A Novel Role for Catalase B in the Maintenance of Fungal Cell-Wall Integrity During Host Invasion in the Rice Blast Fungus *Magnaporthe grisea*

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Asexual spores of the rice blast fungus germinate to produce a specialized and melanized infection structure, the appressorium, which is pivotal to successful plant penetration. To investigate whether *Magnaporthe grisea* counteracts the toxic burst of H₂O₂ localized beneath the site of attempted invasion, we examined the temporal expression of five candidate antioxidant genes. Of these, the putatively secreted large subunit catalase CATB gene was 600-fold up-regulated in vivo, coincident with penetration, and moderately up-regulated in vitro, in response to exogenous H₂O₂. Targeted gene replacement of CATB led to compromised pathogen fitness; the *catB* mutant displayed paler pigmentation and accelerated hyphal growth but lower biomass, poorer sporulation, fragile conidia and appressoria, and impaired melanization. The *catB* mutant was severely less pathogenic than Guy 11 on barley and rice, and its infectivity was further reduced on exposure to H₂O₂. The wild-type phenotype was restored by the reintroduction of CATB into the *catB* mutant. We found no evidence to support a role for CATB in detoxification of the host-derived H₂O₂ at the site of penetration. Instead, we demonstrated that CATB plays a part in strengthening the fungal wall, a role of particular importance during forceful entry into the host.

Additional keywords: hydrogen peroxide, melanin, ROS, stress, turgor.

All aerobes face exposure to endogenously derived reactive oxygen species (ROS), formed by the incomplete reduction of molecular oxygen during respiration and metabolism. In addition, pathogens must contend with the exogenous oxidative burst generated by the host during invasion (Aguirre et al. 2005; Torres et al. 2006). Irrespective of their origin, ROS can oxidize cell components such as DNA, protein, and lipids, thus damaging cell organelles and compromising cell function. To counteract these potentially lethal effects, cells balance ROS levels in a variety of ways, such as boosting the antioxidant

enzyme activities of superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide (H₂O₂), and catalase, which converts H₂O₂ to water and molecular oxygen.

In plants, the accumulation of ROS at the site of pathogen invasion is thought to have an antimicrobial, cell-wall strengthening or signaling role (Lamb and Dixon 1997; Mellersh et al. 2002; Torres et al. 2005, 2006). In animals, pathogen attack heralds ROS accumulation and triggers innate immunity (Giles et al. 2006). There is some evidence to suggest that pathogens survive the harsh environment of the host using their antioxidant defense systems for self-protection or detoxification of host-derived ROS. For example, the necrotrophic fungal pathogen *Botrytis cinerea* has been shown to suppress defense-related O₂⁻ production in bean leaf tissue (Unger et al. 2005).

Fungi carry varying numbers of catalases with seemingly disparate cellular functions. In *Aspergillus fumigatus*, which has two mycelial and one conidial catalase, deletion of a single catalase had no effect on virulence, but a mutant lacking both mycelial catalases showed reduced virulence (Calera et al. 1997; Paris et al. 2003). The single catalase in *Candida albicans* plays a particular role in protecting the fungus from peroxide stress and neutrophil damage (Nakagawa et al. 2003; Wysong et al. 1998). In *Cryptococcus neoformans*, deletion of any one of four catalases or quadruple gene disruption gave mutants showing no loss of pathogenicity in mice (Giles et al. 2006). In phytopathogenic fungi, catalase knock-out mutants in *Botrytis cinerea* (Schouten et al. 2002), *Cochliobolus heterostrophus* (Robbertse et al. 2003), and *Claviceps purpurea* (Garre et al. 1998) are as virulent as the wild-type strains. However, in *Claviceps purpurea*, deletion of the transcription factor *CPTF1*, which encodes a protein controlling overall catalase activity, had a significant impact on virulence (Nathues et al. 2004). Lastly, there is evidence that barley powdery mildew, *Blumeria graminis* f. sp. *hordei*, scavenges host-derived H₂O₂ specifically at sites of fungal invasion, and the expression and secretion of a catalase coincides temporally and spatially with this (Zhang et al. 2004).

Magnaporthe grisea causes the most destructive disease of cultivated rice world wide as well as infecting a wide range of grass hosts, including barley, wheat, and finger millet (Talbot 2003). Disease is spread by prolific numbers of asexual conidia that are discharged from sporophores. Given the correct cues, a tube emerges from the spore and swells at its distal tip to form a dome-shaped appressorium (de Jong et al. 1997; Howard and Valent 1996; Howard et al. 1991). Subsequently, glycerol levels rise within the appressorium to around 3 M and facilitate the generation of sufficient turgor pressure (8 MPa) for the emerging penetration peg to breach the host cuticle and so effect infection (Howard et al. 1991; Talbot 2003). The

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appressorium cell wall is an ordered chitin-rich structure (Bourett and Howard 1990; Tucker and Talbot 2001) that carries a veneer of melanin on its inner surface. In *M. grisea*, melanin is made via a pentaketide pathway leading to 1,8-dihydroxynaphthalene (DHN), which is polymerized by phenol oxidases to form melanin (Kurahashi 2001; Thompson et al. 2000). Several *M. grisea* mutants, such as albino, buff, and rosy (Howard et al. 1991; Money and Howard 1996), carry specific lesions in melanin biosynthesis and are nonpathogenic because the appressoria are 'leaky' (Talbot 2003) and cannot support high turgor pressures.

Much research has focused on the study of signal transduction pathways that regulate appressorium formation in *M. grisea* (Talbot 2003) and on the various biochemical processes that underpin the generation of turgor (Wang et al. 2005). However, save for the work of Tucker and associates (2004) on the putative redox-sensing wall-associated metallothionein 1 (MMT1) and the observation that many antioxidant genes are abundant in the published *M. grisea* genome sequence (Dean et al. 2005), little is known about antioxidant enzymes in this fungus.

