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ORIGINAL PAPER

Characterisation of the laccase-encoding gene *abr2* of the dihydroxynaphthalene-like melanin gene cluster of *Aspergillus fumigatus*

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Abstract *Aspergillus fumigatus* is an important pathogen of the immunocompromised host. Previously, it was shown that the polyketide synthase encoded by the *pksP* (*alb1*) gene represents a virulence determinant. *pksP* is part of a gene cluster involved in dihydroxynaphthalene (DHN)-like melanin biosynthesis. Because a putative laccase-encoding gene (*abr2*) is also part of the cluster and a laccase was found to represent a virulence factor in *Cryptococcus neoformans*, here, the Abr2 laccase was characterised. Deletion of the *abr2* gene changed the gray-green conidial pigment to a brown color and the ornamentation of conidia was reduced compared with wild-type conidia. In contrast to the white *pksP* mutant, the susceptibility of the Δ*abr2* mutant against reactive oxygen species (ROS) was not increased, suggesting that the intermediate of DHN-like melanin produced up to the step catalysed by Abr2 already possesses ROS scavenging activity. In an intranasal mouse infection model, the Δ*abr2* mutant strain showed no reduction in virulence compared with the wild type. In the Δ*abr2* mutant, overall laccase activity was reduced only during sporulation, but not during vegetative growth. An *abr2*p-*lacZ* gene fusion was expressed during sporulation, but not during vegetative growth confirming the pattern of laccase activity due to Abr2.

Keywords Dihydroxynaphthalene-like melanin · *Aspergillus fumigatus* · Gene cluster · Laccase

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Introduction

The improvement in transplant medicine and the therapy of hematological malignancies is often complicated by the threat of invasive aspergillosis. *Aspergillus fumigatus* accounts for approximately 90% of invasive aspergillosis cases. Specific diagnostics are still limited, as are the possibilities of therapeutic intervention, leading to a high mortality rate of 30 to 98% for invasive aspergillosis (reviewed in [8, 12, 22]). An important question concerning *A. fumigatus* is the identification of pathogenicity determinants and their regulation. The group of J. Kwon-Chung and our group had identified a gene that encodes a pathogenicity determinant. It was designated *pksP* (or alternatively *alb1*) for polyketide synthase involved in pigment biosynthesis [14, 15, 19, 20, 38]. Conidia of a *pksP* mutant strain are white. Based on genetic and biochemical data the conidial pigment consists of dihydroxynaphthalene (DHN)-like melanin [6, 9, 21, 39, 40].

The complete absence of DHN-like melanin, as in the case of *pksP* mutants resulted in a severe reduction in virulence. *PksP* mutant conidia of *A. fumigatus* were significantly more sensitive to hydrogen peroxide and sodium hypochlorite than wild-type conidia. As in other cases, it was shown that melanin-containing conidia are able to quench ROS derived from human granulocytes [14, 15]. These results indicated that conidial DHN-like melanin of *A. fumigatus* is involved in protecting conidia from the host immune response in which ROS are important for eliminating fungal conidia (reviewed in [21, 22]). However, because *A. nidulans* conidia are also protected by a green pigment, resistance against ROS does not explain why *A. fumigatus* conidia can be pathogenic while this is rarely the case with *A. nidulans* conidia. One attractive hypothesis is that besides the pigment, the *pksP* gene product of *A. fumigatus* is involved in the production of another compound that is immunosuppressive [5]. This hypothesis was further supported by the notion that the presence of a functional *pksP* gene in *A. fumigatus* conidia is associated with an inhibition of the fusion of phagosomes and lysosomes in human monocyte-derived macrophages [13, 16, 33]. Other pathways involving polyketide synthases have been shown to synthesize two different active products (reviewed in [21]).

The *pksP* gene is part of a gene cluster which consists of six genes. One of the genes designated *abr2* encodes a putative laccase [39]. As mentioned above, our previous data led to the hypothesis that only the *pksP* gene is involved in virulence and not the other genes of the DHN-like melanin pathway. On the other side, a cell wall bound laccase was found to be required for virulence of the human-pathogenic fungus *Cryptococcus neoformans* [27, 28, 30, 43, 44]. Therefore, we characterised the putative laccase Abr2 of *A. fumigatus* in order to study its impact on virulence.

Materials and methods

Fungal and bacterial strains, media and growth conditions

Fungal strains used in this study are listed in Table 1. *A. fumigatus* ATCC46645 was used to generate an *abr2* knock-out strain. *A. fumigatus* KHΔ*pyrG* is a uracil-auxotrophic mutant of strain ATCC46645. The uracil-auxotrophic strain *A. fumigatus* KHΔ*pyrG* contains a deletion of 144 bp in the 3′-coding region of the *pyrG* gene and therefore, codes for a truncated, nonfunctional orotidine-5′-monophosphate decarboxylase (see below).

Table 1 *Aspergillus fumigatus* strains

Strain	Genotype and/or phenotype	Reference
ATCC46645	Wild type	ATCC
KHΔ*pyrG*	Derived from ATCC46645; *pyrG::hph*[a], *pyrG*, Hyg^R	This study
AfΔ*abr2*	Δ*abr2*, Hyg^R	This study
Af*pksP*	*pksP*	[14, 19]
KH*pksP*p-*lacZ*	*pyrG1::pyrG2, pksP*p-*lacZ*, $PyrG^+$	This study
Kh*abr2*p-*lacZ*	*pyrG1::pyrG2, abr2*p-*lacZ*, $PyrG^+$	This study

[a] *hph*: *E. coli* hygromycin B phosphotransferase gene

Strain KHΔ*pyrG* was used to generate strains KH*pksP*p-*lacZ* and KH*abr2*p-*lacZ*. *A. fumigatus* strains were cultivated at 37°C in *Aspergillus* minimal medium (AMM) as previously described [41]. As solid media, malt extract medium (2% (w/v) malt extract, 0.2% (w/v) yeast extract, 1% (w/v) glucose, 5 mM ammonium chloride, 1 mM di-potassium hydrogenphosphate) or AMM containing 3% (w/v) agar were used. Uridine (5 mM) or uracil (5 mM) were added to the media when required. In case the *hph* or *ble* gene were used as a selection marker gene for transformation of *A. fumigatus*, 100 μg hygromycin B and 100 μg phleomycin per ml, respectively, were added to agar plates.

