Lack of mitochondrial citrate synthase discloses a new meiotic checkpoint in a strict aerobe

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Mitochondrial citrate synthase (mCS) is the initial enzyme of the tricarboxylic acid (TCA) cycle. Despite the key position of this protein in respiratory metabolism, very few studies have addressed the question of the effects of the absence of mCS in development. Here we report on the characterization of 15 point mutations and a complete deletion of the *cit1* gene, which encodes mCS in the filamentous fungus *Podospora anserina*. This gene was identified genetically through a systematic search for suppressors of the metabolic defect of the peroxisomal *pex2* mutants. The *cit1* mutant strains exhibit no visible vegetative defects. However, they display an unexpected developmental phenotype: in homozygous crosses, *cit1* mutations impair meiosis progression beyond the diffuse stage, a key stage of meiotic prophase. Enzyme assays, immunofluorescence and western blotting experiments show that the presence of the mCS protein is more important for completion of meiosis than its well-known enzyme activity. Combined with observations made in budding yeast, our data suggest that there is a general metabolic checkpoint at the diffuse stage in eukaryotes.

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Introduction

Mitochondrial citrate synthase (mCS) is the initial enzyme of the tricarboxylic acid (TCA) cycle, which plays a central role in aerobic energy production and metabolite interconversions. One of the key compounds synthesized in the TCA cycle is α -ketoglutarate, a precursor of glutamate which is itself a precursor for the synthesis of other amino acids. Lack of CS activity leads to glutamate auxotrophy and to respiratory defects. Thus, in strict aerobes, a functional TCA cycle is required for viability. In plants and fungi, a CS activity (pCS) is also found in peroxisomes (glyoxysomes) in which it catalyzes the first step of the glyoxylate cycle, which participates in gluconeogenesis. There is a tight metabolic interaction between the two organelles due to the shuttling of some

metabolites between peroxisomes and mitochondria (Tolbert, 1981; Gancedo and Serrano, 1989). The key position of mCS in respiratory metabolism and its high level of conservation through evolution explain why the genes (or cDNAs) encoding this enzyme have been cloned in many eukaryotes from fungi to plants and animals. This protein was also crystallized nearly 20 years ago (Remington *et al.*, 1982). It is thus surprising that inactivation of these genes, or inhibition of their expression, have been reported so far in only two eukaryotes: the yeast *Saccharomyces cerevisiae* and potato plants.

The *S.cerevisiae* nuclear genome contains three genes encoding CS isoforms (Suissa *et al.*, 1984; Kim *et al.*, 1986; Jia *et al.*, 1997). The *CIT2*-encoded protein (Cit2p) is located in peroxisomes and participates in the glyoxylate cycle (Lewin *et al.*, 1990). The *CIT1* (Suissa *et al.*, 1984; Rickey and Lewin, 1986) and *CIT3* (Jia *et al.*, 1997) products (Cit1p and Cit3p, respectively) are located in mitochondria. Cit1p is the major mitochondrial isoform (Jia *et al.*, 1997) and participates in the TCA cycle. It is worth noting that *S.cerevisiae* $\Delta cit1$ strains do not show any significant respiratory defects when grown on non-fermentable carbon sources other than acetate (e.g. glycerol and lactate). The precise role of Cit3p has not yet been elucidated. Deletion of *CIT3* does not cause any respiratory defect, and the $\Delta cit1 \Delta cit3$ strains are still able (albeit very slowly) to grow on glycerol medium (Jia *et al.*, 1997). The fact that strains devoid of mCS maintain respiratory abilities may be explained by metabolic cross-feeding of citrate produced by pCS (Cit2p): expression of the *CIT2* gene is strongly increased in the absence of *CIT1* (Liao *et al.*, 1991). However, the presence of pCS is not able to suppress the inability of *cit1* mutants to grow on acetate-containing media.

In potato plants, mCS activity was reduced to 6% of wild-type activity by using an antisense RNA approach (Landschutze *et al.*, 1995). Surprisingly, despite this strong reduction in mCS activity, no significant changes were observed during vegetative growth and development. Furthermore, there were no differences in respiration rates between wild-type and transgenic plants. In contrast, a severe effect on flower formation was observed. In the most strongly inhibited lines, flowers were either not formed or were aborted at an early stage of development. In these latter cases, the ovaries appeared disintegrated (Landschutze *et al.*, 1995). These striking features of plants displaying a dramatic reduction of mCS activity remain unexplained.

We report here a third example of mCS defects in a eukaryotic organism. The *cit1* gene encoding mCS in the filamentous fungus *Podospora anserina* has been characterized. Sixteen mutant strains bearing either a point mutation or a complete deletion of the gene have been submitted to extensive analyses combining molecular,

immunological, cytological and genetic approaches. The striking and unexpected property of the *cit1* mutants is their developmental defect: in mutant $\times$ mutant crosses, meiosis hardly proceeds beyond a particular stage of the meiotic prophase, the diffuse stage. As stressed in the Discussion, this stage is a major landmark of oogenesis for a number of animal species and of meiosis commitment for *S.cerevisiae*. Altogether, these observations and our data lead us to propose that there is a general metabolic checkpoint at the diffuse stage in eukaryotes and that lack (or decreased production) of mCS activates this checkpoint.

Results

Identification and cloning of the *cit1* gene

In *P.anserina*, the *pex2* gene (formerly *car1*) encodes the PEX2 protein, involved in peroxisome biogenesis (Distel *et al.*, 1996). The *pex2* mutants are unable to grow on long-chain fatty acids (e.g. oleic acid) as sole carbon sources (Berteaux-Lecellier *et al.*, 1995). A systematic search for mutations able to restore growth of the *pex2* mutants on oleic acid led to the isolation of 63 extragenic suppressors of the *pex2* mutations. These suppressors fall in six genes called *suo*, for suppressors on oleic acid (Ruprich-Robert *et al.*, 2002). Twenty-five percent (16/63) of the *suo* mutations lie in the *suo4* gene, which was re-named *cit1* after its sequencing (see below). In a *pex2*⁺ background, most *cit1* mutations cause a developmental defect: they impair development of fruiting bodies (perithecia) and sporulation when homozygotes in a cross (see below for a detailed description). In addition, with the exception of two alleles, mutant ascospores, obtained from heterozygous crosses, do not ripen and exhibit a low germination level. Otherwise, these mutants have no visible vegetative defects. In particular, their growth rates are similar to those of wild-type strains on current media, including those containing oleic acid as sole carbon source (Ruprich-Robert *et al.*, 2002).