The starting point for our investigation was the search for genes whose products may protect against or detoxify the host oxidative burst, as visualized in rice (Pasechnik et al. 1998). We showed that the gene encoding the large subunit catalase, CATB, is highly up-regulated in vivo, coincident with penetration. The loss of function mutant has compromised wall integrity and this, in turn, leads to attenuated virulence. Thus, CATB plays a novel and pivotal role in the infection process of *M. grisea*.

RESULTS

CATB expression is elevated

during penetration in vivo and in response to H₂O₂ in vitro.

The *M. grisea* genome contains two true heme catalases, catalase A (CATA) and catalase B (CATB), plus catalase peroxidase A (CPXA) and catalase peroxidase B (CPXB). The cytochrome c peroxidase B (CCPB) sequence was also identified. To assess whether the expression patterns of any of these genes suggest their involvement in the management of the host oxidative burst, quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR) was performed on wild-type strain Guy11 inoculated onto barley leaves. Transcript levels were monitored in ungerminated conidia (0 h postinoculation [hpi]), germlings stripped from barley leaves during appressorium formation (5 hpi), coincident with penetration (12 hpi), and during subcuticular growth (20 hpi). Figure 1A shows the dramatic upregulation of CATB gene expression at 12 hpi, when transcript abundance is around 600-fold higher than the level in ungerminated conidia. At the same timepoint, CPXA and CPXB showed a small increase in expression of about twofold, as compared with that found at 0 hpi (Fig. 1C and D), whereas CATA and CCPB transcripts are most abundant in ungerminated spores (Fig. 1B and E). As catalases are potent H₂O₂-detoxifying enzymes, we also looked at CATA and CATB transcript levels following exposure to varying concentrations of H₂O₂ in vitro. CATA expression varied little with time and H₂O₂ concentration, whereas CATB transcript abundance showed a moderate increase, in line with increasing H₂O₂ concentrations (Fig. 1F and G). Collectively, the upregulation of CATB in vivo coincident with penetration of the host and the H₂O₂ dose-dependent rise in expression in vitro implies that CATB plays a role in the infection process that may be associated with detoxification of the host oxidative burst.

Targeted replacement of CATB in *M. grisea* and complementation of *catB* mutant with CATB.

To determine the functional significance of the CATB gene in *M. grisea*, a deletion mutant was created by replacing the CATB coding region with a bacterial hygromycin phosphotransferase resistance gene (Fig. 2A). The gene replacement vector was introduced into rice pathogenic strain Guy11 and hygromycin-resistant transformants were selected. Southern blot analysis confirmed successful gene replacement (Fig. 2B, transformant T6), with a single integration event (Fig. 2C). Verification that CATB gene replacement had resulted in gene inactivation was demonstrated by RT-PCR, which showed complete absence of CATB transcript in the *catB* mutant (Fig. 2D), as compared with persistent β -TUB gene expression. To confirm that the various phenotypes shown by the *catB* mutant were associated with gene inactivation, the CATB gene was reintroduced in a plasmid conferring resistance to sulphonylurea (Carroll et al. 1994). A transformant carrying a single-copy integration of the CATB gene was identified by Southern blot analysis and designated *catB*/CATB (data not shown).

Loss of CATB does not lead to overexpression of CATA or related antioxidant genes.

We investigated whether deletion of CATB affects expression of CATA, CPXA, CPXB, and CCPB by monitoring transcript activity during germling morphogenesis in the *catB* mutant (Fig. 1B through E). There was little overall change in the pattern of transcript accumulation in *catB* and Guy11. The exception was a moderate twofold upregulation of CCPB in ungerminated conidia as compared with Guy11. These data suggest CATA, CPXA, CPXB, or CCPB are unlikely to compensate for the absence of CATB and point towards a distinct role for CATB in *M. grisea* pathogenesis.

Loss of CATB function attenuates virulence.

To determine the role of CATB during disease progression, blast-susceptible barley cultivar Pastoral and rice cultivar CO-39 were inoculated with wild-type Guy11, *catB* mutant, and the recomplemented strain *catB*/CATB. Mean lesion densities on barley were as follows: Guy11, 17.8 $\pm$ 7.8; *catB*, 10.6 $\pm$ 6.3; and *catB*/CATB, 16.2 $\pm$ 6. Mean lesion densities on rice were Guy11, 40.26 $\pm$ 18.7; *catB*, 14.46 $\pm$ 11.5; and *catB*/CATB, 37.1 $\pm$ 8.5. In summary, the *catB* mutant gave rise to around 40% fewer lesions than did Guy11 on barley (Fig. 3A and C, $P < 0.001$) and 65% fewer lesions on rice (Fig. 3B and D, $P < 0.001$). This demonstrated that virulence is attenuated in the *catB* strain but full virulence is restored in strain *catB*/CATB (Fig. 3A).

Loss of CATB function reduces conidiation and compromises hyphal development in vitro.

Mutant colonies show a paler pigmented phenotype than the darker gray of Guy11. The *catB* mutant shows reduced sporulation. For example, at 16 days postinoculation (dpi), the mutant produced 30-fold fewer spores than Guy11 (Fig. 4A). Conidiation was restored to wild-type levels in the recomplemented strain *catB*/CATB (Fig. 4A). The collapsed sporophore and hyphae in the *catB* mutant contrast with the more turgid sporophore and hyphae seen in Guy11 (Fig. 4B).

Loss of CATB function impairs penetration.

We compared *catB* mutant and Guy11 germling differentiation on inductive hydrophobic coverslips, which permit normal appressorium formation in Guy11. Both strains form melanized appressoria (Fig. 5A). Appressorium-mediated cuticle penetration was assessed by counting the number of appressoria forming successful penetration hyphae on barley epidermal

Fig. 1. Relative transcript abundance of **A**, *Magnaporthe grisea* wild-type strain Guy11 *CATB*, **B**, Guy 11 and *catB* mutant *CATA*, **C**, *CPXA*, **D**, *CPXB*, and **E**, *CCPB* following fungal differentiation during infection of barley leaves at 0, 5, 12, and 20 h postinoculation (hpi) and of **F**, Guy 11 *CATB* and **G**, *CATA* expression levels in vitro following exposure to 1 to 5 mM H_2O_2 for up to 3 h. Each profile was normalized against β -*TUB* and was calibrated to 0-hpi, ungerminated conidia. Polymerase chain reaction assays were carried out in triplicate. Template controls and no-reverse-transcriptase controls did not show amplification.

peels. The penetration success rates were twofold higher in Guy11 (61%) than *catB* (32%, $P < 0.05$), demonstrating that the mutant is less able to breach the host than Guy11.