For induction of conidiophore formation of *A. fumigatus* (developmental cultures), conidia were used to inoculate liquid cultures which were grown for 24 h at 37°C. Mycelia were filtered and exposed to air as previously described for *A. nidulans* [41]. For transformation of *Escherichia coli*, XL1-Blue (Stratagene, USA), INVαF′ or TOP10F′ (Invitrogen, The Netherlands) were used. *E. coli* strains were grown at 37°C in LB medium supplemented, when required, with 100 or 50 μg per ml of ampicillin or kanamycin, respectively.

Colony radial growth rate determination

Colony diameters of *A. fumigatus* were measured twice a day on both malt extract and AMM agar plates over a period of 94 h. At least 10 colonies of each strain were analysed. Agar plates had been point-inoculated centrally with a 2.5 μl drop of a suspension of 1×10^6 spores per ml. Colony radial growth rates (C_r) [37] were calculated from the slope of the line between 40 and 72 h from a plot of colony radius versus time starting from the time of inoculation. Data were processed by least square regression analysis.

Analysis of conidial germination

AMM (50 ml) were inoculated with 1×10^7 conidia. Cultures were incubated with 180 rpm at 37°C. Over a period of 16 h, samples were taken and deposited on microscope slides. To determine germination, at least 100 conidia of each sample were counted.

Standard DNA techniques

Standard techniques in the manipulation of DNA were carried out as described by Sambrook et al. [31]. Chromosomal DNA of *A. fumigatus* was prepared as previously described for *A. nidulans* [1]. For Southern blot analysis, chromosomal DNA of *A. fumigatus* was cut by different restriction enzymes, as indicated. DNA fragments were separated on an agarose gel and blotted onto Hybond N^+ nylon membranes (Amersham Pharmacia Biotech, UK). Labeling of the DNA probe, hybridization and detection of DNA-DNA hybrids were performed using the DIG High Prime Labeling and Detection System (Amersham Pharmacia Biotech, UK) according to the manufacturer's recommendations.

Sequence analysis

Plasmid DNA was sequenced on both strands by primer walking using the Big Dye™ Terminator Cycle Sequencing Kit (Applied Biosystems, UK). Sequencing reactions were separated on an Applied Biosystems ABI 310 sequencer. DNA sequence data were edited by the programs "Sequence Navigator" and "Auto Assembler" (Applied Biosystems, UK). The analysis of sequences was carried out using "Gene Works 2.2" (IntelliGenetics Inc., USA).

Generation of recombinant plasmids

For generation of plasmid pKH1*pyrGhph* an upstream flanking region (1020 bp) of the *A. fumigatus pyrG* gene with an introduced *Xba*I restriction site at its 3′end was generated by PCR using oligonucleotides PyrG1 and PyrG2XbaI (Table 2), and genomic DNA from the *A. fumigatus* wild-type strain as a template. The downstream fragment of *pyrG* with a size of 1032 bp with an introduced *Xba*I restriction site at its 5′end was synthesised by PCR, employing PyrG3XbaI and PyrG4 oligonucleotides (Table 2), and genomic DNA from the wild-type strain as a template. The upstream and downstream fragments of the *pyrG* gene were cloned independently into the pCR2.1 vector (TA cloning kit, Invitrogen, The Netherlands), yielding plasmids pTAFR1 and pTAFR2, respectively. After linearisation of pTAFR1 with *Xba*I, the *hph* selection marker gene conferring hygromycin B resistance, was introduced. A DNA fragment encoding the *hph* gene was obtained via double restriction of plasmid pUC*hph*1 [25] with *Xba*I and *Spe*I. In the *Xba*I site of the resulting vector designated pTAFR1*hph*, the downstream fragment was transferred from the vector pTAFR2, by excision with *Xba*I restriction endonuclease.

Table 2 Oligonucleotide primers used in the study

Primer	Sequence[a]
Abr2del_for	TAAGGAATCGCACCATCGCC
Abr2del_rev	GAGTGGTCATATGGCAGTGC
Abr2P_for	GTGGATCCCTCAACCGGTGC
Abr2P_rev	GGATCCCATCCTGTTGTCGTGTAT
Abr2.2_rev	TGCTGGGATCCGACTAGTACG
Abr2disr_for	GGCGAGCAGATACTTATGGG
Abr2disr_rev	GAGTGGTCATATGGCAGTGC
PyrG1	TTGACCCCACAGTCGGAGGC
PyrG2XbaI	GGAGTCTAGAATTGCTGTCC
PyrG3XbaI	CCTCTAGAAGCAAAAGTGTAGTGC
PyrG4	TCCTTCCCCTCATCTGTTGG

[a] All sequences are depicted in the 5′→3′ direction

To construct the *abr2* deletion plasmid **pUCΔ*abr2***, a 4125 bp PCR product was generated using oligonucleotides Abr2del_for and Abr2del_rev (Table 2), and chromosomal DNA of the *A. fumigatus* wild-type strain ATCC46645 as a template. The resulting PCR fragment spanned the *abr2* gene and its upstream and downstream flanking region with a size of 1,035 bp and 1,020 bp, respectively. This PCR fragment was cloned into the pCR2.1 TOPO vector (TA Cloning Kit, Invitrogen, The Netherlands) to give plasmid pCR2.1*abr2*. The PCR fragment was re-isolated by restriction digest of plasmid pCR2.1*abr2* with *Eco*RI. The obtained DNA fragment of 4,125 bp was ligated into pUC18, also digested with *Eco*RI, yielding plasmid pUC*abr2*. As a selection marker, the *hph* gene was used. It was obtained from plasmid pUC*hph*1 [25], digested with *Nsi*I and *Xmn*I. The resulting DNA fragment of 2,792 bp was cloned into plasmid pUC*abr2* after restriction with *Nsi*I, which allowed the replacement of the *abr2*-encoding region by *hph*. Thus, the resulting plasmid pUCΔ*abr2* contained the *E. coli hph* gene under control of the *A. nidulans gpdA* promoter, flanked by fragments encoding upstream and downstream sequences of the *abr2* gene. Plasmid pUCΔ*abr2* was linearised by digestion with *Eco*RI. The resulting 4.8 kbp DNA fragment was used for transformation of the *A. fumigatus* wild-type strain ATCC46645.