The *cit1* mutants are recessive, and their developmental defect was used to clone the *cit1* gene by complementation of the *cit1-1* mutant (see Materials and methods). Sequence analysis revealed that this gene encodes an mCS, the initial enzyme of the TCA cycle. The *P.anserina* protein shows 69, 60 and 45% identity with the mCS from *S.cerevisiae* (Cit1p), pig and *Arabidopsis thaliana*, respectively. The highest similarities were observed with the enzymes from filamentous fungi. The *Neurospora crassa* (Ferea *et al.*, 1994) and *P.anserina* polypeptides exhibit >90% identity across an overlap of 469 amino acids. This strong similarity extends into the putative mitochondrial targeting sequences (67% identity across a 27 amino acid overlap). Furthermore, the *P.anserina cit1* gene contains three introns located at the same positions as three of the four introns observed in the *N.crassa cit1* gene (see DDBJ/EMBL/GenBank accession Nos M84187 and AJ296102 for the *N.crassa* and *P.anserina* sequences, respectively). The *P.anserina* CIT1 polypeptide shows 79% identity with its *Aspergillus nidulans* homolog (Park *et al.*, 1997; accession No. U89675). The *P.anserina* genome probably contains a single copy of the *cit1* gene as

suggested by hybridization experiments at low stringencies (Southern and northern blots, data not shown).

The abundance of the *cit1* mRNA was measured in northern blots after growth of the wild-type strain in current culture conditions and in the presence of oleic acid. We observed that *cit1* is highly expressed: the level of its mRNA does not differ significantly from the level of the *AS1* messenger which encodes a protein of the cytosolic ribosomes (Dequard-Chablat and Sellem, 1994). Moreover, oleic acid does not increase the transcription level of *cit1* (data not shown).

Molecular mapping of 16 *cit1* mutations and construction of a $\Delta cit1$ strain

To ascertain that *cit1* was indeed the relevant complementing gene, it was sequenced in the 16 mutants available. They all contain a mutation in the *cit1* coding region. As shown in Figure 1B, the *cit1* mutations are scattered along the coding part of the gene from codon 91 to codon 459. Half of the mutations are either frameshift (5/16) or nonsense (3/16) mutations; they are located between codons 124 and 201. Surprisingly, although all mutants were recovered independently, one frameshift (F176) and one nonsense (N175) mutation were found twice. This may reveal a hot spot for UV mutations: in particular, the frameshift mutations (loss of 2 bp) occurred in a stretch of six pyrimidines. In the following analyses, we used one of the two N175 and F176 mutants, *cit1-39* and *cit1-1*, respectively. With the exception of *cit1-2* (codon 91), the missense mutations lie in the second half of the gene (codons 244–459). These positions correspond to amino acid residues highly conserved throughout evolution: at the seven positions, the same residues are found in the wild-type mCS sequences of *P.anserina*, *N.crassa*, *A.nidulans* and pig, while, at six positions, the same residues are also observed in the mCS of *S.cerevisiae* (CIT1 product) and *A.thaliana*. The *cit1-32* allele was different: it corresponds to the loss of one of two adjacent lysines (positions 324–325). This residue at position 325 is conserved in the wild-type mCS sequences of the three filamentous fungi and the pig. The conclusion of this analysis was that none of these mutants was a bona fide null mutant. In fact, all the mutant polypeptides keep the mitochondrial targeting sequence. Thus, if the mutant proteins are stable enough, they could be imported into mitochondria and disturb the organelle functions independently of their lack of activity. The developmental defects could be explained in at least two, non-exclusive, ways: lack of CS activity and/or poisoning of a cellular function due to the presence of the altered proteins. This conclusion prompted us to obtain a *cit1*-null mutant ($\Delta cit1$).

A strain carrying a complete deletion of the gene was constructed through gene substitution. The *cit1* coding sequence was replaced *in vitro* by the bacterial *hph* (hygromycin resistance) gene under the control of a eukaryotic promoter (Materials and methods; Figure 2). Our rationale was that, like the original *cit1* mutations, the null allele should act as a metabolic suppressor of the *pex2* mutants. A linear DNA fragment containing the deleted gene was thus used to transform a *pex2-1* strain. Hygromycin-resistant transformants were recovered and

Fig. 1. Fine analysis of the *cit1* mutants. (A) Ascus phenotypes: + indicates that the stage is normal when compared with wild type; +/- means that part of the asci are abnormal as, for example, abnormal nuclear migration during post-meiotic mitosis (PMM); ~ + indicates that several cells show abnormalities as illustrated in Figure 3; A = anaphase. Percentages were calculated from 100–200 perithecia. A double bold bar indicates the stage when all asci are blocked. (B) Mutation map. M, N and F denote missense, nonsense and frameshift mutations, respectively. Numbers show the codon positions. Δ325 refers to loss of a lysine residue at position 324 or 325. Δ*cit1* is a complete deletion of the gene (see Figure 2). The *cit1* sequence has been submitted to DDBJ/EMBL/GenBank under the accession No. AJ296102.

tested for their ability to grow on oleic acid as sole carbon source. Twelve transformants (from 240) displayed this property. Southern blot analysis of six of them confirmed that the resident *cit1* gene was indeed replaced by the Δ*cit1* construct (data not shown). It was also ascertained that crosses between these Δ*cit1* strains and the original *cit1-1* mutant yielded barren perithecia.

Crosses between the *pex2-1* Δ*cit1* transformants and a wild-type (*pex2⁺ cit1⁺*) strain led to the recovery of *pex2⁺ Δcit1* strains, which were submitted to extensive phenotypic analyses. The Δ*cit1* mutation does not lead to any visible vegetative defect. As *P.anserina* is a strict aerobe, it is worth noting that growth, respiration rates and life spans of the Δ*cit1* strains are similar to those of the wild type (data not shown). Moreover, the mutant mitochondria

do not differ from the wild-type organelles with respect to number, size and distribution (Materials and methods). However, as observed for most of the original mutants, Δ*cit1* ascospores (obtained from heterozygous crosses) do not ripen: they are green (while the wild-type ascospores are black) and their germination efficiency is low (10% instead of near 100% for the wild-type ascospores). Although glutamate auxotrophy is a classic CS-deficient phenotype (Carls and Hanson, 1971; Kim *et al.*, 1986), the *P.anserina* Δ*cit1* grows as well as the wild-type strains on minimal medium. The germination defect of the mutant is not due to a conditional (developmental) auxotrophy with respect to this compound: when glutamate was added to the germination medium, the level of Δ*cit1* germination remained unchanged.

Fig. 2. *cit1* gene replacement. The upper line shows the restriction map of the 5 kb genomic fragment containing the *cit1* gene with the length of the restriction fragments indicated in base pairs. The lower line shows the DNA fragment in which *cit1* was replaced by the bacterial hygromycin resistance gene (*hph*) under the control of the *A.nidulans trpC* promoter (*PtpC*). The positions of the oligonucleotides used to amplify the 3'-flanking region of *cit1* are shown by arrows. See Materials and methods for more details.

