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Silencing of mitochondrial alternative oxidase gene of *Aspergillus fumigatus* enhances reactive oxygen species production and killing of the fungus by macrophages

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Abstract We previously demonstrated that conidia from *Aspergillus fumigatus* incubated with menadione and paraquat increases activity and expression of cyanide-insensitive alternative oxidase (AOX). Here, we employed the RNA silencing technique in *A. fumigatus* using the vector pALB1/*aoxAf* in order to down-regulate the *aox* gene. Positive transformants for *aox* gene silencing of *A. fumigatus* were more susceptible both to an imposed *in vitro* oxidative stress condition and to macrophages killing, suggesting that AOX is required for the *A. fumigatus* pathogenicity, mainly for the survival of the fungus conidia during host infection and resistance to reactive oxygen species generated by macrophages.

Keywords *Aspergillus fumigatus* · Alternative oxidase · RNAi · Macrophage killing

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Abbreviations

AOX	alternative oxidase
aoxAf	alternative oxidase gene from *A. fumigatus*
dsRNA	double-stranded RNA
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole)
PKS	polyketide synthase
RNAi	RNA interference
ROS	reactive oxygen species
SHAM	salicylhydroxamic acid

Introduction

Aspergillus fumigatus is one of the most important airborne pathogenic fungus. In the last two decades, invasive aspegillosis has increased drastically, in particular in patients immunocompromised by organ transplant, leukemia and AIDS (Brakhage 2005).

We previously described the respiratory chain of *A. fumigatus*, demonstrating the presence of an alternative oxidase (AOX) (Tudella et al. 2004). Mitochondrial respiration in higher animals is strongly inhibited by cyanide, which blocks the cytochrome-mediated electron transport to oxygen at the Complex IV (cytochrome *c* oxidase). In addition to the normal cytochrome-mediated pathway, this alternative cyanide-insensitive metabolic pathway behaves as a short-circuit in the main respiratory chain, and it is specifically inhibited by salicylhydroxamic acid (SHAM) (Schonbaum et al. 1971; Veiga et al. 2003). The electron flux to AOX branches from the classical electron-transport chain at the level of the ubiquinone pool, bypassing Complex III and IV, and leads to

the direct reduction of oxygen to water (Vanlerberghe and McIntosh 1997). AOX-mediated O_2 reduction is not coupled to either proton pumping or oxidative phosphorylation.

AOX contributes to both reduction of the reactive oxygen species (ROS) generated by mitochondria and regulation of the energy production and metabolism in some organisms (Hattori et al. 2008). In *A. fumigatus*, AOX may play a role in the antioxidant defense mechanism (Magnani et al. 2007), so it constitutes a potential novel chemotherapeutic target for aspergillosis treatment.

In order to demonstrate new potential targets in filamentous fungi, knockout of genes by conventional disruption has been widely employed. However, this strategy is time consuming and greatly hampered by the poor efficiency of homologous recombination in these organisms (Nakayashiki 2005; Weld et al. 2006). In this context, the RNA interference technique has the advantage of producing cells with reduced expression of essential genes and of yielding viable cells with intermediate phenotypes (Mouyna et al. 2004).

RNA interference is based on a natural phenomenon by which a double-stranded RNA (dsRNA) induces enzymatic degradation of mRNAs in a sequence specific-manner. This post-transcriptional inhibition pathway was firstly described in the nematode *Caenorhabditis elegans* (Fire et al. 1998), and it has more recently been described in a wide variety of eukaryotic organisms, including filamentous fungi such as *Magnaphorthe oryzae*, *Colletotrichum lagenarium, Aspergillus nidulans* and *A. fumigatus* itself (Yamada et al. 2007). The presence of RNA silencing in *A. fumigatus* has led to the successful application of the RNAi technique to down-regulate both essential and nonessential genes in this organism (Khalaj et al. 2007).

We therefore reported here the silencing of the AOX gene of *A. fumigatus* in order to identify its role in the virulence and oxidative defense of this fungus.

Materials and methods

Strain and culture conditions

Aspergillus fumigatus strain CEA 017 was used for transformation experiments in minimal medium. *Escherichia coli* DH5α was used for plasmid propagation. Plasmid TOPO® was used in subcloning procedures. Ampicillin (100 μg/ml) was added to the growth medium when required.