Loss of CATB function impairs management of host-derived H₂O₂ oxidative stress.

The effect of exposure to H₂O₂ on *catB* and Guy11 was investigated in vitro and in vivo. The mutant *catB* showed accelerated growth on agar plate assays carrying 5 to 20 mM H₂O₂ (Fig. 5B and C) compared with that of Guy11 and *catB*/CATB. However, liquid-culture growth assays revealed *catB* had significantly less biomass than Guy 11 (and *catB*/CATB) at equivalent concentrations of H₂O₂ (Fig. 5D).

We investigated whether *catB* or, indeed, Guy11 made appressoria when exposed to exogenous H₂O₂ throughout germling differentiation on hydrophobic plastic coverslips. Around 75% of *catB* and Guy11 germlings formed melanized appressoria in the presence of 1 mM H₂O₂. However, at 10 mM H₂O₂, 80% of *catB* germlings formed melanized appressoria, while only 1% Guy11 germlings progressed to this stage of development, with the majority forming short abnormal germ tubes (Fig. 5A).

We assessed whether the exposure of spores to exogenous H₂O₂ affects appressorium function and, thus, infectivity. Treatment of spores with 1 or 10 mM H₂O₂ for 1 h prior to infection of barley plants revealed an immediate and dose-dependent decline in infectivity of *catB* mutant in increasing concentrations of H₂O₂ ($P < 0.001$) (Fig. 5E). By contrast, Guy11 was not significantly changed in its capacity to infect barley after exposure to 1 mM H₂O₂ but was reduced in virulence after treatment with 10 mM H₂O₂ ($P < 0.05$). This dramatic reduction in infectivity of *catB* in vivo points to a role for CATB in protecting *M. grisea* from host-imposed oxidative stress during invasion.

Loss of CATB function does not influence the accumulation or detoxification of the host-imposed oxidative burst.

First, we monitored the oxidative burst in barley in response to infection by visualizing the cumulative accumulation of H₂O₂ by DAB (3,3'-diaminobenzidine) staining (Fig. 6A, i and ii). Staining was never observed beneath germ tubes or spores, whereas the frequency of polymerized DAB beneath Guy11 and *catB* mutant appressoria increased significantly between 4 and 5 hpi, rising from 5% to 60%. The frequencies of DAB staining beneath Guy11 and *catB* appressoria were not significantly different at either 5 or 12 hpi (Fig. 6B). Second, we used a modified DAB-H₂O₂ assay (Zhang et al. 2004) to reveal areas of putative fungal scavenging of H₂O₂ about appressoria (Fig. 6A, iii). There was no significant difference in the numbers of zones of clearing, which occurred beneath around 38% of appressoria, or their diameters, which measured $90 \pm 11 \mu\text{m}^2$, between Guy11 and *catB* H₂O₂ appressoria at 12 hpi. These findings provoke speculation about an alternative role for CATB in the management of H₂O₂-imposed stress.

Loss of CATB function does not affect conidial longevity or sexual fecundity.

To assess whether CATB plays a role in spore survival and longevity, we compared the infection rates on barley inoculated with *catB* mutant or Guy11 conidia, harvested from 7- and 17-day-old plate-grown cultures. The *catB* mutant was severely impaired in its ability to cause disease lesions compared with that of Guy11. However, there was no statistically significant reduction in the disease levels caused between 7- and 17-day *catB* conidia nor between 7- and 17-day Guy 11 conidia ($P > 0.05$) (data not shown), showing that reduced virulence in *catB*

is not due to compromised spore survival. Sexual crosses with opposite mating-type strain TH3 showed that loss of CATB does not affect the formation of perithecia or ascospores.

Loss of CATB function compromises fungal wall integrity.

We compared the relative integrity of the conidial, mycelial, and appressorium cell walls. Under hyperosmotic stress, *catB* conidia collapsed at lower concentrations than did Guy11 conidia. At 1 M glycerol, collapse rates were 17% for *catB*, 1% for Guy11, and 2% for *catB*/CATB; at 2 M glycerol, 59% for *catB*, 19% for Guy11, and 16% for *catB*/CATB; at 3 M glyc-

Fig. 2. Targeted gene replacement and complementation of *Magnaporthe grisea* CATB. **A**, Organization of the CATB locus, before and after homologous recombination. Translation start (arrow) and stop (asterisk) codons for CATB gene. The restriction enzymes are *Hind*III (*H*), *Kpn*I (*K*), *Nde*I (*N*), *Sal*I (*S*), and *Spe*I (*Sp*). Scale bar = 1kb. **B**, Southern blot analysis of DNA from wild-type strain Guy11 and selected transformants digested with *Nde*I and hybridized to a 1,946-bp *Nde*I fragment containing the CATB gene. Guy11 and putative transformants T3 and T10 contain the intact CATB gene. Transformant T6 does not hybridize to the native CATB gene but to a 1,122-bp fragment, resulting from the gene replacement by the introduction of the hygromycin B-selectable marker. **C**, Southern blot analysis of DNA from Guy11 and T6 transformant digested with *Hind*III and hybridized to the 1.4-kb hygromycin B gene. Only one band of the correct size (5.7 kb) is detected in the T6 transformant, confirming the single integration of the knockout plasmid. **D**, Reverse transcript-polymerase chain reaction used to monitor the expression of CATB in Guy 11 (G), T3, T6 (*catB* mutant), and T10 transformants, using β -TUB as control. Gene replacement in T6 has resulted in complete loss of *catB* transcript.

erol, 90% for *catB*, 40% for Guy11, and 37% for *catB/CATB*. Exposure of mycelium to Glucanex reveals the *catB* mutant releases 60% more protoplasts than does Guy11 and 52% more than *catB/CATB*, by 3 h posttreatment with this cell-wall-degrading enzyme (Fig. 7A). We compared the robustness of *catB* and Guy11 appressorium walls by incipient cytorrhysis. At 24 h, appressoria formed on hydrophobic coverslips were exposed to 1 to 4 M glycerol. The *catB* mutant was hypersensitive to this treatment; for example, at 2 M glycerol, twice as many *catB* appressoria collapsed as did Guy11 appressoria ($P < 0.05$) (Fig. 7B). Together, these data point to a defect in prepenetration germing growth, notably in wall integrity, upon deletion of *CATB*.