Developmental defects of the *cit1* mutants

In heterozygous crosses, *cit1* mutant strains can be used as either a male or female partner without any obvious consequences, except that fruiting body (perithecium) development is slightly delayed when the mutants are used as the female partner. In contrast, all perithecia from homozygous mutant crosses are barren, with the exception of three mutants (see below).

Detailed analyses of the mutant fruiting bodies showed that the first steps of sexual reproduction are normal and that meiosis proceeds normally until late prophase (Figure 1A). Wild-type perithecia contain >150 meiocytes which are not synchronous in their development. Therefore, progression of the successive stages from fertilization to mature asci with four ripe ascospores can be easily observed (Figure 3A). In the mutant perithecia, dicaryotic cells are formed, caryogamy and entry into meiosis occur normally but, as pachytene (Figure 3B) is the most prominent stage seen in mutant perithecia, we infer that pairing and synapsis are slower than in the wild-type strain. Moreover, most meiocytes remain blocked at the diffuse stage (Figure 3C). This stage is characterized by a decondensed appearance of the chromosomes and the loss of their stainability with standard techniques. While the preceding pachytene condition is characterized by a rigid configuration of chromatin, during the following stage, the chromatin exhibits a fuzzy appearance in which individual chromosomes are no longer visible (compare Figure 3B and C). In wild type, this diffuse stage is followed by the re-emergence of condensed, individualized chromosomes in the classical diplotene condition (see Discussion for additional information). The arrest observed in mutant perithecia is not due to a delay in exiting this stage because asci remain blocked even in perithecia followed over 10 days after the end of wild-type perithecium development.

Ten of the 14 different mutants exhibit this general picture with a more or less pronounced leakiness (Figure 1A). Three of them, *cit1-3*, *cit1-14* and *cit1-69*, which correspond to the two earliest frameshift mutations and to a missense mutation, respectively, show no leakiness at all: 100% of asci are blocked at the diffuse

stage. Seven other mutants belong to a second subclass in which 70–99% of asci do not proceed further than the diffuse stage. The other asci evolve to later stages and, with the exception of *cit1-1* (F176), even form ascospores. However, most of them are abnormal (Figure 1A). Four mutants clearly stand out: *cit1-32* ($\Delta 325$), *cit1-30* (M407), *cit1-39* (N175) and *cit1-58* (M396). *cit1-32* perithecia develop normally and almost all asci form ripe (black) ascospores which are ejected from the fruiting bodies and germinate as observed in the wild type. *cit1-30* perithecia develop very slowly but asci do not show any specific arrest and all form ascospores. The ascospores, which are ejected 3 days later than in the wild-type condition, are black and germinate normally. Almost half of the *cit1-39* asci also contain four ascospores (Figure 3D) but they are green (unripe) and unable to germinate. In contrast to all other mutants, *cit1-58* perithecia contain very few asci, which show normal prophase but are blocked before ascospore formation (Figure 1A). Although the diffuse stage arrest appears as the most prominent landmark of the *cit1* mutants, it is worth noting that most mutants also exhibit abnormalities before and/or after this stage, i.e. during crozier development, during nuclear migration events and finally during ascospore formation (Figures 1A and 3E).

Amazingly, there is no clear-cut correlation between the developmental phenotypes of the mutants and the type of the mutations (Figure 1). Cytological analysis of the perithecia from $\Delta cit1$ homozygous crosses partially solved this problem. In these crosses, the asci are all arrested at the diffuse stage (Figure 3F and G). Therefore, the leakiness exhibited by some of the *cit1* point mutations is likely to be due either to a residual activity of the mutant enzyme or to the presence of an inactive CIT1 protein still able to perform an unknown function required for progression of meiosis (see Discussion). To clarify this point, we constructed a strain able to express a catalytically inactive but structurally unchanged form of CIT1. In *S.cerevisiae*, a *cit1* mutant with the substitution mutation H313G was shown previously to produce such an mCS form (Kispal *et al.*, 1989). The *S.cerevisiae* Cit1p H313 residue (i.e. H274 in the mature pig enzyme) is one

Fig. 3. Meiosis and sporulation of *cit1* mutants. (A) Wild type: the upper ascus contains ascospores with two nuclei each (small arrowhead); the nucleus of the lower ascus is at the diffuse stage (large arrowhead). (B) *cit1-3*: note that the four asci are at the pachytene stage; arrowheads point to two nuclei in which the synapsed homologs are clearly visible. (C) Three asci of *cit1-1* at the diffuse stage; the arrowhead points to the prominent nucleolus, characteristic of this stage. (D and E) *cit1-39*: this mutant forms either normal (wild type-like) (D) or abnormal ascospores (E). (F and G) Δ *cit1* is completely blocked at the diffuse stage; (F) note the fuzzy appearance of the nucleus in which individual chromosomes are no longer visible (compare with B); (G) lower magnification of a Δ *cit1* group of eight asci. Bars: 5 μ m.

of the conserved residues thought to be essential for mCS activity (Karputas *et al.*, 1990). It corresponds to H309 in *P.anserina* CIT1. The *cit1-H309G* allele was obtained by *in vitro* mutagenesis and introduced into a Δ *cit1* background (Materials and methods). This new mutant displays the same phenotypic properties as those observed in the original mutants. The *cit1-H309G* ascospores, from heterozygous crosses, are unripe and exhibit a low germination level. Perithecia from homozygous crosses are barren but contain asci, of which 85% are arrested at the diffuse stage while 15% contain ascospores among which rare wild-type-like four-spored asci are found (Figure 1A). To ensure that *cit1-H309G* indeed contained an inactive mCS form, we checked the status of the CIT1 protein in this mutant, in comparison with Δ *cit1* and a subset of the original mutants.

Status of the CIT1 protein in the *cit1* mutants

Immunofluorescence experiments using an antibody directed against the *S.cerevisiae* Cit1p protein (Vélot *et al.*, 1999) were performed on the wild-type strain and a selected set of *cit1* mutants. We chose the mutants that exhibit the two extreme developmental phenotypes, i.e. Δ *cit1* and *cit1-32* (Δ 325), as well as the *cit1-39* nonsense (N175) mutants. In addition, crude extracts of these strains were used for CS activity measurements and immunoblot analysis. As shown in Table I, global CS activity is reduced to 25% of the wild-type activity in both Δ *cit1* and *cit1-39* strains, while it reaches 50% of the control in the *cit1-32* mutant. These data lead to three conclusions. First, as deduced from the residual CS activity observed in Δ *cit1*, the *cit1* gene does not control all the cellular CS activity.