Construction of the double constructs *pALB1/aoxAf*

A pALB1 plasmid was a kind gift of Isabelle Mouyna (Institute Pasteur, Paris). For the double construct, a portion of the alternative oxidase gene (*aoxAf*) was PCR amplified from *A. fumigatus* genomic DNA using *primers* AOXRNAi-F (5'-GTTTACTATCCG**GTCGAC**CGC**AAGCTT**AGC-3') and AOXRNAi-R (5'-GCAT**GGGCCC**TTTGTTAGCTTCC**TCTAGA**AGAC-3'). The antisense *aoxAf* fragment was PCR amplified from genomic DNA to incorporate a *Xba*I and a *Hind*III restriction site (in bold), and it was cloned into pALB1 digested with the same enzymes. A sense fragment was amplified to incorporate a *Sal*I and an *Apa*I restriction site (in bold) and cloned into pALB1 containing the antisense *aoxAf* fragment.

Transformation

The circular plasmids described above were used to transform *A. fumigatus* following procedures previously described (Mouyna et al. 1998). After overnight expression of *hygromycin phosphotransferase* (*hph)* gene, transformants were selected on minimal medium containing 200 μg/ml of hygromycin B for 7 days, at 25°C.

RNA extraction and reverse transcription (RT)-PCR

Transformants and wild type strains were grown in liquid minimal medium containing 2% xylose (to repress the pGla promoter) or 2% maltose (to induce the pGla promoter) (Mouyna et al. 2004). After growth for 24 h at 37°C, RNA was isolated using the Trizol® reagent (Invitrogen).

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized using the SuperScript™ first-strand synthesis system (Invitrogen). All RT-PCR reactions were performed using a Mastercycler Realplex4 (Eppendorf). Taq-Man™ Universal PCR Master Mix (Applied Biosystems) and primers LUX (Invitrogen) were used for the reactions. The thermal cycling conditions comprised an initial step at 50°C for 2 min, followed by 30 min at 60°C for reverse transcription, 95°C for 5 min, and 40 cycles at 94°C for 20 s and 60°C for 1 min. The measured mRNA levels were normalized using the beta-tubulin gene. The reactions and calculations were performed according to Semighini et al. (2002).

Measurement of intracellular ROS

Intracellular ROS levels were measured in conidia using CM-H_2DCFDA [5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester] (Molecular Probes) using a F4500 Hitachi Fluorescence Spectrophotometer. Conidia ($5x10^6$ cells) were incubated with 5 μM CM-H_2DCFDA (Molecular Probes, Eugene, OR, USA) for 30 min, at 37°C, in a medium containing 1.7 mM KCl, 5.0 mmol/L $MgCl_2$, 0.5 mmol/L EGTA, 10 mmol/L

KH_2PO_4, 2% glucose (w/v), 50 mmol/L HEPES-KOH pH 7.4 and 1 mmol/L KCN. The fluorescence intensity was detected using excitation 503 nm and emission 529 nm.

Conidia viability

A total of 5×10^6 conidia of CEA, pALB1 and pALB1/*aoxAf* strains were incubated in a minimal medium containing 2% maltose and 25 μM antimycin A, for 1 h at 30°C with shaking. 50 mM paraquat and 80 mM H_2O_2 were then added to the medium and incubated for 5 h. Conidia were harvested by centrifugation and washed twice with 1× PBS solution to remove the pro-oxidant agents.

Conidia were suspended again in 200 μL GH solution (2% Glucose (w/v), 10 mM Na-HEPES) containing 5 μM FUN® 1 probe (Molecular Probes) and incubated in the dark with shaking for 30 min at 37°C. The addition of 0.5 μM Propidium Iodide was done just at the moment of the determination. Conidia viability was determined by flow cytometry, which was performed in a FACSCalibur (BD Biosciences, Franklin Lakes, NJ). Thirty thousand cells per sample were analyzed. Data were acquired and analyzed with ELLQuest PRO 3.3 (BD Biosciences).

Conidial killing assay

The ability of conidia to survive inside cells following phagocytosis was assessed using a tetrazolium dye (MTT) reduction assay (Peck 1985), that followed absorbance at 570 nm using a micro plate reader. Macrophages (4×10^5 cells) were incubated into each well of a 96-well plate RPMI complete medium for 12 h at 37°C in 5% CO_2. After this time, $4\text{x}10^6$ conidia were added to each well and incubated for 30 min, 2, 4 and 8 h.

After incubation, the cell suspension was centrifuged at 2,000 *g* for 10 min. The pellet was washed with 100 μL of 1% Triton X-100 (w/v), incubated for 10 min, and centrifuged. The pellet was washed and suspended in RPMI medium containing 0.5 mg/mL of MTT and incubated at 37°C for 2 h. After that, the pellet was recovered by centrifugation and suspended in 200 μL acidic isopropanol in each well. Plates were swirled until the blue color dissolved. The percentage of cells killed was calculated as:

$$\% = 1 - [\,DO_{570nm}\ \text{sample}/DO_{570nm}\ \text{positive control}] \times 100$$

Results

Suppression of *aoxAf* expression by RNA interference

We constructed the RNAi silencing vector in *A. fumigatus* based on a hairpin design. Two 500 bp inverted repeated sequences of conserved region of the gene of interest were amplified by PCR. These fragments were cloned in pALB1 plasmid as inverted repeats separated by a 250-bp spacer of GFP sequence and *alb1* gene cloned sequences.