For complementation of the *abr2* deletion mutant, a PCR fragment of 3,641 bp comprising the *abr2* gene and 1 kb of promoter region, was generated by the use of primer pair Abr2P_for and Abr2.2_rev (Table 2). The resulting *abr2*-encoding PCR fragment was used for a co-transformation approach. Co-transformation was carried out by the use of the plasmid pAN8-1 which contains the *ble* gene, conferring resistance to phleomycin [29]. The *ble* gene is under control of the *A. nidulans gpdA* promoter. Transcription of *ble* is terminated by the *A. nidulans trpC* terminator. For the increase of transformation frequency plasmid HELP1 [19] was simultaneously added to the transformation mix. Transformants were selected on AMM agar plates containing phleomycin. Phleomycin-resistant transformants producing gray-green conidia were checked by PCR for the presence of the *abr2* gene using primers Abr2disr_for and Abr2disr_rev (Table 2) and chromosomal DNA of the transformants as the template.

To measure *abr2* expression, an *abr2*p-*lacZ* gene fusion was generated. For this purpose, a 1.23 kbp DNA fragment spanning the putative promoter region of *abr2* was amplified by PCR using the oligonucleotides Abr2P_rev and Abr2P_for (Table 2), each of which encoding *Bam*HI restriction sites. As the template, chromosomal DNA of *A. fumigatus* wild type was used. The PCR fragment obtained was cloned into the pCR2.1 TOPO vector (TA Cloning Kit, Invitrogen, The Netherlands). After restriction with *Bam*HI, the DNA fragment spanning the promoter region was cloned into the *Bam*HI site of plasmid pUC*pyrG2lacZ* [23], which carries the non-functional *pyrG2* allele. The resulting plasmid pUC*pyrG2abr2*P*lacZ* encoded an in frame *abr2*p-*lacZ* gene fusion. This was checked by DNA sequence analysis across the junctions. All *lacZ*-containing plasmids carried the *pyrG2* allele as the selection marker. It encodes a nonfunctional *pyrG* gene of *A. fumigatus*, which forced site-specific integration of plasmids into the chromosomal *pyrG* locus [42].

Transformation of *A. fumigatus* and generation of the *pyrG* deletion strain KHΔ*pyrG* and of strain KH*pksP*p-*lacZ*

Transformation of *A. fumigatus* was carried out using protoplasts as previously described [42]. The *pyrG* deletion strain KHΔ*pyrG* was generated as follows: the vector pKH1*pyrGhph* was cut with *Spe*I and *Bgl*II yielding a DNA fragment of 5.9 kbp, which was used for transformation of the *A. fumigatus* wild-type strain ATCC46645. The selection of *pyrG* deletion strains occurred on AMM agar plates containing uracil and hygromycin B. Uracil-auxotrophic, hygromycin B-resistant transformants were checked by Southern blot analysis (data not shown). One of the resulting uracil-auxotrophic, hygromycin-resistant transformants was designated KHΔ*pyrG*. It contained a deletion of 144 bp in the 3′ coding region of the *pyrG* gene. When uracil-auxoptrophy was used, the *A. fumigatus* strain KHΔ*pyrG* (Table 1) was applied. When selection for hygromycin B resistance was used, the wild-type strain ATCC46645 was employed. Strain KH*pksP*p-*lacZ* was generated by transformation of strain KHΔ*pyrG* with plasmid pUCpyrG2pksP-lacZ [23] encoding a *pksP*p-*lacZ* gene fusion. By Southern blot analysis, a transformant strain designated KH*pksP*p-*lacZ* was identified encoding a single copy of plasmid pUCpyrG2pksP-lacZ integrated at the chromosomal *pyrG* gene locus (data not shown).

Field emission scanning electron microscopy (FESEM)

FEMES was carried out according to Maerker et al. [26]. In brief, the conidia were harvested with sterile water, containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$ to a final concentration of 3×10^8 conidia per ml. For fixation of the conidia, 35% (v/v) formaldehyde was used in a final concentration of 5% (v/v). After incubation on ice for 10 min, glutaraldehyde was added to a final concentration of 2% (v/v). After washing with cacodylate buffer and subsequently with TE buffer, the samples were placed onto poly(L-lysine) coated glass cover slips.

ß-Galactosidase (ß-GAL) activity assays

A. fumigatus strains were grown in AMM at 37°C. ß-GAL activities were measured in protein extracts obtained from three *A. fumigatus* cultures grown in parallel. Specific activities were calculated as previously described [25].

Laccase activity assay and determination of protein concentrations

Aspergillus fumigatus strains were grown in AMM at 37°C. After harvesting at different time points as indicated, mycelia were frozen in liquid nitrogen and ground to a fine powder. Mycelia were suspended in extraction buffer (Tris/HCl 0.1 M, pH 7.0). Samples were centrifuged at 4°C with 13,000 rpm for 10 min. The supernatant was retained. N,N-Dimethyl-*p*-phenylendiamine (DMP) was used as the substrate. Laccase activity was determined in 900 µl laccase buffer (37 mM citric acid monohydrate, 126 mM Na_2HPO_4, pH 6.0), 50 µl DMP (147 mM stock solution) and 50 µl enzyme solution. Extinction was followed at 550 nm at

25°C and the enzyme activities were calculated using a molar extinction coefficient of 1.8 mM^{-1} cm^{-1}, as previously described [32]. Laccase activities were measured in protein extracts obtained from three *A. fumigatus* cultures grown in parallel. Protein concentrations were determined according to Bradford [3].