Secondly, the *cit1-32* mutant, which exhibits a wild-type phenotype, shows a CS activity intermediate between the wild-type and Δ *cit1* values. This suggests that the mutant protein is still partially active. Thirdly, the CS activity observed in the nonsense (*cit1-39*) mutant does not differ significantly from the residual activity present in the null (Δ *cit1*) mutant (ratio 0.9 for *cit1-39*/ Δ *cit1*). As expected, the same ratio was observed between the *cit1-H309G* and Δ *cit1* activities. This demonstrates that the stringency of the meiotic phenotypes is not related to the level of the residual CS activity.

Immunofluorescence clearly revealed a mitochondrial protein akin to CS in young wild-type asci (Figure 4A), while no staining was detected in the Δ *cit1* asci observed at the same developmental stage (Figure 4B). The same result was obtained with the *cit1-1* (F176) frameshift mutant (data not shown). In contrast, the staining was similar to wild-type asci all through their development, including ascospores, in *cit1-32* (compare Figure 4C and F), *cit1-30* (M407) and *cit1-H309G* (data not shown). The nonsense *cit1-39* mutant exhibits two major differences when compared with the wild type. First, in the young mutant asci, staining is weak and limited to a few patches instead of the typical network observed in the young wild-type asci. The second difference concerns the older asci, which contain ascospores: while, in wild type, the mitochondrial network was stained by anti-CIT1 in both the ascus compartment and the ascospores, this network was only seen in the ascus compartment of *cit1-39*, and not inside the mutant ascospores (compare Figure 4G and I with H and J).

Additional data were obtained with western blotting (Figure 5). Although the polyclonal anti-Cit1p antibody

Table I. Summary of data obtained on the most relevant strains studied

Strain	Mutation	CS activity (%) in crude extracts ^a	In situ mCS detection		Developmental phenotype ^b	
			Asci	Spores	Asci containing spores (%)	Ejected spores
Wild type		100	+	+	100	Ripe
$\Delta cit1$	Deletion	25	–	–	0	
<i>cit1-1</i>	F176	ND	–	–	0	
<i>cit1-39</i>	N175	25	+	–	60	Unripe
<i>cit1-30</i>	M407	ND	+	+	100	Ripe
<i>cit1-32</i>	$\Delta 325$	50	+	+	100	Ripe

^aSpecific activities were measured on crude extracts and are expressed as a percentage of wild-type activity. Average values were determined from either two (wild-type strain) or three (mutant strains) independent experiments. Measurements were reproducible within 15%.

^bSee Figure 1 for more details.

ND, not determined.

Fig. 4. In situ staining with anti-mCS antibody. (A) A wild-type ascus at the pachytene stage: the clear mCS staining reveals a large mitochondrial network. (B) A $\Delta cit1$ ascus: no labeling is observed. (C) A wild-type ascospore: a dense mitochondrial network is observed. (D and E) DAPI staining corresponding to (A) and (B), respectively. (F) A *cit1-32* mutant ascospore: the staining pattern is similar to the wild type (compare with C). (G and H) Asci containing three (G, wild type) or four (H, *cit1-39*) young ascospores: the mitochondrial network is seen in both cases but note that it is not detected inside the *cit1-39* ascospores. (I and J) DAPI staining corresponding to (G) and (H), respectively. Bars: 1 μ m.

from *S.cerevisiae* reveals few unspecific bands, the *cit1* product can be identified unambiguously by its presence at the expected molecular size (52 kDa) in the wild-type and *cit1-32* extracts, and its absence in $\Delta cit1$. Neither a truncated form nor a readthrough product can be seen in the *cit1-39* extracts. However, in the four extracts, the antibody reveals, just below the CIT1 signal, a faint band, which might correspond to another isoform of CS.

Autonomous ascus development due to intragenic complementation and recombination

mCS is known to act as a dimer. We thus addressed the question of possible cases of intragenic complementation

Fig. 5. Immunoblot analysis of crude extracts from *cit1* mutants and wild-type strains. Crude protein extracts (80 μ g) from the indicated strains were subjected to immunoblot analysis with an anti-Cit1p polyclonal antiserum as described in Materials and methods. The large arrowhead and the thin arrow indicate mCS and a putative isoform, respectively. Numbers on the right indicate the size (in kDa) of the SDS-PAGE standards of lane M (BioLabs).

between *cit1* mutants. Mutant $\times$ mutant crosses were performed in all possible combinations of the 15 mutants, including $\Delta cit1$. In addition to crosses involving $\Delta cit1$, homoallelic crosses (in which each mutant was crossed with itself) were used as controls. Intragenic complementation, occurring inside the fruiting bodies after fertilization, should lead to the production of ripe, black ascospores, while lack of complementation should give the same results as those observed in the homoallelic crosses. The *cit1-32* ($\Delta 325$) allele appeared dominant with respect to all other mutants: all crosses involving this mutant produced large amounts of black ascospores. The leakiness of *cit1-30* (M407) and *cit1-39* (N175) in crosses with other mutants (except *cit1-32*) was never increased and sometimes decreased. Complementation was observed only in crosses between missense mutants. The amounts of ascospores from these crosses varied, according to the mutant combinations, from a few percent to 100% of the wild-type controls. In all cases, the ascospores exhibited a wild-type phenotype. The highest levels of complementation were observed in combinations involving *cit1-12* (M459), i.e. the most C-terminal missense mutant. As expected for a mutant expressing a structurally intact protein, *cit1-H309G* showed complementation with *cit1-12*.

In the course of this study, we carefully analyzed all crosses, even those that clearly did not show complementation. Among the latter, we observed the following remarkable data. A number of crosses led to the production of $\sim 10^{-4}$ – 10^{-5} asci, which could be explained only by

intragenic recombination for the following reasons. First, these asci were absent in the negative controls, i.e. homocaryotic crosses and crosses involving *Δcit1*. Secondly, these amounts were in the range of the intragenic recombination (conversion) frequencies observed in *P.anserina* (Touré, 1972). Thirdly, the observed ripe (black) ascospores germinated and their analysis demonstrated that they contained a wild-type (*cit1*⁺) nucleus. The explanation is that intragenic recombination, which occurs during the early meiotic prophase (before the meiotic block characteristic of the *cit1* mutants), leads to a wild-type *cit1* allele which is fully expressed (probably during the diffuse stage) and permits an autonomous development of the relevant ascus up to its normal end, namely ascospore formation. However, in these asci, the ripening of the ascospores is limited to those containing the recombinant wild-type nucleus. Genetic analysis of the recombinant ascospores also showed that the *mat* locus, which does not map on the same chromosome as *cit1*, exhibited the same second division segregation frequency as in a wild-type cross (proof of the occurrence of at least one crossing-over between the centromere and the *mat* locus) and segregated normally in the recombinant *cit1*⁺ nuclei. Also, the number of chiasmata visible in the mutants that are not completely blocked is similar to that observed in wild type. These observations led to the conclusion that the general patterns of recombination and chromosome segregation were normal in the meiocytes that underwent intragenic recombination. However, to ensure that recombination occurs before the mutant arrest, even in the *Δcit1* meiocytes, we used an antibody raised against the Rad51 protein. In yeast, where all meiotic recombination events are initiated by double-strand breaks, immunostaining foci of Rad51p appear only when those breaks have occurred. The *Δcit1* strain shows the same amount of foci and the same temporal order of appearance and disappearance as wild type.

cit1 and peroxisomes

All *cit1* mutations were screened as partial suppressors of the metabolic defect of *pex2* mutants, i.e. as mutations alleviating their inability to grow on oleic acid as sole carbon source. The *Δcit1* mutation also exhibits this property. To shed light on the relationship between *cit1* and peroxisomes, two experiments were performed.