We evaluated the structural integrity of the mutant cell wall by comparing the effects of various inhibitors of cell-wall turnover on Guy11 and the *catB* mutant. Comparative growth assays showed no difference in sensitivity of Guy11 and *catB* strains to exposure to sodium dodecyl sulfate (SDS) (0.0001 to 0.05%), to exposure to the chitin synthase inhibitors nikkomycin Z (Cabib 1991) and polyoxin D (Debono and Gordeev 1994) or the 1,3-beta glucan synthase inhibitor caspofungin (Kurtz et al. 1996) at rates up to 100 $\mu\text{g ml}^{-1}$, or to exposure to the fluorochrome chitin-binding dye Calcofluor White (data not shown) at rates up to 2.5 mg ml^{-1} . This demonstrates that while *catB* walls are weaker than those of Guy11, the chitin and glucan synthesis inhibitors and wall modifiers do not differentially affect strain growth.

To gain insight into the cause of the compromised structural integrity of the *catB* mutant, we used Raman spectroscopy to compare the spectral profiles of the *catB* and Guy11 cell walls.

Raman shift wave number 1,085 cm^{-1} , assigned as a C-O bond stretch (Maquelin et al. 2002), reveals a clear difference between the strain profiles, with *catB* showing a peak intensity considerably greater than Guy11 (Fig. 8). The peak intensity demonstrates a greater prevalence of C-O functional groups (e.g., C-OH) such that the oxidation status of *catB* mutant may be greater than that of Guy11.

Loss of CATB function impairs melanization.

The paler pigmented phenotype of the *catB* mutant suggests that melanin biosynthesis is compromised. We compared the effect of a melanin biosynthetic reductase inhibitor, the fungicide Tricyclazole (TCZ), at concentrations of 1 to 150 $\mu\text{g ml}^{-1}$, on *catB* and Guy11 and *catB/CATB* colony growth. TCZ at a concentration of 100 $\mu\text{g ml}^{-1}$ (Fig. 9A) consistently reduced the growth of *catB* by some 1.7-fold more than that of Guy11 ($P < 0.05$). We compared laccase activity, hypothesizing that polyphenoloxidase function may be impaired in *catB*. The oxidation of the laccase substrate 2, 2'-azino-di-3-ethylbenzothiazoline-6-sulfonate (ABTS) in plate assays showed that laccase activity was reduced in *catB*, with a halo consistently 1.4 times smaller than the darker-staining Guy11 ($P < 0.05$) (Fig. 9B). Since copper sulphate is known to stimulate melanogenesis through laccase activity, we looked at the effect of copper sulphate on colony growth. Exposure of the two strains to 1 to 5 mM copper sulphate revealed that *catB* consistently grew better in the presence of copper concentrations than Guy11. For example, *catB* grew 1.1-fold faster than Guy11 in 1 mM copper sulphate and 1.4-fold faster in 5 mM copper sulphate than did Guy11 and *catB/CATB* ($P < 0.05$) (Fig. 9C). Moreover,

Fig. 3. Pathogenicity of Guy 11, *catB*, and *catB/CATB* on barley and rice. **A**, Barley and **B**, rice plants were sprayed with conidial suspensions (10^4 spores ml^{-1}) in 0.25% gelatin. At 6 days postinoculation, 40 leaves picked at random were scored for the number of lesions on 5-cm leaf lengths, in three independent experiments. Mean lesion density recorded **C**, on barley and **D**, on rice. Error bars represent the standard deviations of the means.

addition of high concentrations of copper sulphate to ABTS plates gave rise to laccase haloes of equivalent diameter with *ΔcatB* and Guy11, suggesting that high exogenous concentrations of copper may boost laccase activity in the mutant (Fig. 9D). Thus, these data suggest that CATB plays a role in the melanization of the fungal cell wall.

DISCUSSION

Fungal catalases.

Recent phylogenetic analysis provides some insight into the origin of fungal catalases (Giles et al. 2006; Johnson et al. 2002). Most recently, Giles and associates (2006) gathered the catalases into four distinct clades, i.e., clade P (peroxisomal catalase), clade C (cytoplasmic catalase), clade A (spore-specific catalase), and clade B (secreted catalase). They speculated that ancestral fungi probably possessed spore-specific, peroxisomal and cytoplasmic catalases but that the secreted catalases, which exist only in euscomycete fungi, arose later. The *M. grisea* genome database yielded two catalase-peroxidase sequences and single copies of catalase A and B. No peroxisomal catalase-like sequence was found. The CATA and CATB sequences from *M. grisea* are homologous to large subunit monofunctional catalases. Within the large subunit enzyme group, those with N-terminal signal peptides, such as *M. grisea* and *Blumeria graminis* CATB (Zhang et al. 2004), *Neurospora crassa* CAT3 (Michan et al. 2003), and *Aspergillus nidulans* CATB (Kawasaki and Aguirre 2001), cluster closely together, while *M. grisea* CATA falls in a branch with other putatively nonsecreted fungal catalases alongside *N. crassa* CAT1 and *A. nidulans* CATA (Giles et al. 2006).

Temporal expression of catalases and other genes encoding putative antioxidant genes.