Sensitivity towards reactive oxygen species

1×10^8 *A. fumigatus* conidia were mixed with 10 ml AMM top agar and poured onto AMM agar plates. In the center of the agar plate, a hole with a diameter of 10 mm was created, which was filled with a solution of H_2O_2 or diamide. After an incubation of the agar plates at 37°C for 16 h, the diameter of the inhibition zone was measured.

Animal infection model

An optimised murine low dose model for invasive aspergillosis was applied [24, 35]. Mice were intranasally infected with a 25 µl drop of a fresh suspension containing 5×10^4 conidia. Survival was monitored daily, and moribund animals were sacrificed by intraperitoneal injection of 200 µl 3.2% (v/v) narcoren (Rhone Merieux, Germany). The drinking water was supplemented with 0.5 mg of tetracycline (Sigma) per ml to prevent opportunistic bacterial infections. A control group (inhalation of PBS) remained uninfected to monitor the influence of the immunosuppression procedure on vitality.

Results

Deletion of the laccase-encoding gene *abr2* of *A. fumigatus*

To analyse the importance of Abr2 for the overall laccase activity of *A. fumigatus*, the *abr2* gene was deleted. For this purpose, plasmid pUCdel*abr2* (Fig. 1a) was generated (see Materials and methods). A DNA fragment obtained by digestion of plasmid pUCdel*abr2* with *Xmn*I which encodes the hygromycin B resistance gene *hph* flanked by upstream and downstream sequences of the *abr2* gene (Fig. 1a), was used for transformation of the *A. fumigatus* wild-type strain ATCC46645. Forty two hygromycin B resistant transformants were isolated. Twenty of them were tested by PCR for the presence of the hygromycin resistance gene. Seven of those, which showed the presence of the selection marker gene, were analysed by Southern blot analysis. Four of these transformants exhibited the expected gene replacement (Fig. 1b, lanes 3, 4, 5, 7, Fig. 1c). One of the mutant strains (lane 3) was designated AfΔ*abr2* and used for further studies. Growth of strain AfΔ*abr2* on agar plates revealed that the *abr2* deletion affected the pigment formation of conidia (Fig. 2a). The colonies showed a brown color. A similar finding was previously reported for an *abr2* mutant by both Tsai et al. [38] and Krappmann et al. [17]. Complementation of the *abr2* deletion mutant using the wild-type *abr2* gene was carried out. For this purpose, the *abr2* gene was amplified by PCR. The generated PCR fragment was applied to a co-transformation experiment, using plasmids pAN8-1 and pHELP1 (see Materials and methods). After transformation of the Δ*abr2* mutant, 17 phleomycin-resistant transformants were isolated. Six of them produced gray-green conidia, i.e., were complemented to the wild type (data not shown). The presence of the *abr2* gene in the transformants producing wild-type conidia was shown by PCR analysis (data not shown).

The germination of conidia (Fig. 2b), the growth rate measured as diameter of colonies on AMM agar (Fig. 2c) and malt agar (Fig. 2d) were the same for both the *abr2* deletion mutant and the wild-type strain, indicating that *abr2* is not essential for vegetative growth. Radial growth rates (Cr) of colonies of *A. fumigatus* wild-type and Δ*abr2* mutant strain on AMM agar plates were 0.46 and 0.48 mm/h, respectively. Cr on malt extract showed values of 0.5 mm/h for *A. fumigatus* ATCC46645 and 0.53 mm/h for the *abr2* deletion strain. SDs were in the range of 0.005–0.02.

Conidia of the strain AfΔ*abr2* were analysed by FESEM (Fig. 3). As previously reported [14] and shown here as a control, wild-type conidia display an ornamented surface (Fig. 3a) which is lacking in *pksP* mutant conidia (Fig. 3b). Conidia of the *abr2* deletion strain displayed some ornamentation which was less pronounced than that observed on wild-type conidia. This finding indicates that formation of the DHN-like melanin intermediate present in the Δ*abr2* mutant is sufficient for production of at least some ornamentation on the surface of conidia.

In contrast to the *pksP* mutant the *abr2* deletion mutant showed the same sensitivity against H_2O_2 and diamide in *vitro* as the wild type

Previously, we showed that mutation of the polyketide synthase gene *pksP* led to increased sensitivity of the respective mutant against ROS generated by immune effector cells [14]. To analyse whether the deletion of *abr2* enhances sensitivity against ROS in the respective mutant or whether the compound produced by the DHN-like melanin pathway to the stage of the Abr2