First, peroxisomes were examined by immunofluorescence in the *Δcit1*, *cit1-1* (F176), *cit1-3* (F124), *cit1-35* (M439) and *cit1-39* (N175) single mutant strains and in the *Δcit1 pex2* double mutant strains (Materials and methods). The data were clear-cut: the *cit1* mutant asci showed the same peroxisomal pattern as wild-type asci (Berteaux-Lecellier *et al.*, 1995), at least until they arrest in the mutant context (see above and Figure 1), and *Δcit1* did not restore peroxisome biogenesis in a *pex2* mutant background (data not shown).

Secondly, catalase activities were measured in wild-type, *cit1-1*, *cit1-35* and *Δcit1* strains according to the following rationale. It was suspected previously that the inability of *pex2* mutants to grow on media containing oleic acid as sole carbon source was due to the lethal effect of H₂O₂ production in the cytosolic compartment of the mutants in which β -oxidation is mislocalized. There are thus three ways to alleviate this *pex2* metabolic defect:

Table II. Catalase activities (per minute per milligram) in cell-free extracts of wild-type and *Δcit1* strains

Strain	Carbon source ^a	Expt 1 ^b	Expt 2 ^b	Expt 3 ^b
<i>cit1</i> ⁺	Dextrin	0.014 (1)	0.019 (1)	0.021 (1)
<i>cit1</i> ⁺	Dextrin + OA	0.025 (1.8)	0.041 (2.2)	0.039 (1.9)
<i>Δcit1</i>	Dextrin	0.026 (1.9)	0.041 (2.2)	0.048 (2.3)
<i>Δcit1</i>	Dextrin + OA	0.082 (5.7)	0.079 (4.2)	0.084 (4.0)

^aOA, oleic acid; it was added to the growth medium 16 h before catalase activity measurements.

^bCatalase activities are expressed in arbitrary units. The values in parentheses indicate the increase factors with respect to the wild-type activity with dextrin as the carbon source. In spite of the variations in the crude values observed between experiments, the increase factors appear highly reproducible.

(i) restoration of a peroxisomal compartment; (ii) a decrease in β -oxidation efficiency; and (iii) an increase in the efficiency of H₂O₂ detoxification through, for instance, an increase in catalase activity (Ruprich-Robert *et al.*, 2002). Clearly, the *cit1* mutations do not belong to the first and second classes of suppressors: peroxisome biogenesis is not restored in the *pex2 cit1* double mutants and the *cit1* mutants grow as well as the wild type on a medium containing oleic acid as sole carbon source. However, as shown in Table II, catalase activity is increased 2-fold in *Δcit1* strains as compared with wild type, in both the presence and absence of oleic acid. The three mutant strains tested gave the same results. Moreover, growth in the presence of oleic acid increases catalase activity in wild-type and mutant strains. As previously shown in budding yeast, such an increase is characteristic of the peroxisomal catalase (Skoneczny *et al.*, 1988).

Discussion

In the filamentous fungus *P.anserina*, mutations of the *cit1* gene, which encodes mCS, result in several noteworthy features with three intertwined facets. First, *cit1* mutant strains are viable and do not show any significant vegetative defects except a low efficiency in ascospore germination. Notably, their respiration rates are similar to wild type. Secondly, *cit1* mutations act as partial suppressors of the peroxisomal assembly *pex2* mutations. Thirdly, they impair meiosis progression downstream of the diffuse stage, and a null mutant (*Δcit1*) is completely blocked at this stage.

A second CS isoform ensures viability of the cit1 mutants

In *S.cerevisiae*, the respiratory abilities of a *Δcit1* strain are explained by the up-regulation of the *CIT2* gene (encoding pCS), which would compensate for the loss of mCS through cross-feeding between peroxisomes and mitochondria. This process, in which changes in mitochondrial functions result in alteration of nuclear gene expression, was called retrograde regulation (Liao *et al.*, 1991; Chelstowska *et al.*, 1995).

The viability of the *P.anserina cit1* mutants is probably due to the activity of a second CS isoform, as suggested by our enzyme assays: they reveal that *Δcit1* strains maintain

a residual CS activity that represents 25% of the wild-type global activity. This second isoform may be either mitochondrial or, more probably, peroxisomal. A second gene for mCS has been reported so far only for *S.cerevisiae* where it seems to play a minor role in the TCA cycle (Jia *et al.*, 1997). The genome of *N.crassa* (<http://www.mips.biochem.mpg.de/proj/neurospora/>), a filamentous fungus closely related to *P.anserina*, contains only two genes for CSs, one encoding the mitochondrial isoform (Ferea *et al.*, 1994; contig 2.66) and the other probably encoding the peroxisomal isoform (contig 2.220). Furthermore, an antibody raised against the mCS (CIT1 product) from *S.cerevisiae* gives no signal in the mitochondria of the *P.anserina* $\Delta cit1$ strains. The fact that *P.anserina* *cit1* mutants do not show glutamate auxotrophy is also consistent with the peroxisomal hypothesis: in budding yeast, glutamate auxotrophy has been reported only in strains carrying a simultaneous deletion of *CIT1* and *CIT2* (Kim *et al.*, 1986). Potato plants also survive and show normal respiration rates when mCS production is strongly reduced (Landschutze *et al.*, 1995). In *P.anserina* and potato plants, the critical experiment for the pCS-controlled survival hypothesis requires the inactivation of the gene encoding the pCS isoform. Unfortunately, all our attempts to find this gene have failed so far. This may be explained by the fact that a small number of pCS have been described to date and that they show low similarities to mCS, with the exception of Cit1p and Cit2p from *S.cerevisiae* (see La Cognata, 1996).

In spite of their ability to grow on several respiratory substrates, yeast $\Delta cit1$ strains fail to grow (Kim *et al.*, 1986; Kispal *et al.*, 1988) or display strongly reduced growth (Jia *et al.*, 1997) on acetate medium. McCammon (1996) suggested a toxic effect of this compound in such a mutant background. The same hypothesis could account for the germination defect observed in the *cit1* mutant ascospores, especially because, in *P.anserina*, germination requires acetate, which cannot be replaced by another carbon source.

