVRTFFSHSNASQYPVYSSTQ
NcAOD5  118 PPEALRPVLGPN--YNPAAAVSVRNGHRHPSNAGSDAGSSIGTFYSQS--TQYPVFSSAA
AnACUK  116 PDGALMPGIGGNNFYNNNSMSNGVPSGGINMNGANTVNSAASTQNSSANFYPTPQSNYSL

PaRSE3  176 SIPHGLTESLPFNSQQSPVSPTFQQT-------------------SSNPPISGMVAPPVS
NcAOD5  174 TQLGSIPENLPFP-QQSPVSPTFQP--------------------SSNPQLGSIGVSSVS
AnACUK  176 YQENPINHQNSFP-SQSPVSPTFSLKTNPTPRNTAPNNNNNNALTSSMPQPATTGVSNAP

PaRSE3  217 S-PMTPFG-LPFDPSDPNIFNFNIDGLNFGSHYGAMEFGMLGHMSSSAADTPPQESGMGQ
NcAOD5  213 S-PMNSFPPALFDPSNPAIFNFNLEGLNFGSQYGAMEFGMLGHMSSGAAETPPRDPSMAQ
AnACUK  235 NQSQNPFAGPFFDPSDPALFNFDLSSMNFENRYGALEFGMLGHMATGAGDSPDSGTHRGS

PaRSE3  275 Q--PGDVHFG-AGLFGS-----------HFDN--RMLPEFLGLDAGAN-----GIY---S
NcAOD5  272 QG-TSDVGFNPSGVFGNGLN----QFEKVYDNNTGLISDFLTLDAHSN-----GLY---S
AnACUK  295 MGRSGSTQFASTPIGGTTTFGESPQNQQPFMFGDPLLNEWPSGQTSGQPHVNVGVYPQSS

PaRSE3  311 QGNLQHG------LPHAYAIPAGPTSLQSPSTENNSPQPTTFGFDDRPSPTMSQYP----
NcAOD5  319 QGNLQHG------LPHAYAIPAGPTSLQSPSTENNSPQPTGFGFESPTATNYTGVPGAAG
AnACUK  355 QGNVIPGHLSKPDAPHAFAIESGPNNFTSPGAAT-SPQINSGGYEDANAFNNVVTK----

PaRSE3  361 NAPGAKSSSNSRPSK--LRKLDKVAILQ--KRQRDPSYIYDTVKKSFDYVGSFHKLFEVL
NcAOD5  373 NQPGSQQPRAQKPKTPALGKLGPQSVLG--KRQRDPSSIYEAVKEPFQYVASFHKLISLL
AnACUK  410 -SNGLSVNGQQRPPTISTPSLKHQSLQMNKRRHRNPSAVYESVKEPYAYTSRFHSLTAFI

PaRSE3  417 SSRFSQPHAARIAKSLAAIRPALLASTRNLTTQDLIFMEQCFQRTLFEYEDFMTQSSSPT
NcAOD5  431 QNRFSGASTISIVRSLASIRPSFMSCMKTLNRADLIFMEKSFQRALFEHEEFMHQSPSPA
AnACUK  469 QRRFSPQKTLQIAKALASIRPSFIATTKTLNRDDLIFMEKCFQRTLWEYEDFINACGTPT

PaRSE3  477 LACRRTGEIAGVNKEFTALTGWTKDVLLGKEPNRNTNLGGTG---------------VRT
NcAOD5  491 IACRRTGEIAAVNKEFTALTGWTKDVLLGKTLNLNANMGGTNSDTLSISSKGGRGGIVGT
AnACUK  529 IVCRRTGEVAAVGKEFSILTGWKKEVLLGKEPNYNVNTGGSS---------------AAN

PaRSE3  522 TPRLKSLNESSAENGGAASG--------PRPVFLAELMDHESAVEFYEDYSQLAFGDSRG
NcAOD5  551 TPRLKPLHPEQGTNADSQQQQSQQHKEQPQPVFLAELMDEASVTQFYEDYAQLAFTHSRG
AnACUK  574 SRNITPRSSVESTGR-------------PHPVFLAELLDDDSVVEFYEDFARLAFGDSRG

PaRSE3  574 RMTRKCRLLKYRTDK--------------PAAGGGGGAGE---EERKPDPSAAPRQQEKD
NcAOD5  611 TVVRKCRLLKYRTQENMDAAAAAAAAASAPTASGGSGSSNGTVVNGGPDSSPAGKTEKER
AnACUK  621 SVTTRCKLLKYKTKE---------------------------------DMEAAQSDDNGQ

                                            *
PaRSE3  617 ------SRHSILSNRVAKIDGEHGISKLE-RDGKLECSYTWTIKRDMFDMPMLFVINVRF
NcAOD5  671 PTGVNVASNSILSNRVAKIDGEHGISKLE-RDGKLECSYTWTIKRDVFDIPMIIMINFLP
AnACUK  648 ------RWNNHLRK--GGIANEAGMNQLGFKDGKVECAYCWTVKRDVFDIPMLIVMNVRL

PaRSE3  670 FFFFSTITMEEGIANGILVFTMLLPES
NcAOD5  730 CYYRSHNQLAV----------------
AnACUK  700 PLP------------------------
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FIGURE S2.—Alignment of RSE3 with homologs from *Neurospora crassa* (Nc), *Aspergillus nidulans* (An) and *Saccharomyces cerevisiae* (Sc). Same legend as in Figure 1A except that the star indicates the position of the mutation G642V.