Of the various patterns of temporal expression of *CATA*, *CATB*, *CPXA*, *CPXB*, and *CCPB* genes during germling differentiation on barley leaves, most notable was the peak in *CATB* transcript accumulation at the mature appressorium stage. This coincided with the host burst of H_2O_2 , as evidenced by DAB staining of the barley or rice and fungus interface (Pasechnik et al. 1998). No such detailed transcript data is available for the early stages of germling morphogenesis of other phytopathogenic fungi. However, in the necrotroph *B. cinerea*, expression of the *CATB* gene is up-regulated during host colonization (Schouten et al. 2002), which was not the case for rice blast infection. *M. grisea* *CATB* transcript abundance increased also in vivo immediately following exogenous application of H_2O_2 , in a dose-dependent manner, albeit to a lower level than in vitro. This finding hints at the importance of the host in triggering *CATB* expression in planta.

The observation that *CATB* transcripts were significantly enhanced in mature appressorium-stage germlings coincident with host penetration in Guy11 provoked the question as to whether genes encoding other antioxidant enzymes would compensate by boosting their own expression levels in the *catB* knockout mutant. However, no dramatic stage-specific increases (i.e., greater than twofold) in transcript abundance of *CATA*, *CPXA*, *CPXB*, and *CCPB* were recorded in the *catB* mutant, suggesting that CATB plays a distinct role in *M. grisea* and that functional redundancy between CATB and these proteins is unlikely.

Expression of *CATA* in the filamentous fungi *A. nidulans* and *A. fumigatus* and of the homologous nonsecreted catalase CAT1 in *N. crassa* is known to be spore-specific (Navarro et al. 1996; Paris et al. 2003) or elevated in conidia (Michan et al. 2002). This may be the case for *M. grisea* CATA, in which transcript abundance is elevated in ungerminated spores but is

not altered by treatment with exogenous H_2O_2 . *M. grisea* CATA may, therefore, be developmentally regulated to specifically protect conidia from endogenously-derived oxidative stress during metabolism, as has been found during *N. crassa* conidiation (Lledias et al. 1999; Peraza and Hansberg 2002).

The role of CATB in pathogenesis.

CATB gene ablation gave a mutant that caused disease of half the severity of its wild-type counterpart Guy11 on rice and barley. Appressorium-mediated cuticle penetration rates were also significantly reduced in the mutant compared with that of Guy11. The native CATB emerges, therefore, as a significant virulence determinant in successful rice blast infection. This is at variance with what has been found in other phytopathogenic fungi studied.

The *M. grisea catB* mutant phenotype displayed several unusual growth characteristics. Compared with the wild-type

Fig. 4. Conidiation and hyphal morphology of Guy11 and *catB*. **A**, Comparison of the number of conidia produced by Guy 11, *catB*, and *catB/CATB* on solid medium (points are from three replicates). **B**, Scanning electron microscopy of Guy11 and *catB* showing conidium and turgid or collapsed hyphae. Scale bar = 10 μ m.

strain, the paler *catB* mutant showed reduced sporulation, conidia and appressoria more prone to collapse under hyperosmotic stress, and accelerated release of protoplasts. Most pronounced of all visible characteristics, however, was the

more rapid radial growth of the $\Delta catB$ mutant in vitro upon exposure to exogenous concentrations of H_2O_2 than that seen in Guy11. However, the biomass of these mutant colonies was less than that of their wild-type counterparts. This particular

Fig. 5. Effect of exogenous H_2O_2 on Guy11, *catB*, and *catB/CATB* growth and virulence. **A**, Strains Guy 11 and *catB* differentiating on hydrophobic coverslips with and without H_2O_2 treatment (0.1 to 10 mM), 10 × 12 germlings were scored at each timepoint. Error bars represent standard deviation from mean. **B** and **C**, Effect of H_2O_2 (0 to 20 mM) on agar plate growth of Guy11, *catB*, and *catB/CATB* at 10 days. Diameters were measured from three replicate experiments. **D**, Effect of H_2O_2 (0 to 10 mM) on liquid culture growth of Guy11, *catB*, and *catB/CATB*. Biomass was recorded at 6 days postinoculation (dpi) with three replicates measured in two independent experiments. **E**, Effect of exogenously applied H_2O_2 (1 and 10 mM, 1-h exposure on 1 ml of 10^4 spores) on the ability of Guy11 and *catB* strains to cause infection on barley plants. Mean disease lesion densities were counted at 6 dpi. Error bars indicate standard deviations. *P* values were calculated comparing untreated with H_2O_2 -treated (1 and 10 mM) spores individually for each strain. * indicates $P < 0.05$, ** indicates $P < 0.001$. All differences between Guy11 and *catB* virulence after all H_2O_2 treatment were statistically significant differences at the 0.001 level.

phenotype has not previously been recorded; for example, *B. cinerea* and *Cochliobolus heterostrophus* CATB-disrupted mutants were more sensitive to exogenous H₂O₂ than their wild-type counterparts (Robbertse et al. 2003; Schouten et al. 2002). Curiously, in the filamentous fungus *N. crassa*, inactivation of CAT3 boosted production of conidia and aerial hyphae, leading to enhanced hyphal adhesion and to higher carotene levels (Michan et al. 2003). No such phenotypic changes were seen in the *M. grisea* *catB* mutant. Several of the *catB* mutant growth characteristics are, however, seen in the *M. grisea* metallothionein mutant, *mnt1* (Tucker et al. 2004). These include decreased sporulation, lighter pigmentation, resistance to H₂O₂ stress in vitro, and weakened fungal walls.

As endogenous oxidative stress is a major determinant of the rate of aging (Melov et al. 2000; Osiewacz 2002), we looked at the infection rates of differently aged *catB* germ-lings. Deletion of CATB did not alter the number of disease lesions. This further supports the idea that the conidially-associated, predicted cytoplasmic CATA, rather than the putatively secreted CATB, is more likely involved in the management of endogenous oxidative stress.

The observation that the *catB* mutant was able to develop normal-looking melanized appressoria in high concentrations of H₂O₂ while Guy11 was halted at the short germ-tube stage by this level of oxidative stress led us to compare infectivity of the two strains in vivo following exposure of ungerminated conidia to varying concentrations of exogenous H₂O. These plant infection assays revealed a dose-dependent loss of infectivity in *catB*, demonstrating a role for CATB in the management of host-produced H₂O₂. In the *catB* mutant, therefore, H₂O₂ affects the viability and proper functioning of appressoria but not their formation.