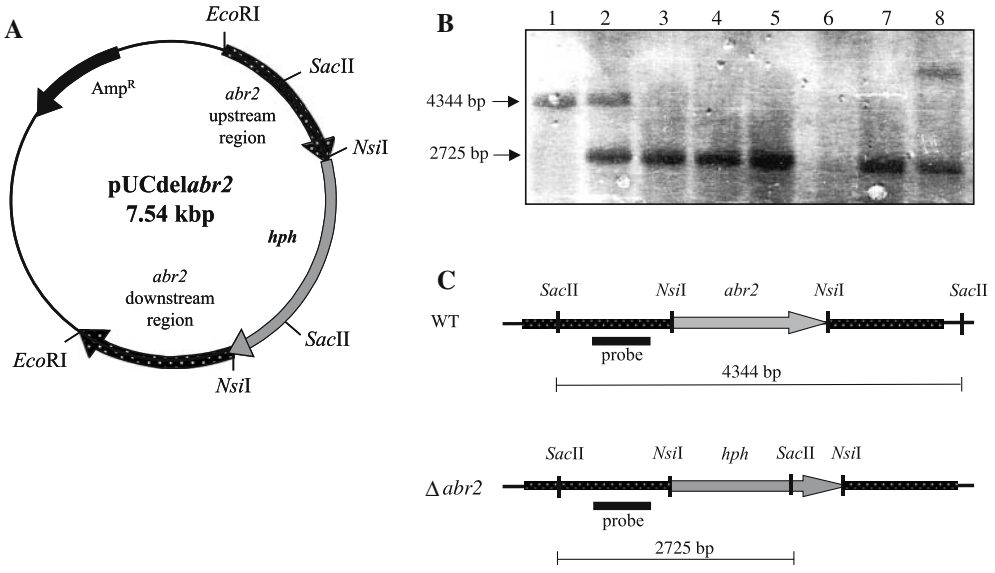

Fig. 1 Deletion of *A. fumigatus abr2* gene. **a** Schematic map of the generation of the *abr2* knock-out plasmid pUCdelabr2. Abbreviations: amp^R, ampicillin resistance gene; *hph*, hygromycin B phosphotransferase gene used as the selection marker gene in *A. fumigatus*. **b** Southern blot analysis. Chromosomal DNA of the wild-type strain ATCC46645 (*lane 1*) and transformant strains (*lanes 2–8*) was cut by *Sac*II. A 641 bp *abr2*-derived PCR fragment was used as the probe. In the AfΔ*abr2* mutant strains, the band characteristic of the wild type (*lane 1*) of 4,344 bp had disappeared. Instead, the band of 2,725 bp characteristic of gene replacement at the *abr2* locus was detected (see **c**). **c** Schematic representation of the chromosomal *abr2* locus of the wild type and the AfΔ*abr2* deletion mutant. Restriction endonuclease cleavage sites, the DNA fragments identified by Southern blot analysis (**b**) and the position to which the probe hybridises, are indicated

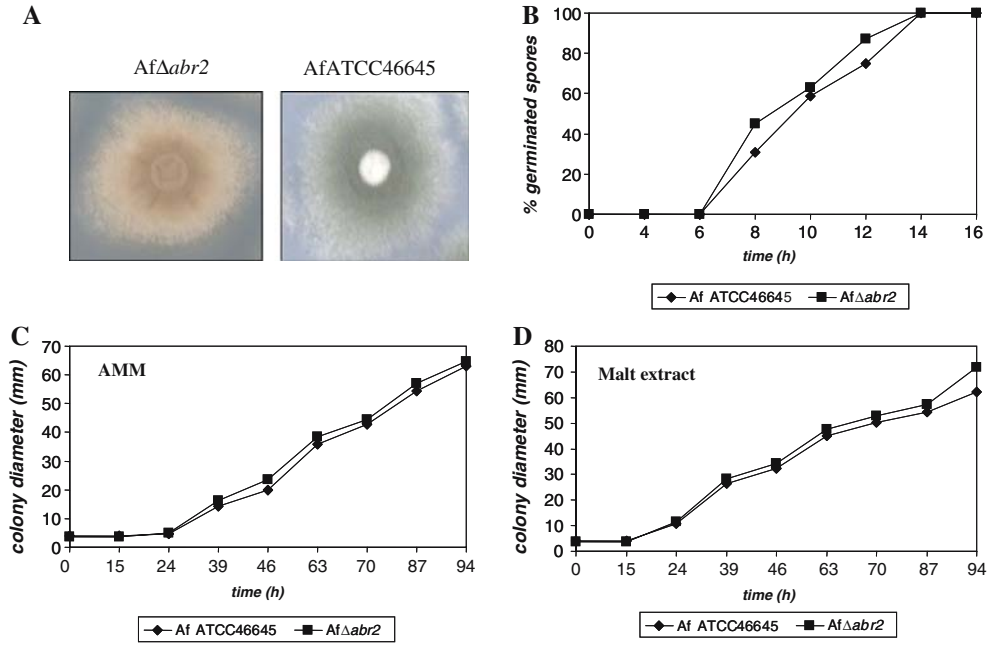

Fig. 2 Phenotypic characterisation of mutant strain AfΔ*abr2* and wild-type strain ATCC46645. **a** Growth and sporulation. Colonies were grown on AMM agar plates for 72 h at 37°C. **b** Kinetics of germ tube outgrowth for *A. fumigatus* conidia incubated in AMM at 37°C. The number of conidia showing a germ tube was recorded at different times of incubation and is presented as the percentage of the total number of conidia. The results are representative of the results of two independent experiments. **c, d** Growth of *A. fumigatus* strains on AMM agar plates and malt agar plates, respectively, at 37°C. The diameter of colonies was determined. Data for each strain represent the mean of at least ten colonies, grown independently. SDs were in the range of 0.2–0.7 mm