Protoplasts of *A. fumigatus* were transformed with the pALB1/*aoxAf* plasmid, and positive transformants were selected by hygromycin B. Subsequently, they were transferred to a culture medium containing maltose for induction of the RNA interference phenotype, or to a medium containing xylose for its repression. In the xylose medium all the transformants formed green colonies (Fig. 1a) whereas in the maltose medium, pALB1 and pALB1/*aoxAf* transformants formed white colonies, attributable to reduction of *alb1* gene expression (Fig. 1b). *Alb1* gene is responsible for synthesis of the *polyketide synthase* (PKS) protein. This enzyme is involved in the melanin biosynthesis required for conidial pigmentation (Langfelder et al. 1998).

To assess whether RNAi of alternative oxidase gene is efficient in the white strains, RNA levels were analyzed by Real-time PCR. The results showed a decrease of 95% in the *aox* gene expression when compared with the wild strain CEA 017 (Fig. 1c).

Measurement of ROS production in transformants

We have used the probe CM-H_2DCFDA to measure ROS production in *A. fumigatus* conidia. Wild strains CEA

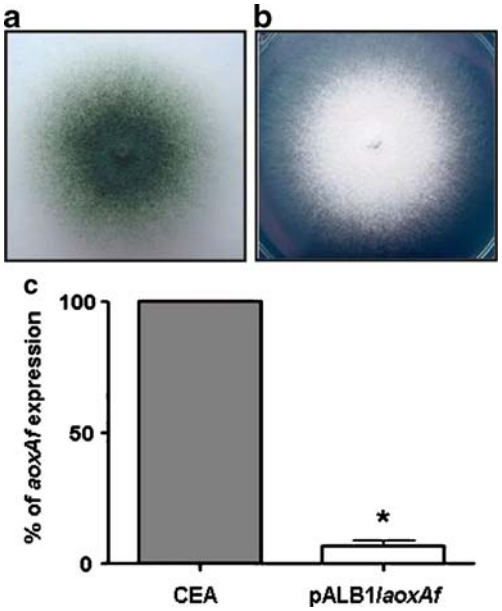

Fig. 1 pALB1/*aoxAf* transformants. A. In minimal medium containing xylose, transformants exhibited green color; B. In maltose medium, white color is due to the RNA interference. C: Quantitative Real time-PCR analysis of *aoxAf* expression for the wild type (CEA) and the complete phenotype (pALB1/*aoxAf*). Results were firstly standardized for β-tubulin, with an wild type expression set arbitrarily to 100. *$p<0.0001$

displayed a constant level of fluoresce throughout the incubation time (Fig. 2a, *line a* and Fig. 2b). In pALB1, the fluorescence increased by 3-fold during the incubation time (Fig. 2a, *line b* and Fig. 2b), whereas in pALB1/*aoxAf* transformed strain, it increased by 5-fold (Fig. 2a, *line c* and Fig. 2b).

Conidia viability

After exposition of *A. fumigatus* conidia to ROS donors, the viability was determined by flow cytometry analyses using FUN® 1 probe and Propidium Iodide. Conidia of *A. fumigatus* exposed to antimycin A (Fig. 3b), which enhances endogenous production of superoxide, and of paraquat (Fig. 3c) or H_2O_2 (Fig. 3d), showed a decrease in the percentage of viability of pALB1/*aoxAf* transformed strain when compared to wild and pALB1 strains under the same conditions (Fig. 3a). Antimycin A was used in order to supplant the antioxidant defense of the fungus.

MTT assay

Alveolar macrophage constitutes the first line of phagocytic host defense against inhaled conidia (Schaffner et al 1982). The phenotypic differences in the double silencing led us to investigate whether pALB1/*aoxAf* transformed strain is less protected against killing than the wild-type and pALB1 strains.