Increased catalase activity accounts for partial suppression of the *pex2* mutations

The inability of the *pex2* mutants to grow on long-chain fatty acids (e.g. oleic acid) as sole carbon source has been explained by the fact that peroxisome biogenesis is impaired in these mutants (Berteaux-Lecellier *et al.*, 1995). In this context, β -oxidation occurs in the cytosol and this misplaced process may be toxic for the cell, due to production of H_2O_2 , which cannot be detoxified efficiently in this compartment (Ruprich-Robert *et al.*, 2002). The fact that *cit1* mutations partially restore growth of *pex2* mutants on oleic acid can be explained neither by restoration of a peroxisomal compartment nor by a decrease in β -oxidation efficiency. In contrast, the ability of the double mutant strains to grow on oleic acid is probably due to the increase in catalase activity observed in the *cit1* mutant context: decomposition of the H_2O_2 produced in the cytosol through β -oxidation of oleic acid would be efficient enough to allow cell survival. As a 4-fold increase in catalase activity is sufficient to counteract the cytotoxic effects of H_2O_2 in mammalian cells (Santanam *et al.*, 1999), the 2-fold increase found here may also be sufficient. However, growth on oleic acid of

the *pex2 cit1* double mutants is far from being as efficient as the wild-type growth. In addition, the double mutant strains die when they are stored for a few days on this medium, while the wild type can survive for months (data not shown). We do not know how catalase activity increases in the *cit1* mutants. We assume that this could be part of a response to mitochondrial dysfunction, akin to the retrograde regulation described in budding yeast (Chelstowska *et al.*, 1995).

The mCS protein *per se* is required for progression of meiosis downstream of the diffuse stage

As for flower development in potato plants (Landschutze *et al.*, 1995), the effect of *P.anserina* *cit1* mutations on ascus differentiation remains puzzling (see Table I). Although appealing, an energy problem cannot explain the data reported in the two systems. First, in plants, mitochondrial dysfunctions mostly impair late events in flower development, especially production of functional pollen (Conley and Hanson, 1995). Secondly, in *P.anserina*, respiration-deficient mutants are unable to differentiate female organs and are thus blocked far upstream of a meiotic defect. This is observed, for example, in a strain carrying an inactivated *cox5* gene, which encodes a subunit of cytochrome *c* oxidase (Dufour *et al.*, 2000). When the respiratory defect of this mutant is partially overcome and, consequently, its energy production enhanced, female fertility is partially restored but most of the asci are arrested at different steps of their development and not at a precise stage (Lorin *et al.*, 2001). Moreover, the meiotic block triggered by the null *cit1* mutation occurs when the ascus has nearly reached its maximum size, and both growth and size of the asci are similar in the wild-type and the $\Delta cit1$ contexts. A limited energy supply would have been expected to cause a less specific or an earlier block during ascus differentiation and elongation. Such a phenotype is encountered only in the *cit1-58* mutant: it differentiates few asci and shows no specific meiotic arrest. This mutant bears a stringent (L→P) mutation in a highly conserved domain (Figure 1B) and the mutant protein might be toxic to mitochondrial metabolism. In contrast, half of the *cit1-39* (N175) asci contain ascospores, although the mutant strain harbors a truncated CIT1 protein. The *cit1-39* leakiness cannot be explained by a readthrough of the stop codon: this process would produce too few mCS molecules to explain the high frequency of ascospore production. More importantly, the catalytically inactive *cit1-H309G* mutant appears leaky with respect to the developmental defect. Thus, a limited energy supply cannot account for the developmental phenotypes of the *cit1* mutants.

Interestingly, several correlations can be deduced from the mutant phenotypes (Table I). First, the *cit1-32* mutant seems to retain enough mCS activity to ensure a near wild-type developmental phenotype. Secondly, the residual CS activity observed in the null mutant ($\Delta cit1$), although sufficient for vegetative life, cannot compensate for the lack of mCS during sporulation. Thirdly, lack of mCS activity *per se* is not responsible for the meiotic block and the subsequent lack of ascospore formation: the same residual CS activity is observed in the null mutant and in *cit1-39* (N175), of which more than half of the asci form ascospores. Last, but not least, the phenotypic properties of

the *cit1-39* mutant lead to the conclusion that the mCS protein is more important than its activity. The truncated protein, which has lost the 300 C-terminal amino acids, is detected in asci containing ascospores but it could not be detected either in the ascospores themselves or during vegetative growth (western blotting). This suggests that the mutant mRNA or the mutant protein are unstable during these phases of the life cycle. However, the possibility that the truncated protein detected *in vivo* would not be stable anymore in the crude extracts cannot be excluded. In any case, the presence of this truncated protein in the asci correlates with their ability to form ascospores, while lack of the protein in the ascospores correlates with their inability to ripen and to germinate. The *cit1-H309G* mutant provides definitive evidence for an unknown function for the CIT1 protein: in this mutant, the presence of a catalytically inactive enzyme, which is probably structurally unchanged, leads to a partial bypass of meiotic arrest. All these observations disclose an unexpected role for the mCS protein, in addition to its well-known enzymatic function.

In budding yeast, pCS cannot fully compensate the lack of mCS: $\Delta cit1$ strains do not grow on acetate medium. Kispal *et al.* (1989) and Vélot *et al.* (1999) have demonstrated that restoration of a full wild-type phenotype (i.e. growth on acetate) requires the presence, inside the mitochondria, of a molecule akin to mCS and able to interact with its partners in the TCA complex: this can be an inactive but structurally unchanged form of mCS (Kispal *et al.*, 1989). A large body of experimental data supports the idea that the sequential TCA cycle enzymes form a supramolecular complex, allowing enzymatic reactions to occur via channeling of metabolites between enzyme active sites (Srere *et al.*, 1997). In addition, using a transdominant genetic approach, Vélot and Srere (2000) have shown that mCS from *S.cerevisiae* (Cit1p) does interact *in vivo* with its sequential enzyme malate dehydrogenase. In *P.anserina*, lack of mCS could alter the TCA complex in a discrete way, impairing a function involved in meiosis progression. Alternatively, mCS *per se* may be a bifunctional protein as previously shown for other mitochondrial proteins. Interestingly, at least four of these proteins function in the TCA cycle: the NAD⁺-dependent isocitrate dehydrogenase (Elzinga *et al.*, 1993), two subunits of α -ketoglutarate dehydrogenase, and aconitase (Kaufman *et al.*, 2000). It was shown that these proteins bind either mitochondrial mRNAs or DNA. The possible partners of the *P.anserina* mCS remain unknown. In any case, with this view, the degree of leakiness of a given *cit1* mutant would reflect the capacity of the relevant CIT1 protein to participate still in the TCA complex or to bind another partner. The *cit1-39* and *cit1-H309G* alleles exemplify this idea.