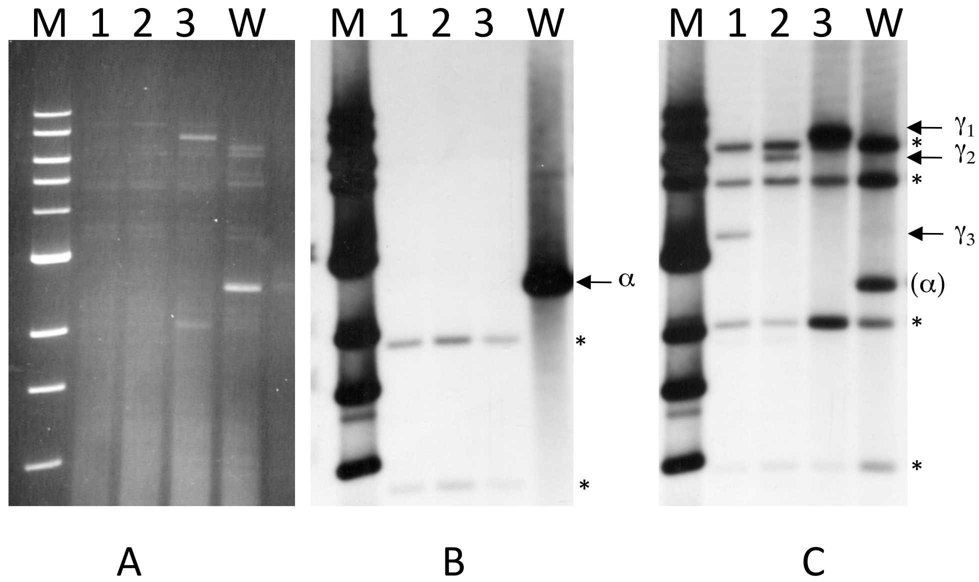

FIGURE S3.—(A) *Hae*III restriction patterns of mtDNA extracted from independent senescent cultures of *cyc1-1 rse3-1* (1, 2, 3) and of wild type (w) strains whose lifespans were 35, 44, 37 and 9,6 cm respectively. (B) and (C) Southern hybridization with ^{32}P labeled probes corresponding to the α (B) or γ (C) region of the *P. anserina* mitochondrial chromosome (Albert and Sellem, 2002). Asterisks point out non-rearranged chromosomal *Hae*III fragments. Arrows point out fragments absent in juvenile wild-type cultures corresponding to junctions of circular senDNAs. Position of senDNAα is indicated by a. The senescent wild-type culture (w) only contained senDNAa whereas the 3 senescent cultures of *cyc1-1 rse3*-1 only contained senDNAsγ. The senDNAα detected on the blot hybridized with the γ probe (α) is the result of the reprobing.

TABLE S1

Characteristics of the 12 SSR markers used for the genetic linkage analysis of *rse2*

Marker	Position on chrm3_SC2	Forward primer	Reverse primer	expected size*	
				in *s* isolate	in *T* isolate
198ks/a	646370-646670	TGTCTCGAAACCCCCTTTAC	GAAAATGTAAGCCCCGTGTT	301	-
539/770	881178-881409	CGTCAACCTCTTCTCTCGTGT	TTGGAACTGGGGATGTTAGC	232	-
440/651	949785-949996	GCCGAGCAGGGTATAAAATG	GATTGTCGTCGCTCTTGAAA	212	-
inc133-1a/b	1015820-1016093	TCCGCATTCATCTTCCACT	AGAAGGCTGAACCGAGGAG	274	-
inc133-2a/b	1195841-1196187	GCACCTCCCATCAAAACCT	TTCCGACTTTTCGAGTTGCT	347	-
inc133-3a/b	1448053-1448395	AAGCAAGGGAGAGGTTGGTAG	GTAACTCGACCGCCCCTTA	343	-
N1	1486762-1487055	ACACGACACAACCGACCA	GCGGCGACAACGAATAAT	260	-
130ks/a	1605394-1605705	AGGGCTTCCTTGAGTGTGTC	CTCACCCTCACCATCAACAC	312	-
N3	1486762-1590877	TGGAGGAGCCGGATATTG	TGGATCATCGCCATTCTG	310	+
415ks/a	1875644-1875954	ACGAACCTCGATAGGTCCAG	AGGAACCACACAGCACACAT	311	+
397ksa/b	2318128-2318436	GTGGTGATGGGCATTGACTA	CCAACAACCACCATCAACAT	309	-
620ksa/b	2540384-2540689	AATACGAATCCTCCCCCTCT	CGGTCGAGGTTTTGTAGCTT	306	-

* Expected size of the PCR amplification (in nucleotides) in the *P. anserina s* strain.
In *P. comata T* strain the amplification is either larger (+) or smaller (-) in size.