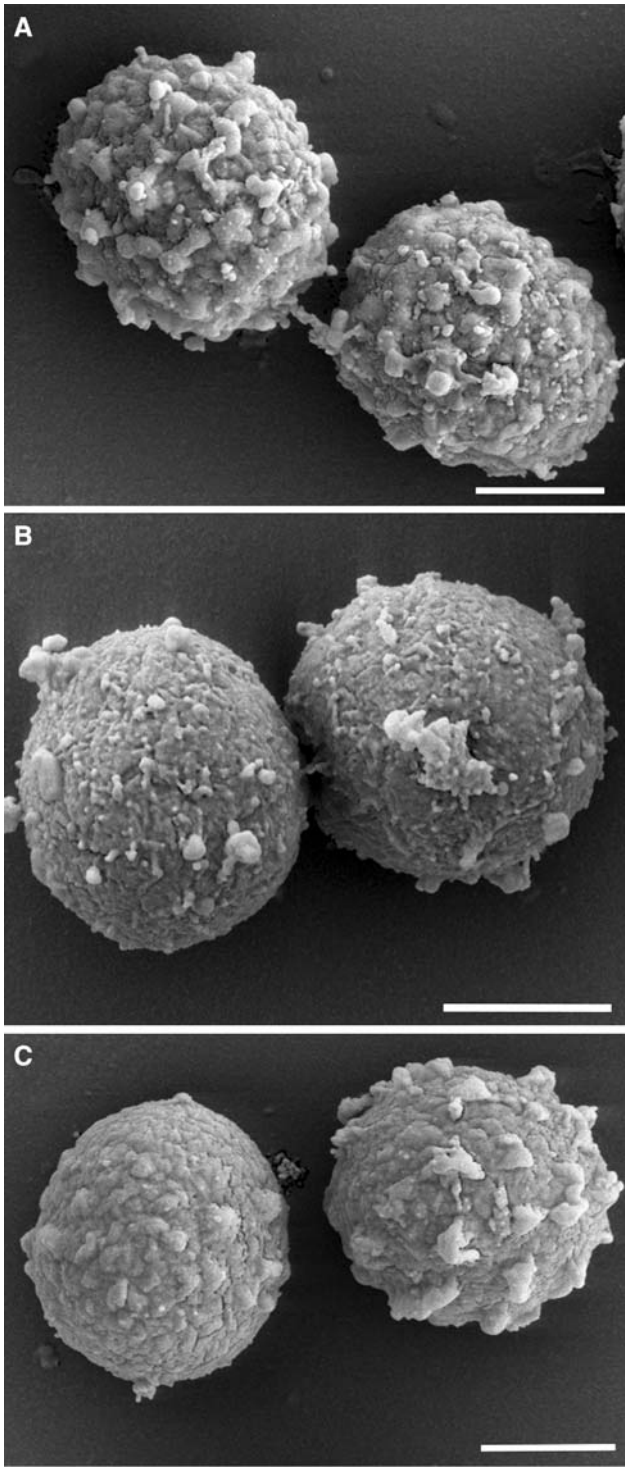

Fig. 3 Field emission scanning electron micrographs of conidia. Bar: 2 µm. **a** *A. fumigatus* wild-type strain ATCC46645. **b** *A. fumigatus pksP* mutant. **c** *A. fumigatus abr2* deletion mutant

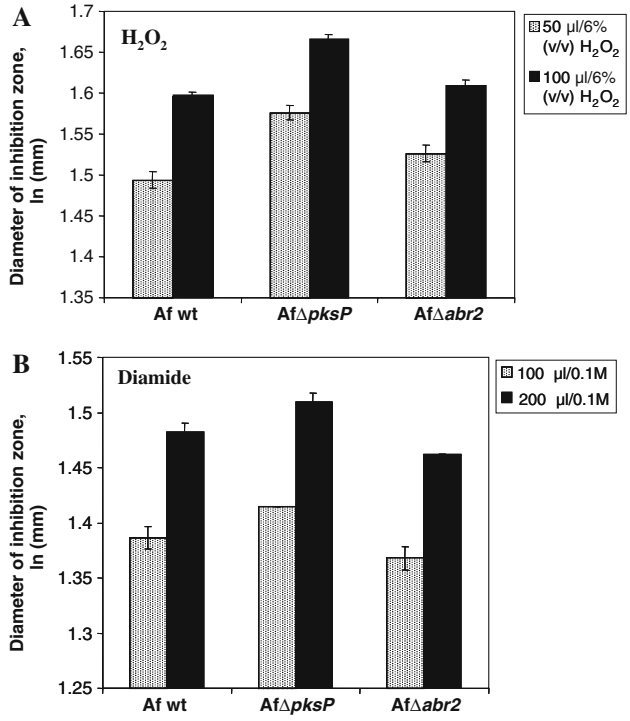

Fig. 4 Sensitivity of Af*Δabr2* towards H_2O_2 and diamide. The strains *A. fumigatus* ATCC46645 (Af wt), Af*pksP* and Af*Δabr2* were analysed. Two concentrations of H_2O_2, 50 µl and 100 µl from a 6% (v/v) H_2O_2 solution, were examined. 100 µl and 200 µl from a 0.1M diamide solution were used in the experiment. Data for each strain and concentration represent the mean and SDs of five independently performed assays. **a** H_2O_2 **b** Diamide

laccase is sufficient to scavenge ROS, the sensitivity of the *Δabr2* mutant against ROS was compared with that of both the *pksP* mutant and the wild-type strain. As shown in Fig. 4a, the *pksP* mutant showed increased sensitivity against ROS whereas there was no significant difference between the *Δabr2* mutant and the wild-type strain. The analysis of the effect of diamide confirmed the results obtained for H_2O_2, but in case of the *pksP* mutant the effects were less prominent (Fig. 4b). Taken together, the *Δabr2* mutant showed the same sensitivity against ROS as the wild type.

Abr2 activity was detectable during sporulation but did not contribute to laccase activity during hyphal growth under standard conditions

To determine the contribution of Abr2 to total laccase activity, cell extracts of both the wild type and the *Δabr2* deletion strain were analysed for laccase activity. Laccase activity was detectable in both strains during vegetative growth after 24 h (Fig. 5). In contrast to the wild-type strain, laccase activity was reduced in the *Δabr2* mutant strain in sporulating mycelia. These findings indicate that the Abr2 laccase is mainly active during sporulation under standard conditions. Furthermore, these results imply that additional laccases are

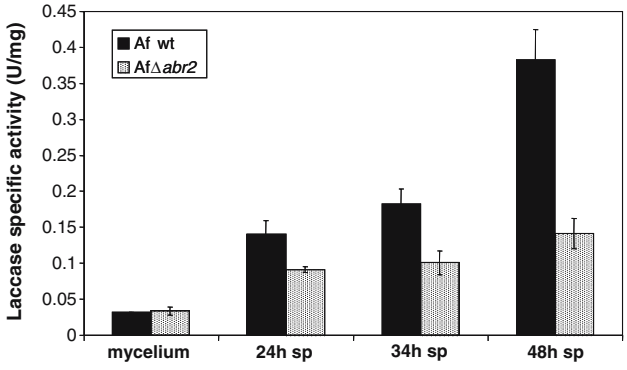

Fig. 5 Laccase activity of mutant strain Af*Δabr2* and wild-type strain ATCC46645 (Af wt). Enzyme activity was monitored during vegetative growth in AMM after 24 h (mycelium) and sporulation after 24, 34 and 48 h (sp)

active during vegetative growth of the fungus under the conditions tested and that their activities also increase during sporulation as seen from the *abr2* deletion strain.