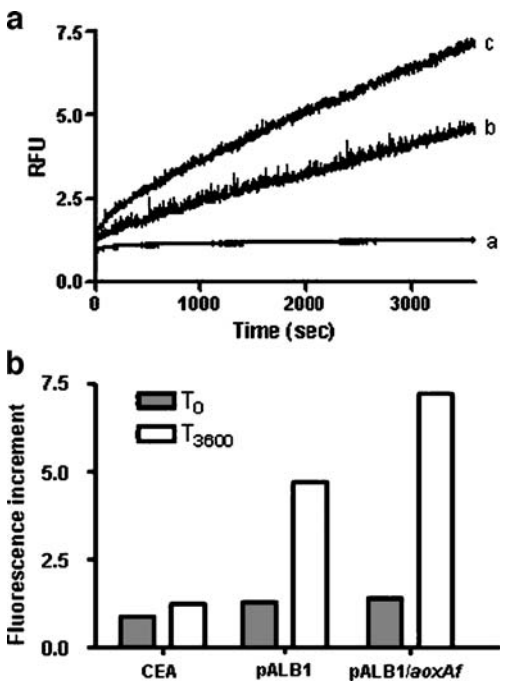

Fig. 2 Detection of ROS production in CEA **a**, pALB1 **b** and pALB1/*aoxAf* **c** strains. A. RFU of conidia incubated for 60 min with 5 mM CM-H_2DCFDA, at 30°C. B. Comparison of RFU in initial (T_0) and final time

After phagocytosis of *A. fumigatus* conidia by alveolar macrophages, a drop in the surviving percentage of the transformed strain of *A. fumigatus* pALB1 (single-silence) or pALB1/*aoxAf* (double silence) in relation to the wild-type strain was observed (Fig. 4).

Discussion

In fungus, gene silencing is mostly induced by RNA silencing in plasmid constructs that express hairpin RNA. Assessments of the silencing ability of different RNA species have shown that double-stranded RNA in the form of a 500–1,000 base pairs (bp) hairpin, with a relatively short intron-containing loop, is the most potent trigger for interference (Nakayashiki 2005; Mouyna et al. 2004; Yamada et al. 2007; Liu et al. 2002; Kadotani et al. 2003; Goldoni et al. 2004; Rappleye et al. 2004). During plasmid-mediated silencing in filamentous fungus, gene silence varies at different degrees among 70–90% (Nakayashiki 2005). Protoplasts of *A. fumigatus* transformed with the pALB1/*aoxAf* showed a decrease of 95% in the *aox* gene expression, indicating efficient *aox* gene silencing using the pALB1/*aoxAf* plasmid construction.

It has been recently reported the efficacy of RNAi construct for *A. fumigatus*. Transformant analysis showed that a full down-regulation is never achieved and that the vector used for RNAi is lost or modified over successive transfers resulting in a silencing inhibition (Henry et al. 2007). However, one of the original advantages of RNAi was the rapidity of the technique to investigate a transformant phenotype following an ectopic integration of the silencing cassette.

The ROS include a range of different molecules including hydrogen peroxide, hydroxyl radical and superoxide anion, capable of affecting the cell redox homeostasis (Perrone et al. 2008). The normal process of mitochondrial respiration is the major source of ROS. In excess, these species can damage nucleic acids, proteins and lipids, frequently resulting in an increase of antioxidants proteins, concomitantly with increase of the AOX protein amount (Dufour et al. 2000).

The decrease in the *aox* gene expression caused an increase in ROS production when compared with wild-type and pALB1 strains, in agreement with previous reports on ROS scavenging by melanin (Jahn et al. 2002; Brakhage and Liebmann 2005). Moreover, the major increase of ROS production (5-fold) in pALB1/*aoxAf* transformed strain suggests that AOX is involved in the antioxidant defense of *A. fumigatus*. In this connection, it should be considered that although mitochondrial uncoupling may impair ATP production of the fungus, it may afford protection against the superoxide radicals generat-