We asked whether CATB acts as a potent antioxidant that directly detoxifies the localized burst of plant H₂O₂ formed as the pathogen attempts penetration. This is an unlikely scenario, as deletion of *M. grisea* CATB did not affect scavenging of

host-derived H₂O₂ by the fungus. Furthermore, the *catB* mutant tolerated and grew in concentrations of H₂O₂ that were toxic to Guy11. It appears more likely that CATB is involved in the formation or the maintenance of a robust fungal cell wall. The evidence in support of this model are the following mutant phenotypes: i) reduced conidiogenesis, ii) conidia more prone to collapse on exposure to hyperosmotic stress, iii) greater sensitivity to protoplasting enzymes, and iv) appressoria more prone to cytorrhysis, i.e., the mutant cannot accumulate high concentrations of glycerol prior to penetration peg emergence. This body of data suggests that the large subunit catalase B in *M. grisea* is involved in cell-wall reinforcement during conidiogenesis and, more importantly, during appressorium maturation at the time of host penetration.

However, CATB is unlikely to be involved in wall turnover, as we showed that inhibitors and wall modifiers of chitin and glucan synthesis do not differentially affect growth of the *catB* mutant or Guy11. An insight into the variation in wall composition of the two strains is demonstrated by the Raman spectroscopic data, which indicated that the *catB* mutant cell walls are likely to be in a hyperoxidant state. This implies a 'blockage,' perhaps in either or both the formation or oxidative cross-linking of DHN melanin in *M. grisea* (Caesar-Tonthat et al. 1995; Henson et al. 1999). We observed that the activity of the secreted blue copper oxidase, laccase, was reduced in *catB* compared with that in Guy11 and pursued this by considering the effect of copper sulphate, known to stimulate the biosynthesis of melanin by inducing laccase activity, on colony growth (Galhaup and Haltrich 2001; Parisot et al. 2002). The *catB* mutant tolerated higher concentrations of copper than did Guy11. Addition of copper sulphate led to restoration of laccase activity and to darker colony pigmentation in *catB* mutant. Taken collectively, these data imply that the *catB* mutant is in a hyperoxidant state, which may, in turn, lead to reduced oxidative cross-linking of melanin. This can be compensated for by a boost in laccase activity in the mutant (as effected by exogenous copper). Surprisingly, the

Fig. 6. Evidence for the host oxidative burst in barley. **Ai** and **ii**, DAB (3,3'-diaminobenzidine) staining reveals a burst of H₂O₂ in barley cv. Pastoral beneath Guy11 appressoria (arrow) from 4 h postinoculation (hpi). **iii**, DAB-H₂O₂ staining reveals clearing of H₂O₂ beneath the appressorium (arrow) at 12 hpi. **B**, Presence of DAB staining in epidermal cells beneath Guy11 and *catB* germ-lings at 5 and 12 hpi. Each timepoint represents 12 × 10 spores scored in two independent experiments. Error bars represent standard deviations from the mean.

melanin biosynthesis inhibitor TCZ, considered to interfere with DHN biosynthesis before the oxidative cross-linking of melanin occurs (Kurahashi 2001), suppressed colony growth more effectively in the *catB* mutant than in Guy11. Other melanin-deficient mutants in *M. grisea* also failed to generate sufficient turgor to cause disease (Howard et al. 1991), but unlike *catB* and *mmt1* mutants, these mutants are more sensitive to ROS than the wild-type strain (Pasechnik et al. 1998).

The fact that the *catB* mutant is severely impaired in virulence but does not show complete loss of pathogenicity suggests that there are additional antioxidant enzymes that critically contrib-

ute to defense from oxidative injury imposed by the host. For example, glutathione peroxidases and peroxidoxins have been demonstrated to neutralize exogenous H_2O_2 and might also take part in ROS-dependent signaling cascades (Aguirre et al. 2005; Torres et al. 2005). The interplay of such proteins is likely to form an extensive antioxidant network in *M. grisea* during the establishment of rice blast disease; much remains to be unmasked.

Taken together, these data suggest that CATB is involved in the melanization and, thus, in the fortification of the fungal cell wall. This role is of particular importance for the genera-

Fig. 7. The *catB* mutant has weakened cell walls. **A**, Release of protoplasts from Guy11, *catB*, and *catB*/CATB mycelium treated with cell-wall-degrading enzyme Glucanex. Error bars represent the standard deviation of the means. **B**, Effect of 10-min exposure of 24-h Guy11, *catB*, and *catB*/CATB appressorium-stage germlings to 0 to 4 M glycerol. A total of 300 appressoria were scored to assess collapse in two independently conducted experiments.

tion of sufficient turgor pressure to enable the penetration peg to pass through the cuticle and breach the plant cell wall. It is unlikely, however, that CATB is involved in detoxification of the host oxidative burst response. The data attests to an important and novel developmental role for CATB in the infection process. We seek now to uncover how CATB influences the oxidative cross-linking of the fungal cell wall.

MATERIALS AND METHODS

Fungal strains, growth conditions, and nucleic acid analyses.

M. grisea wild-type rice-pathogenic strain Guy11 was used throughout this work; strain TH3 was used in the sexual crosses.

Fig. 8. Raman spectroscopy data of Guy 11 and *catB* cell walls. The clear peak at wavelength $1,085\text{ cm}^{-1}$ indicates the prevalence of C-O groups in the *catB* wall.

Strain maintenance, composition of media, DNA and RNA extraction, and transformation protocols were as described by Talbot and associates (1993). Standard procedures were adopted for restriction-enzyme digestion, gel electrophoresis, gel blots, and sequencing (Sambrook and Russell 2001).

Monitoring gene-expression levels by qRT-PCR profiling.

