The Role of Catalase-Peroxidase Secreted by *Magnaporthe oryzae* During Early Infection of Rice Cells

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The biological role of a secretory catalase of the rice blast fungus *Magnaporthe oryzae* was studied. The internal amino acid sequences of the partially purified catalase in the culture filtrate enabled us to identify its encoding gene as a catalase-peroxidase gene, *CPXB*, among four putative genes for catalase or catalase-peroxidase in *M. oryzae*. Knockout of the gene drastically reduced the level of catalase activity in the culture filtrate and supernatant of conidial suspension (SCS), and increased the sensitivity to exogenously added H₂O₂ compared with control strains, suggesting that *CPXB* is the major gene encoding the secretory catalase and confers resistance to H₂O₂ in hyphae. In the mutant, the rate of appressoria that induced accumulation of H₂O₂ in epidermal cells of the leaf sheath increased and infection at early stages was delayed; however, the formation of lesions in the leaf blade was not affected compared with the control strain. These phenotypes were complemented by reintroducing the putative coding regions of *CPXB* driven by a constitutive promoter. These results suggest that *CPXB* plays a role in fungal defense against H₂O₂ accumulated in epidermal cells of rice at the early stage of infection but not in pathogenicity of *M. oryzae*.

The generation of reactive oxygen species (ROS) such as the superoxide anion radical (O₂⁻), hydroxyl radical (OH[•]), and hydrogen peroxide (H₂O₂) in plant cells is one of the most rapid and drastic defense reactions activated following pathogen attack (Doke 1983; Lamb and Dixon 1997). H₂O₂ is involved in the cross-linking of cell wall proteins, induction of expression of defense-related genes, and production of phytoalexins (Bradley et al. 1992; Chai and Doke 1987; Torres and Dangi 2005; Torres et al. 2006). It has been suggested that H₂O₂ mediates mutual interactions between pathogens and host plants through its fungicidal and bactericidal effects (Lu and Higgins 1999; Peng and Kuc 1992). Hyphal growth of some biotrophic and hemibiotrophic fungal pathogens is inhibited in the host cells because of H₂O₂ produced by the host (Mellersh et al. 2002; Shetty et al. 2007; Thordal-Christensen et al. 1997; Vanacker et al. 2000), whereas the growth of necrotrophic fungal pathogens is enhanced in the presence of H₂O₂ (Able 2003; Govrin and Levine 2000; Kumar et al. 2001). In such oxidative environments, ROS-degrading enzymes could play crucial roles for the survival of pathogens. In fact,

catalase-deficient mutants of *Claviceps purpurea*, *Botrytis cinerea*, and *Cochliobolus heterostrophus* show increased sensitivity to H₂O₂ in vitro. However, none of these mutants showed altered pathogenicity; inoculation of these mutants resulted in the formation of lesions of normal size and frequency (Garre et al. 1998; Robbertse et al. 2003; Schouten et al. 2002).

The hemibiotrophic fungus *Magnaporthe oryzae* causes the most serious disease of rice worldwide (Talbot 2003). After the conidia of *M. oryzae* contact the surface of a rice leaf, germ tubes emerge from the conidia, and the tip of the tube swells to form an appressorium, which is a penetration structure into rice cells. The hyphal penetration peg elongates from the mature appressorium into the rice cell (Howard and Valent 1996; Tucker and Talbot 2001). During this process, H₂O₂ accumulates in the rice tissues infected with *M. oryzae* (Kato et al. 2009; Kawasaki et al. 1999). It remains to be elucidated how H₂O₂ accumulation is involved in the interaction between *M. oryzae* and rice cells at infection sites.

The genome sequence of *M. oryzae* (Dean et al. 2005) predicts four catalase genes: that is, two monofunctional catalase genes (*CATA*, MG100061.6 and *CATB*, MG06442.6) and two bifunctional catalase-peroxidase genes (*CPXA*, MG04337.6 and *CPXB*, MG09834.6), as designated by Skamnioti and associates (2007) who showed that a putative secretory catalase, *CATB*, is involved in pathogenicity not via detoxification of host-produced H₂O₂ but by maintaining the integrity of the cell wall (Skamnioti et al. 2007). Therefore, it remains unclear whether *M. oryzae* has H₂O₂ detoxification systems in order to cope with the host oxidative burst and how these systems are involved in the host–fungus interaction.

In this study, we detected a catalase encoded by the *CPXB* gene in the culture filtrate of *M. oryzae*. To elucidate the role of *CPXB* at the early stage of infection, a knock-out mutant of this gene was generated, and its sensitivity to H₂O₂ and pathogenicity were analyzed.

RESULTS

Partial purification of a secreted catalase and identification of the encoding gene.