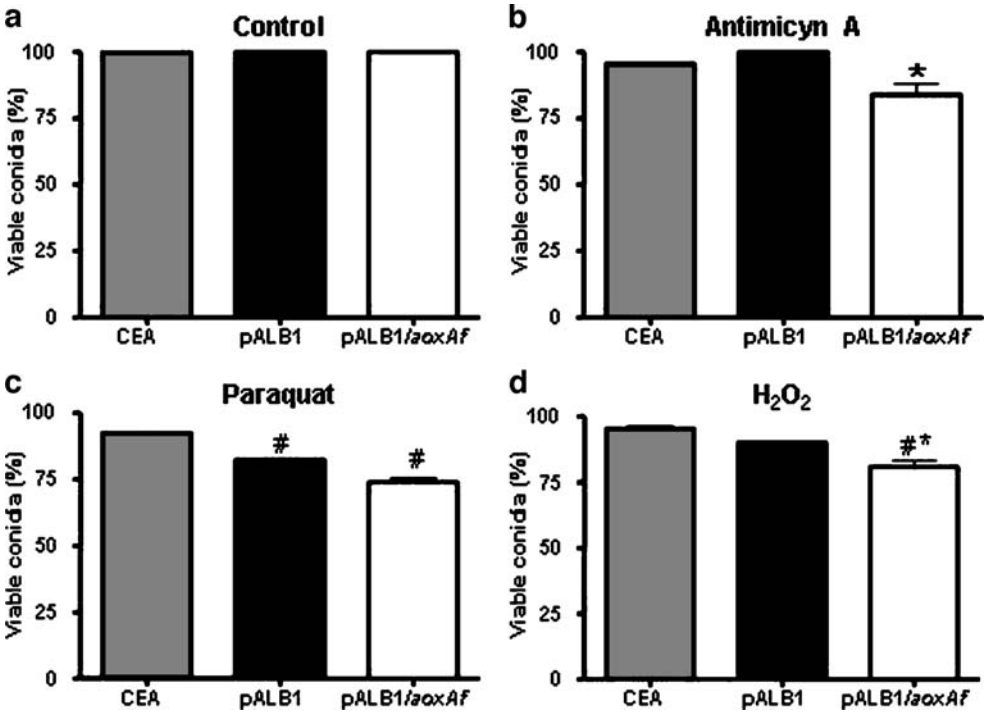

Fig. 3 Conidia viability of CEA, pALB1 and pALB1/*aoxAf* strains, before **a** or after exposition to antimycin A **b**, paraquat **c** or H_2O_2 **d**. *$p<0.05$; #$p<0.01$

ed by the host defense mechanism (Helmerhorst et al. 2001).

ROS are part of a host defense mechanism against many pathogens, including *A. fumigatus*. Therefore, to survive, this fungus triggers a counteracting antioxidant response. In many fungi, it has been observed an increase of AOX mRNA levels as a consequence of oxidative stress (Magnani et al. 2007; Joseph-Horne et al. 2001). AOX would prevent the auto-oxidation of reduced quinone and the subsequent formation of ROS either when the electron transport flow through the cytochrome pathway becomes limited (Wagner and Moore 1997) or in response to the oxidative burst generated by mammalian defense cells during the antifungal defense (Helmerhorst et al. 2001).

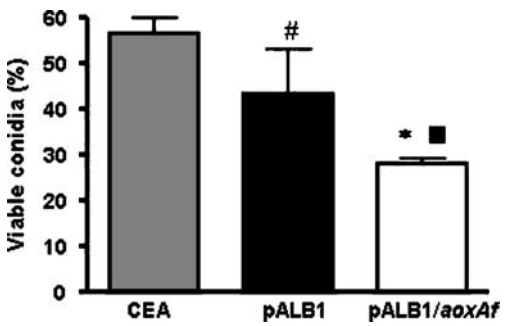

Fig. 4 MTT assay of CEA, pALB1 and pALB1/*aoxAf* strains, indicating the % of MTT reduced by live conidia. #$p<0.05$ (CEA X pALB1); *$p<0.001$ (CEA X pALB1/*aoxAf*); $p<0.01$ (pALB1 X pALB1/*aoxAf*)

A higher susceptibility to oxidants was observed because of the decrease in the percentage of viability of pALB1/*aoxAf* transformed strain, supporting our previous proposal that this pathway, in addition to other antioxidant systems, plays a role in the antioxidant defense mechanism of *A. fumigatus* (Magnani et al. 2007).

Macrophage-mediated killing is critical for the host resistance during *A. fumigatus* infection, and ROS production by alveolar macrophages plays an essential role in the killing of *A. fumigatus* conidia (Shibuya et al. 2006). A drop in the surviving percentage of the transformed strain of *A. fumigatus* pALB1 (single-silence) is in agreement with previous reports demonstrating that the presence of a functional *polyketide synthase* protein in *A. fumigatus* conidia is associated with inhibition of phagolysosome fusion in human monocyte-derived macrophages (Brakhage and Liebmann 2005), as well as with an increase of intracellular killing in white *pks* mutant conidia (Jahn et al. 2002). In addition, a drop in the surviving of the transformed strain of *A. fumigatus* pALB1/*aoxAf* (double silence) in relation to the wild-type strain and pALB1 suggests that the double silencing strain conidia of *alb1* and *aox* gene are less protected against killing than the single silencing one.

Therefore, the present results suggest that AOX is required for the *A. fumigatus* pathogenicity, mainly by surviving of the fungus conidia during the host infection, and reactive oxygen species generation by macrophages, opening new perspectives for the treatment of aspergillosis, having AOX as a potential target.

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