We used barley as the host for two reasons: i) we were able to routinely peel off inoculated adaxial barley epidermal strips and so reduce the plant component in RNA preps, but rice epidermal peels proved problematic, and ii) these tissues were amenable to appressorium-mediated penetration assays. Production of good-quality rice epidermal peels proved more problematic. Considerable care was taken to monitor the timing of stage-specific germling development on barley, so as to synchronize maturation of appressoria and subsequent penetration events. Stage-specific material was collected from wild-type strain Guy11 or *catB* mutant at 0, 5, 12, and 20 hpi of detached barley leaves, for RNA extraction. The abaxial epider-

Fig. 9. The *catB* mutant has compromised melanization. **A**, Sensitivity of Guy11, *catB*, and *catB/CATB* colonies to exposure to the fungicide Tricyclazole at the rate of $100\text{ }\mu\text{g ml}^{-1}$. **B**, Laccase activity monitored in 0.2 mM 2'-azino-di-3-ethylbenzothiazoline-6-sulfonate at 5 days postinoculation of equivalent numbers of Guy11, *catB*, and *catB/CATB* conidia. **C**, Sensitivity of Guy11, *catB*, and *catB/CATB* colonies to exposure to 5 mM copper sulphate. **D**, Laccase activity of Guy11, *catB* mutant, and *catB/CATB* in the presence of 5 mM copper sulphate.

mal surface was removed. First-strand cDNA was synthesized from total RNA using the RETROscript first strand synthesis kit (Ambion, Austin, TX, U.S.A.). Real-time PCR was performed on first-strand cDNAs, prepared as above, using Qiagen QuantiTect SYBR green RT-PCR kit (Qiagen Ltd., Hybaid, Cambridge). Primers were designed to amplify approximately 100 bp and to bridge introns of *CATA* (accession number MG100061), *CATB* (MG06442), *CPXA* (MG04337), *CPXB* (MG09834), and *CCPB* (MG04545). Transcript abundance of the target genes relative to the constitutively expressed normalizer β -*TUB* gene (MG06604) was quantified, taking account of primer efficiencies as described by Pfaffl (2001). A second constitutive control gene, elongation factor 1 (*EGF1*) (MG03641) was also used as an independent normalizer. Expression analysis of *CATB* and *CATA* in in vitro-grown Guy11 was with mycelium harvested from liquid cultures exposed to 1 to 5 mM H₂O₂ for up to 3 h. The forward (F) and reverse (R) primers were as follows.

βTUB: F: 5' CTCTGCCATCTTCCGTGGA 3' and
R: 5' ACGAAGTACGACGAGTTCTTGTCT 3'
CATA: F: 5' GGTGGACGCCGTGTACGT 3' and
R: 5' CCGTTCCTTGGACAATGTCTTGA 3'
CATB: F: 5' GGTCACCGTCCACAACAATAATC 3' and
R: 5' TCAGGGCACTGGGTGTATAAGG 3'
CPXA: F: 5' TTGCTCCTTCCAAGCTTATCCA 3' and
R: 5' CTTGTACCTCCACGGAATGT 3'
CPXB: F: 5' CGTGGCGGTGGTGAAT 3' and
R: 5' TGATGGGCCAAATCAGCC 3'
CCPB: F: 5' GGGATGGTCCTAAGCAGTACGTC 3' and
R: 5' TGTCCTCGATGAGGCACATG 3'
EGF1: F: 5' CCTTGTACCAAGGAGCGTTAGG 3' and
R: 5' TCAACGGTGACCACATGATCTC 3'

Real-time quantification was performed using the ABI Prism 7300 sequence detection systems (Applied Biosystems, Foster City, CA, U.S.A.), cycling 50°C, 2 min for 1 cycle, 95°C, 10 min for 1 cycle, 95°C, 15 s and 60°C 1 min for 40 cycles. No template controls were monitored for the presence of primer dimers, and no-reverse-transcriptase controls were included for each cDNA sample. PCR were carried out in triplicate and mean values were determined.

Targeted gene replacement and complementation of *CATB*.

The full-length *CATB* gene and its 1.1-kb upstream and 0.9-kb downstream flanking sequences were amplified by PCR and were cloned into the *EcoRI* site of pT7Blue vector (Novagen, Inc., Madison, WI, U.S.A.) to form plasmid pZZ100. Partial digestion of pZZ100 with *SalI* released the *CATB* gene sequence, which was replaced by the *SalI*-hygromycin B resistance gene (1.4 kb) released from plasmid pCB1636 (Sweigard 1997), forming the *CATB*-replacement plasmid pZZ101. Protoplast-mediated transformation of *M. grisea* Guy11 was carried out following the method of Talbot and associates (1993). To confirm *CATB* replacement, *NdeI*-digested genomic DNA from hygromycin-resistant *catB* transformants was gel blotted and hybridized with a 1,946-bp fragment from the Guy11 *CATB* gene, which was α^{32} P dCTP radiolabeled with the Prime-It II random primer labeling kit (Stratagene, La Jolla, CA, U.S.A.). To assess whether a single integration event had occurred, Guy11 and *catB* mutant were digested with *HindIII*, were gel blotted, and were hybridized to the 1.4-kb hygromycin B gene amplified from pCB1632. For complementation of the *catB* mutant, a 4,943-bp *KpnI-SpeI* fragment spanning the *CATB* locus was excised from pZZ101 and was ligated into *KpnI-SpeI*-digested pCB1532, which carries the sulphonylurea resistance selectable marker (Carroll et al. 1994). The resulting plasmid pPS300 was sequence-verified and reintroduced into the *catB*

mutant, with transformants selected on 100 μ g ml⁻¹ chlorimuron ethyl; the complemented strain was designated *catB/CATB*. To ensure that single integration of *CATB* into *catB* had occurred, *HindIII*-digested Guy11 and *catB* DNA was subjected to Southern blot analysis and was hybridized with a 5.7-kb *HindIII* fragment carrying the *CATB* sequence.

Plant infection and infection-related germing development assays.