We previously reported that the supernatant of conidial suspension (SCS) prepared from *M. oryzae* grown on a nutritional medium includes catalase activity (Tanabe et al. 2008). The results prompted us to seek catalase activity in culture filtrates of *M. oryzae*. Preliminary experiments indicated that H₂O₂-degrading activity was detected at 10 to 15 days postinoculation

(dpi) in yeast-glucose (YG) medium (data not shown), and we partially purified the protein by column chromatography. Coomassie staining of the gel after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single band whose mobility was indistinguishable from that of a catalase-active band. This single band was excised, digested with lysyl endopeptidase to obtain a partial amino acid sequence, and analyzed. We obtained two amino acid sequences from a single digested fragment, LVGEEGPQLYVSHGG (major signal; peptide number 1) and ATMLTSDLALINDPE (minor signal; peptide number 2). A BLAST search for these peptide

Fig. 1. In-gel staining of catalase activity. **A**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of partially purified catalase from culture filtrate and in-gel detection of the activity. The partially purified fraction from P91-15B containing catalase activity was divided into two aliquots and separated by SDS-PAGE on a 5 to 20% polyacrylamide gel (5 µg of protein/lane) together with prestained molecular weight markers. After electrophoresis, the gel was separated into two parts and stained with Coomassie Brilliant Blue R-250 (left panel) and stained for catalase (right panel), respectively. Lane 2 in each panel shows the molecular weight standard as indicated on the right. Arrows indicate the catalase-active band. **B**, Primary amino acid sequence of CPXB. Dotted, underlined, and doubly underlined amino acids indicate regions of putative signal peptide, a region removed in the knock-out mutants, and the amino acid sequence obtained by Edman degradation method, respectively. Black- and gray-shadowed letters are peroxidase motif and potential N-glycosylation site, respectively.

sequences showed that peptides numbers 1 and 2 corresponded to residues 791 to 804 on the predicted amino acid sequence of *M. oryzae* β-glucosidase 1 (accession number MG09272.6) and residues 418 to 432 of catalase-peroxidase (Fig. 1B, CPXB; accession number MG09834.6), respectively. These results showed that both β-glucosidase 1 and CPXB co-migrate on SDS-PAGE and indicate that CPXB encodes the catalase secreted into the culture medium.

The apparent molecular weight (MW) estimated from the mobility on SDS-PAGE was 125 kDa, which remained unchanged after boiling or treatment with dithiothreitol at 50 mM before electrophoresis (data not shown). CPXB encodes 786 amino acids, and the calculated MW is 85,585 Da in *M. oryzae* isolate 70-15. In silico analysis of the cellular localization (PSORT) predicted that CPXB is an extracellular protein with a single cleavage site of the signal sequence at positions 16 and 17 in the N-terminal region (Fig. 1B).

Generation of CPXB disruption mutants.

To analyze the biological role of CPXB, CPXB disruptants were generated by the *Agrobacterium*-mediated transformation method (Saitoh et al. 2008). We amplified the two genomic DNA fragments corresponding to positions 1 to 194 and 414 to 786 in the amino acid sequence and constructed a plasmid pCAMBIA-CPXB-KO that was introduced into Ina86-137 via *Agrobacterium* spp. to induce homologous recombination (Fig. 2A). Total DNA was isolated from the hygromycin-resistant transformants, digested with *EcoRI/EcoRV* or *NcoI*, and subjected to Southern blot analysis. The sizes of detected DNA fragments were consistent with those predicted from the restriction map of CPXB and pCAMBIA-CPXB-KO (Fig. 2B), indicating that the targeted replacement of CPXB occurred correctly in the five candidates (ΔCPXB; numbers 14-1, 17-1, 22-3, 28-1, 111-1) (Fig. 2B). The putative amino acid sequence of CPXB contains two peroxidase motifs at 132 to 143 (GGLFVRMAWHA) and 306 to 316 (TVALIAGGHAF) (Fig. 1B). Therefore, in these strains, the DNA sequence encoding the second peroxidase motif was expected to have been deleted. Among these, number 22-3 was transformed with pCAMBIA-cCPXB constructed for the complementation of CPXB, in which the genomic DNA fragment of CPXB relevant to the putative protein coding region, including 219 bp of the 5'-flanking region, was fused with *PtpC* promoter and *Tnos* terminator (Fig. 2C). We used this promoter because we could not amplify the 5'-upstream region of CPXB of Ina86-137. A bialaphos-resistant line, R12-3 (designated as cCPXB), was subjected to Southern blot analysis, and the probed size of DNA fragments after digestion with *EcoRI/EcoRV* indicated that the chromosomal insertion of the constructed gene properly occurred (Fig. 2D).

The five strains of ΔCPXB were almost normal with respect to conidiation, conidial germination, rates of appressorium formation on plastic slips, and rate of penetration into onion epidermal cells (data not shown).

Extracellular activity of catalase and sensitivity to H₂O₂ in ΔCPXB.