Evidence for a meiotic checkpoint at the diffuse stage

Checkpoints were defined by Hartwell and Weinert (1989) as control mechanisms which ensure the proper order of events in the cell cycle by arresting or delaying progression through the cycle in response to defects in cellular processes. In both mitosis and meiosis, checkpoints avoid the production of defective cells. For instance, meiocytes

that fail to complete recombination are arrested at the pachytene stage (Roeder and Bailis, 2000).

Beyond pachytene, the diffuse stage is seen in several animal, plant and fungal species (Barry, 1969; Klasterska, 1976; Zickler, 1977). This stage coincides with dissolution of the synaptonemal complex and is followed by the diplotene stage, with homologs widely separated except at the positions of chiasmata. In many organisms, the diffuse stage is transient, but in other species it can be of very long duration as in amphibian and mammalian oocytes. In the latter cases, it correlates with nuclear growth and/or the development of giant lampbrush chromosomes. In *S.cerevisiae*, the end of prophase is also the stage when cells are committed to completion of the meiotic division (Esposito and Klapholz, 1981). Lee and Honigberg (1996) have shown that, at least in budding yeast, separate nutritional controls act at initiation of meiosis and for meiosis commitment. When the nutritional conditions required for the transition to meiotic completion are not fulfilled, the cells arrest after synaptonemal complex formation and dissolution (Lee and Honigberg, 1996), thus precisely at the diffuse stage. Thus, completion of meiosis progression requires not only fulfillment of the well-known checkpoints but also appropriate nutritional signals (Honigberg and Lee, 1998). Whether *S.cerevisiae cit1* mutants, which are unable to sporulate in an acetate-containing medium, exhibit a precise meiotic arrest in this condition and/or in a sporulation medium containing another respiratory substrate unfortunately remains unknown.

To our knowledge, the *P.anserina cit1-3* (F124), *cit1-14* (F133), *cit1-69* (M310) and $\Delta cit1$ are the first mutants specifically blocked at the diffuse stage. As it appears unlikely that a mitochondrial enzyme would be a positive regulator of meiosis progression, we propose that this specific arrest discloses a checkpoint, which is activated in the absence of the mCS protein. Lack of CIT1 could cause a specific defect in meiosis, which, in turn, could be sensed by an unknown protein triggering the arrest. The critical experiment would be to identify this protein through mutations allowing $\Delta cit1$ cells to complete meiosis but with defective ascospore formation. However, the checkpoint hypothesis is already supported by a fact that fulfills one of the major criteria in the definition of checkpoints (Hartwell and Weinert, 1989): when the arrest is overcome, in the leaky mutants, the late events in ascospore formation are impaired (Figure 1A). *cit1-H309G* is a paradigm for this problem: all spores are abnormal. Obviously, the catalytically inactive but structurally intact protein encoded by this allele is able to elude the checkpoint and thus permits progression of meiosis. The diffuse stage checkpoint would thus avoid the formation of progeny unable to survive, by arresting the cycle in response to a metabolic deficiency, here lack of mCS, required for ascospore germination. The presence of a control mechanism activated by the absence of CIT1 is demonstrated by our recombination data. In *P.anserina*, each fertilized female organ (perithegium) is able to differentiate >100 asci. Strikingly, when a wild-type *cit1* allele is restored in a cell, the production of mCS is sufficient for the full development of this cell among all others blocked at the diffuse stage. This fact shows that the development of asci containing a wild-type *cit1* gene

through recombination is completely autonomous. This means that each meiocyte is able to sense the signal required for meiosis progression.

The existence of mechanisms that prevent meiosis progression when metabolic requirements are not met is suggested in other organisms. The diffuse stage arrest in amphibian oocytes is probably also an important control point. Indeed, this step is needed for synthesis and storage of mRNAs and proteins, which are required in the early stages of embryogenesis before the late expression of the zygotic genome. Arrest at this precise stage, when the decondensed state of the chromatin favors transcription, makes sense. Our intragenic recombination data show that transcription can also occur at this stage in other organisms, followed by translation and proper targeting of the proteins. One can suggest that a transient arrest at the diffuse stage could allow the meiocytes to express some of the genes required for viability of the progeny: if this requirement is not achieved, the checkpoint is activated. However, as stressed by Roeder and Bailis (2000) for the pachytene checkpoint, the critical question that remains to be addressed in all systems is the nature of the signal(s) that trigger arrest.

Most studies have focused on checkpoint activation by impairment of the meiotic process *per se*: defects in DNA replication, homolog synapsis, recombination and chromosome segregation. Our data strongly suggest that the meiocytes also sense their metabolic state and avoid progression of meiosis if this could lead to unproductive progeny.

Materials and methods

Strains and media

Podospora anserina is a filamentous ascomycete whose life cycle and general methods for genetic analysis have been described (Rizet and Engelmann, 1949). The *car1* mutants were characterized previously (Simonet and Zickler, 1972, 1978; Berteaux-Lecellier *et al.*, 1995). The *car1* gene was re-named *pex2* in accordance with the unified nomenclature for peroxisome biogenesis factors (Distel *et al.*, 1996). The *cit1* mutants were isolated as revertants of the *pex2-3* mutant, as growing sectors which appeared, after UV mutagenesis (100 J/m²), on medium containing oleic acid as sole carbon source. The culture and spore germination media have been reviewed recently (Berteaux-Lecellier *et al.*, 1995). When required, sodium glutamate was added to the spore germination medium in the range of 0.02–0.2% (w/v). Life spans were measured as previously described (Marcou, 1961; Contamine and Picard, 1998) for five subcultures from two strains exhibiting a given genotype.

Cosmids, plasmids and bacterial strains

The genomic library used for the transformation experiments has been described (Berteaux-Lecellier *et al.*, 1995). The integrative cosmid vector (pMOCosX) carries as selectable marker the bacterial (*hph*) hygromycin-resistant gene under the control of a eukaryotic promoter (Orbach, 1994). Subcloning of the *cit1* gene was performed using pUC18, pBluescript (Stratagene). Cloning and plasmid preparations were carried out in either *Escherichia coli* DH5 α (Hanahan, 1983) or XL1-Blue (Stratagene).

Cloning procedures

The *cit1* gene was cloned by complementation of the *cit1-1* sexual defect using the SIB selection method (Akins and Lambowitz, 1985). The library contained ~6000 cosmids from the entire genome, divided into 60 pools. In the 20th pool tested, among 360 hygromycin-resistant transformants, one presented a wild-type phenotype. Two successive rounds of SIB selection allowed the isolation of the cosmid carrying the *cit1* gene. Localization of the gene was obtained according to the procedure developed by Turcq *et al.* (1990). An *EcoRI*–*HindIII* fragment of 2.7 kb competent to complement the *cit1-1* mutant was recovered (Figure 2). Transformation experiments were performed as previously

described (Picard *et al.*, 1991) except that protoplasts were made with Glucanex (Novo Nordisk Ferment AG) instead of Novozym.