Plant infection assays were performed on blast-susceptible 14-day-old seedlings of rice (*Oryza sativa*) cultivar CO-39 or 7-day-old seedlings of barley (*Hordeum vulgare*) cultivar Pastor. A suspension of 10⁴ conidia per milliliter in 0.25% (vol/vol) gelatin water was applied, using an artist's airbrush with high-pressure air from a Kenair duster (Kenro Ltd., Wiltshire, U.K.) as described in Talbot and associates (1993). Disease lesions were counted from 40 randomly chosen 5-cm leaf tips, as detailed in Valent and associates (1991), in three independent experiments. Mean disease-lesion densities were calculated and compared.

Phenotypic analyses of *catB* mutant.

Vegetative growth of Guy11 and *catB* mutant was monitored using conidia harvested from 12-day cultures adjusted to 10⁴ spores per milliliter or by plug inoculum. Exposure to exogenous 0.75 to 20 mM H₂O₂, 0.0001 to 0.05% (wt/vol) SDS, 0.01 to 5 mM copper sulphate, nikkomycin Z (Sigma, St. Louis), polyoxin D (Calbiochem, San Diego, CA, U.S.A.), caspofungin (Merck Sharp, Amsterdam) at rates up to 100 μ g ml⁻¹, Calcofluor White (Sigma) at rates up to 2.5 mg ml⁻¹, or fungicide TCZ, 5-methyl-1,2,4 triazole(3,4-6) benzothiazole (a gift from A. Corran, Syngenta, Jealott's Hill, Berkshire, U.K.) at rates up to 1 to 150 μ g ml⁻¹ was assessed by measuring colony diameter at 10 dpi. Biomass was recorded in shake flask cultures (150 rpm) grown for 48 h at 250°C, whereupon 0, 1, 5, and 10 mM H₂O₂ was added and cultures were grown for a further 24 h. Fungal biomass was recorded by dry weight determination of three replicates per treatment in two independent experiments. Laccase activity was monitored on 0.2 mM ABTS agar plate assays of mycelial plugs at 2 dpi.

Infection-related appressorium development was assessed by following germing differentiation and appressorium formation on hydrophobic plastic coverslips up to 24 hpi, by light microscopy. Germing exposure to 0, 0.1, 1, and 10 mM H₂O₂ on hydrophobic coverslips was scored for effects on germination and development.

Sensitivity to the protoplasting enzyme β -1,3-glucanase, Glucanex (Sigma) was assayed by incubating 48-h grown mycelium in 12.5 mg Glucanex per milliliter in OM buffer (1.2 M MgSO₄, 10mM Na₂HPO₄/NaH₂PO₄, pH 5.8) for 3 h at 30°C. At 30-min intervals, protoplasts were withdrawn and counted by hemocytometry.

Conidial collapse was observed following exposure of conidia to 1 to 3 M glycerol for 10 min. Appressorium turgor was assessed by a cell-collapse assay (Dixon et al. 1999); germings differentiating in water droplets on hydrophobic coverslips were left for 24 h under high humidity conditions. The water was replaced by 0 to 4 M glycerol, germings were left for 10 min, and 300 appressoria per treatment were scored for cell collapse, in two independent experiments.

Perithecia and ascospore formation was followed by crossing the *catB* mutant (Guy 11 background) with opposite mating type strain TH3. Plug inoculum of the two strains were placed 4 cm apart on oatmeal agar and were incubated under constant fluorescent light at 18°C for 28 days. Perithecia were gently crushed to reveal asci and ascospores.

The host oxidative burst and staining for H₂O₂ scavenger activity.

DAB staining of host tissues was with leaves placed in 1 mg of 3,3'-diaminobenzidine tetrahydrochloride per milliliter and incubated for 8 h at room temperature. At specific timepoints up to 24 hpi, leaves were placed in a clearing solution of ethanol/acetic acid (vol/vol) and, then, mounted in 30% glycerol and 30% lactic acid, and were examined for DAB staining by light microscopy. The percentage of germings showing DAB staining of the underlying host tissues were recorded by scoring 10 germings on each of 12 leaves in two independent experiments.

Staining for Guy11 and *catB* mutant H₂O₂ scavenger activity was with abaxial epidermis barley leaf peels, at specific timepoints postinoculation, clamped between the jaws of hairgrips, and then, immersed in staining solution (1 mM H₂O₂, 2.5 mM DAB, 0.1% Triton X100) for 1 h at room temperature, rinsed twice in distilled water, and mounted in 30% glycerol. The percentage of appressoria overlying H₂O₂ clearing was recorded.

SEM and Raman microscopy.

Scanning electron microscopy (SEM). Small squares of sporulating mycelia on agar were mounted on copper stubs at room temperature using Leit C carbon conductive cement. The stubs were rapidly inserted under a stream of dry argon into precooled stub holders (at -186°C) withdrawn from the SP2000A sputter-cryo system. The sample was introduced to the cold stage of the JEOL JSM840A scanning electron microscope at about -150°C to inspect for frost. As no serious frosting was observed, they were returned to the sputter-cryo system and, after recooling to -186°C, were coated with gold and palladium under argon. The samples were returned to the microscope and digital images were acquired using SemAfore.

Raman microscopy. Raman microscopy was performed using a LabRAM 300 microscope (Jobin-Yvon Ltd., Stanmore, Middlesex, U.K.). Scattering was excited by a frequency-doubled 532-nm Nd:YAG laser. Ten-day-old plate cultures of *M. grisea catB* mutant and Guy11 strains were harvested and filtered through two layers of Miracloth, were ground with pestle and mortar to a fine powder in 20 mM Tris, 50 mM EDTA, pH 8, in liquid nitrogen, were centrifuged at 3,000 × *g* for 10 min, and were lyophilized. A 10-μl sample was spread on a quartz slide and was targeted by confocal optics. Spectra were acquired between 544 and 1,972 cm⁻¹ at approximately 5 to 8 mW for 60 to 90 s for three samples of each preparation, were baseline corrected and normalized by Labspec software (Jobin-Yvon Ltd.), and were imported into MVSP 3.12d (Kovach Computing, Pentraeth, Anglesey, U.K.) and MatlabR12 (Maths Works, Natick, MA, U.S.A.) for multivariate analysis.

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