Sporulation-dependent expression of an *abr2*p-*lacZ* gene fusion

To analyse the regulation of *abr2* expression and correlate this data with the Abr2 activity during sporulation, *A. fumigatus* strains were generated carrying an *abr2*p-*lacZ* gene fusion integrated in single copy at the *pyrG* gene locus. For this purpose, plasmid pUC*PyrG*2*abr2*P*lacZ* was used (Fig. 6a). It encodes the *abr2* gene promoter fused in frame with the *E. coli lacZ* gene. Transformation of *A. fumigatus* strain KHΔ*pyrG* using this plasmid resulted in the isolation of eight transformants. Southern blot analysis indicated the presence of the gene fusion in single copy at the *pyrG* gene locus (Fig. 6b, c). The 8 kbp band characteristic of the wild-type *pyrG* gene (Fig. 6a, lane 1) had disappeared in the transformants (Fig. 6a, lanes 2 and 3). Instead, they showed a 10 kbp band due to the integration of the plasmid at the *pyrG* gene locus. The transformants shown in Fig. 6c were designated

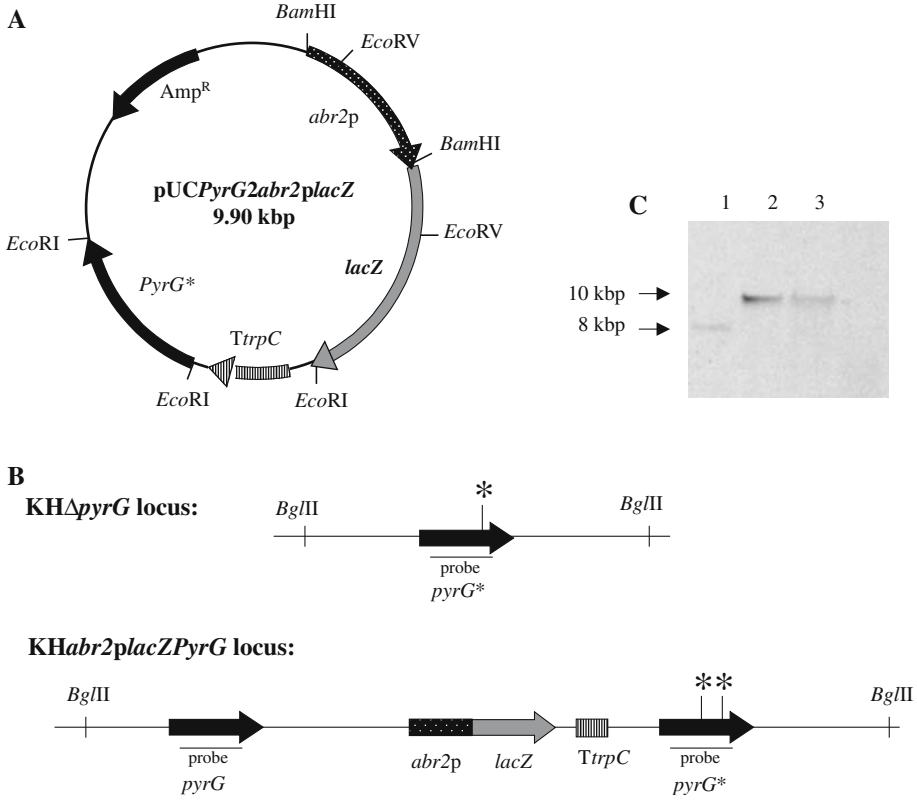

Fig. 6 Integration of an *abr2*p-*lacZ* gene fusion in single copy at the *A. fumigatus* chromosomal *pyrG* gene locus. **a** Schematic map of plasmid pUC*PyrG*2*abr2*P*lacZ*. Abbreviations: *abr2*P, promoter region of the *abr2* gene; Amp^R, ampicillin resistance gene; lacZ, *E. coli lacZ* gene; *PyrG*, orotidine 5′-monophosphate decarboxylase gene of *A. fumigatus*, used as the selection marker gene. The asterisks indicate mutations. **b** Schematic representation of the chromosomal *pyrG* gene locus of strain KHΔ*pyrG* carrying a deletion of part of the *pyrG* gene and of strain KH*abr2placZ-PyrG* carrying the *abr2*p-*lacZ* gene fusion integrated at the *pyrG* gene locus. Restriction endonuclease cleavage sites and the position, to which the probe hybridizes, are indicated. **c** Southern blot analysis. Chromosomal DNA of the *A. fumigatus* wild-type strain KHΔ*pyrG* (*lane 1*) and strains KH*abr2*p*lacZPyrG*-2 and KH*abr2*p*lacZPyrG*-4 (*lanes 2* and 3) was digested by *Bgl*II. A 450 bp PCR fragment encoding a part of the *A. fumigatus pyrG* gene (**b**), was used as the probe

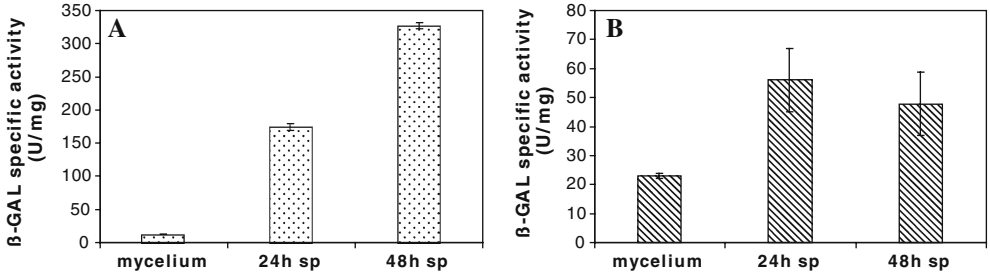

Fig. 7 Expression of *abr2*p-*lacZ* (**a**) and *pksP*p-*lacZ* (**b**) gene fusions during vegetative growth and sporulation. The β-GAL activity was measured after 24 h of growth in liquid culture, and after 24 and 48 h after induction of sporulation. Data for each condition represent the mean and SDs of three independently grown cultures

KH*abr2*p*lacZ*-2 and KH*abr2*p*lacZ*-4 and used in further studies.