Sequencing

The 2.7 kb fragment was sequenced on both strands with the universal primer and synthetic oligonucleotides. For gene substitution, a larger *XhoI*–*XhoI* fragment of 5 kb was also sequenced (Figure 2). The sequences of the mutant alleles were determined after PCR amplification of the gene in each strain. The PCR fragments were prepared for direct sequencing as described (Rosenthal *et al.*, 1993). Sequencing was performed using an automatic sequencing machine (373A DNA sequencer, Applied Biosystems) using a Dye Terminator, Cycle Sequencing Kit (Abi-Prism, Perkin Elmer).

Cytology

Mitochondria were stained in the vegetative cells (mycelium) with the mitochondrion-specific dye 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI, Sigma) using the procedure described for yeast (McConnell *et al.*, 1990) and adapted for *P. anserina* (Jamet-Vierny *et al.*, 1997). Staining of meiocytes and immunofluorescence detection of peroxisomes were performed as described (Berteaux-Lecellier *et al.*, 1995). Here also, we used an antibody against the trifunctional peroxisomal enzyme FOX2 of *N. crassa* (a kind gift of Dr W.-H. Kunau). Immunodetection of mCS was performed using a polyclonal antibody directed against the yeast Cit1p (Vélot *et al.*, 1999) and diluted 3000-fold. The RAD51 antibody (Ab1, Oncogene), raised against a recombinant human protein, was diluted 400-fold. The secondary antibody was fluorescein isothiocyanate-conjugated goat anti-rabbit IgG diluted 80-fold (Sigma).

DNA procedures

Total DNA was extracted by a miniprep method (Lecellier and Silar, 1994). Standard procedures for Southern blotting on neutral membrane (Appligene) were used. The probes were prepared with a T7 Quick Prime labeling kit (Pharmacia). Low-stringency hybridizations were performed at 37°C, according to Church and Gilbert (1984), and the membranes were washed at 50°C, in 2 $\times$ SSC.

RNA procedures

Total RNA was isolated from mycelia as described (Lockington *et al.*, 1987). Standard procedures for northern blotting on positive membrane (Appligene) were used. For quantitative northern analyses, autoradiograms were quantified against the levels of *AS1* mRNA with a phosphorImager (STORM) using ImageQuant software. The RT–PCR experiment was performed with the Titan one tube RT–PCR kit (Roche Diagnostic). The two oligonucleotides used were chosen in order to hybridize, respectively, on the sequence following the start codon and, on the other strand, before the stop codon. This 1.4 kb template for direct sequencing was prepared as described (Rosenthal *et al.*, 1993).

Construct for gene replacement (Figure 2)

The *HindIII*–*EcoRI* fragment of 2.7 kb containing the *cit1* gene, in the B-XE plasmid (pBluescript vector containing the 3.9 kb *XhoI*–*EcoRI* fragment with the *cit1* gene and its 5'-flanking region), was substituted by the 1.4 kb fragment containing the *trpC::hph* construct from pUCHygro. This last vector was constructed by cloning, at the *SmaI* site of pUC18, the 1.4 kb *HpaI* fragment carrying the *trpC::hph* construct from pCB1004 (Carroll *et al.*, 1994). The 3'-flanking region of the *cit1* gene was amplified by PCR from the B-XH plasmid (pBluescript vector containing the 3.8 kb fragment *HindIII*–*XhoI*) (Figure 2). A *NotI* restriction site was added at the end of the 3' primer to permit cloning in the B-XE plasmid between the *EcoRI* and *NotI* sites, after digestion of the PCR product, which was then sequenced (Figure 2).

Obtaining a strain bearing the *cit1H309G* allele

To create the B-XE H309G plasmid, site-directed mutagenesis was performed on the B-XE plasmid, according to the procedure described by Briand *et al.* (2001). The replacement of the CAC (histidine) codon by the GGC (glycine) codon creates an *AciI* restriction site. We used a PCR approach on putative mutant clones to amplify the region encompassing the mutated codon. The amplified fragment was then digested by *AciI*, which allowed the selection of mutant clones. The whole *cit1* gene was then sequenced to ensure that no additional mutations were introduced during the mutagenesis. This plasmid was introduced into the Δ *cit1* strain by co-transformation with the Ppable plasmid (Coppin and Debuchy 2000) which carries, as selectable marker, the phleomycin resistance gene (*ble*). Transformants were thus selected on medium containing 10 μ g/ml

phleomycin and then crossed with a wild-type strain to allow their purification. These crosses provided $\Delta cit1^+B-XE H309G^+$ ascospores whose genotype was determined according to the following rules. First they had to grow on media containing both hygromycin and phleomycin and, secondly, the mutant protein was expected to lead to intragenic complementation in a cross with *cit1-12*. Several transformants were examined to ensure that the integration sites of the construct did not influence the phenotype of the transformants.

Respiration

Measurements were made polarographically on protoplasts in a Gilson oxygraph equipped with a Clark type O₂ electrode in 0.6 M sorbitol, 7.5 mM MgCl₂, 10 mM KH₂PO₄, 10 mM imidazole pH 7.4, 0.2% bovine serum albumin (BSA). The percentage of cyanide (KCN)-insensitive and of salicyl hydroxamic acid (SHAM)-insensitive respiration was measured. KCN and SHAM were added to final concentrations of 1 and 2.5 mM, respectively, to inhibit the cytochrome *c* oxidase or the alternative pathway (Dufour *et al.*, 2000).

Enzyme assays

Crude extracts were obtained as follows: mycelia were harvested from liquid cultures after 36 h growth and crushed in liquid nitrogen. The resulting powder was suspended in homogenization buffer (10 mM Tris pH 7.5, 1 mM EDTA, 76 mM glycine). After centrifugation at 11 000 r.p.m. at 4°C for 10 min, the supernatant was kept on ice. Enzyme activities for catalase and CS were determined spectrophotometrically (Zeiss spectrophotometer) as described by Baudhuin *et al.* (1964) and Srere *et al.* (1963), respectively. Protein concentrations were determined at 562 nm using the BCA protein assay (kit from Pierce) as described by Smith *et al.* (1985), with BSA as the standard.

Immunoblot analysis

Proteins were separated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose (Potran BA, Schleicher and Schuell). The nitrocellulose blot was treated strictly as described previously (Vélot *et al.*, 1999) with the same rabbit anti-Cit1p polyclonal antibody (diluted 3000-fold). The secondary antibody (anti-rabbit IgG coupled to alkaline phosphatase) was from Sigma and was diluted 1000-fold.

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