The expression of the *abr2*p-*lacZ* gene fusion was determined. Results are shown in Fig. 7a. As expected, the expression of the gene fusion was detectable during sporulation and increased up to 48 h. There was hardly *abr2*p-*lacZ* expression during vegetative growth of the fungus. These data well agree with the results on the laccase specific activity (Fig. 5) indicating that Abr2 mainly contributes to overall laccase activity during sporulation but not during vegetative growth under the conditions applied. Interestingly, the expression pattern of the *pksP*p-*lacZ* and *abr2*p-*lacZ* gene fusions apparently differed. The *abr2*p-*lacZ* expression increased seventeen-fold during sporulation after 48 hcompared with the expression during vegetative growth, whereas for the *pksP*p-*lacZ* gene fusion, this increase during sporulation only was 2.5-fold (Fig. 7b).

The Δ*abr2* mutant showed no reduction in virulence compared with the wild type

To assess a possible role of Abr2 in pathogenesis, the corresponding deletion mutant was tested in an intranasal mouse infection model of invasive aspergillosis. Groups of 10 immunosuppressed mice were infected by intranasal inhalation with 5×10^4 conidia of the wild-type or the Δ*abr2* mutant strain. Results of a representative experiment are shown in Fig. 8. In the groups infected with wild-type conidia (strain ATCC46645), mortality was 80% after 11 days (Fig. 8). When mice were infected with conidia of the Δ*abr2* mutant strain AfΔ*abr2*, mortality was 80% after 13 days. Hence, the overall-mortality of the *abr2* deletion strain was similar to that of the wild type. Moreover, at the beginning of the experiment more of the mice infected with conidia of the AfΔ*abr2* strain died compared with mice infected with wild-type conidia. Taken together, these data indicate that Abr2 does not contribute to virulence of *A. fumigatus*.

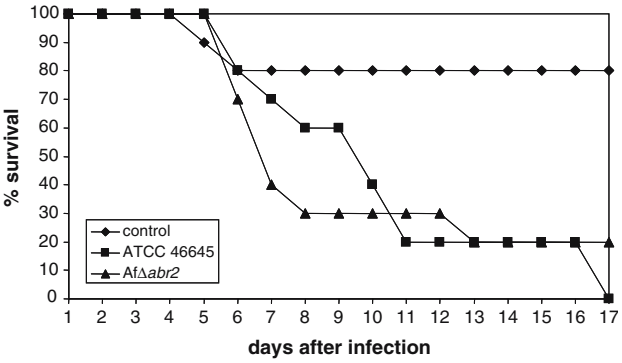

Fig. 8 Virulence of *A. fumigatus* wild type (ATCC46645) and Δ*abr2* mutant strain in mice. Groups of ten BALB/c mice were analysed, each infected intranasally with 5×10^4 *A. fumigatus* conidia, as indicated. Survival was monitored for 17 days

Discussion

Under the conditions applied, laccase activity of *A. fumigatus* was detected during vegetative growth, which strongly increased during sporulation. The analysis of the *abr2* deletion mutant led to the conclusion that Abr2 only contributes to overall laccase activity during sporulation. It is worth to notice that the substrate for determinating laccase activity, DMP, can be oxidised not only by laccases but also by other enzymes as peroxidases [32]. However, because Abr2 clearly used DMP as the substrate and its amino acid sequence resembles that of a typical fungal laccase, it is very likely that Abr2 represents a laccase. The finding that Abr2 only contributes to overall laccase activity during sporulation well agrees with the analysis of an *abr2*p-*lacZ* gene fusion which was mainly expressed during sporulation. By contrast, there was expression of a *pksP*p-*lacZ* gene fusion during vegetative growth which also increased during sporulation, but the increase was less than that observed for the *abr2*p-*lacZ* gene fusion. In fact, the *abr2*p-*lacZ* expression increased steadily up to seventeen-fold during sporulation compared with the

expression during vegetative growth, whereas for the *pks*p-*lacZ* gene fusion, this increase during sporulation only was 2.5-fold and reached its maximum after 24 h. This finding implies that differential regulation of genes from the same cluster occurs. A similar observation was made for genes of other clusters in filamentous fungi, e.g., genes belonging to the penicillin biosynthesis cluster in *A. nidulans* are, at least in part, differentially regulated [4, 7]. Consistently, the promoter regions of *pksP* and *abr2* apparently contain different *cis*-acting elements. Computer analysis revealed that the *pksP* promoter region encodes a single putative stress response element (STRE) [11, 36], three putative AbaA binding sites [2], a potential cAMP-responsive element originally described in the *S. cerevisiae* SSA3 gene promoter and two putative stunted A (StuA) binding sites [10]. By contrast, in the promoter region of *abr2* a single putative AbaA site, a single potential STRE and two putative copper signalling elements (CuSE) were detected. CuSE are defined by the consensus sequence 5′-DWDDHGCTGD-3′ (D = A, G, or T; H = A, C, or T and W = T, or A). They are bound by Cuf1 (copper-sensing transcription factor) and were described for *Schizosaccharomyces pombe* [18]. Whether these elements contribute to *abr2* expression remains to be tested.

Even in liquid medium *abr2*p-*lacZ* expression increased during later stages of the cultivation (data not shown) when most likely sporulation was induced even in liquid medium [34]. Based on the analysis of the *abr2* deletion mutant it can be concluded that the laccase activity measured during vegetative growth was due to other laccases of *A. fumigatus*. In the genome of *A. fumigatus*, there are at least three additional candidate genes (Afu1g15670, Afu4g14280, Afu2g17540; www.tigr.org). Abr2 activity is mainly, if not exclusively, required for the production of the gray-green spore pigment. Interestingly, in contrast to the *pksP* mutant, conidia of the Δ*abr2* mutant did not show increased sensitivity against ROS or diamide. Therefore, the intermediate of DHN-like melanin produced up to the stage of Abr2 is apparently sufficient to protect conidia against ROS. This assumption was further supported by the observation that in a low dose intranasal mouse infection model there was no difference in virulence of the *abr2* mutant compared with the wild type. Furthermore, the surface of Δ*abr2* conidia looked more similar to that of wild-type conidia, i.e., it displayed at least some ornamentation, whereas the *pksP* mutant showed a smooth surface [14]. Taken together, these data support the model that mainly the *pksP* gene of the DHN-like melanin biosynthesis gene cluster is important for virulence.

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