The mutants were tested for extracellular activity of catalase. Hyphae of the ΔCPXB, cCPXB, and the control strain were grown on nylon membranes laid on potato dextrose agar (PDA) plates for 5 days, the membranes were removed, and the agar plates were stained for catalase activity. In the control strain and cCPXB, catalase activity was detected as a light-green stain whereas, in the ΔCPXB strains, the activity was significantly diminished (Fig. 3A). We previously reported that SCS includes catalase activity, and it was not clear whether the activity in SCS was due to CPXB. We examined the H₂O₂-de-

grading activity not only in culture filtrates in YG medium but also in SCS of $\Delta CPXB$ strains, all of which showed much less activity of catalase in vitro than the control strain or $cCPXB$ (Fig. 3B and C). The sensitivity of $\Delta CPXB$ to H_2O_2 was examined. Hyphae of $\Delta CPXB$, $cCPXB$, and the control strain were incubated on PDA agar plates containing H_2O_2 , and the diameter of the colonies was measured after 5 days. The $\Delta CPXB$ strains showed growth comparable with that of control strain in the absence of H_2O_2 . At 1 mM H_2O_2 , the growth of $\Delta CPXB$ strains was slightly inhibited compared with the control strains. H_2O_2 at 5 mM strongly inhibited the growth of $\Delta CPXB$ strains, while the growth of the control strain and $cCPXB$ strain exhibited approximately 50% inhibition (Fig. 3D and E). These results strongly suggested that CPXB is a major secreted catalase of *M. oryzae* for the degradation of extracellular H_2O_2 , conferring resistance against H_2O_2 during vegetative growth. In these experiments, ectopic strains showed the same results as those of the control and $cCPXB$ strains (data not shown). Because the five $\Delta CPXB$ strains exhibited the same levels of extracellular catalase activity, we used numbers 14-1, 17-1, and 22-3 in the following experiments.

Role of CPXB on infection of *M. oryzae* to epidermal cells of rice leaf sheaths.

We previously reported that the level of H_2O_2 in the epidermal cells of rice leaf sheath is reduced after inoculation of conidia in the presence of SCS that includes catalase activity (Tanabe et al. 2008, 2009). Catalase activity in SCS was due to CPXB (Fig. 3C); thus, it was indicated that preexisting CPXB in the conventionally prepared conidia suspension is responsible for the degradation of H_2O_2 during infection in rice. To evaluate the role of CPXB in the quenching of H_2O_2 during the infection, conidia of $\Delta CPXB$ s and the control strain were washed by successive centrifugation to remove preexisting CPXB, resuspended in water, and inoculated on leaf sheaths of rice. The accumulation of H_2O_2 was examined around the penetration sites in the epidermal cells of leaf sheaths at 24 h postinoculation (hpi) by 3',3'-diaminobenzidine tetrahydrochloride (DAB) staining. The pattern of DAB staining in response to the inoculation with Ina86-137 was indistinguishable from those inoculated with $\Delta CPXB$ strains (Fig. 4A). Inoculation of $\Delta CPXB$ strains induced a slightly but significantly higher rate of DAB signal-positive cells than in the control

Fig. 2. Targeted gene disruption of *CPXB*. **A**, Schematic diagram of genomic organization of the *CPXB* locus before (wild-type strain [WT]) and after (mutant) disruption. Organization of the exon (closed bar) and intron (open bar) with the translational start (arrow) and termination (asterisk) sites indicated. Closed and open triangles indicate the putative heme-binding motifs. Restriction sites used for Southern analysis are indicated as N (*NcoI*), V (*EcoRV*), and E (*EcoRI*). HPT; hygromycin phosphotransferase. **B**, Southern blot analysis of the genomic DNA of the WT strain and selected transformants ($\Delta CPXB$). Genomic DNA (1 μ g) was digested with *EcoRI*/*EcoRV* (upper panel) or *NcoI* (lower panel), separated by agarose gel electrophoresis, and blotted onto a nylon membrane, which was hybridized with a 32 P-labeled 770-bp fragment ("probe" in A and C). **C**, Schematic diagram of *pCambia-cCPXB* for the construction of the complemented strain. Organization of the exon (closed bar) and intron (open bar) with the translational start (arrow) and termination (asterisk) sites indicated. Restriction sites used for Southern analysis are indicated as V (*EcoRV*) and E (*EcoRI*). **D**, Southern blot analysis of the genomic DNA of the recipient of *pCambia-cCPXB* (no. 22-3), transformed strain (R12-3), and the control strain (WT; Ina86-137). Genomic DNA (1 μ g) was digested with *EcoRI*/*EcoRV*, separated by agarose gel electrophoresis, and blotted onto a nylon membrane, which was hybridized with a 32 P-labeled 770-bp fragment ("probe" in A and C).

strain or *cCPXB* (Fig. 4B). These results indicate that CPXB is secreted from the infectious hyphae and reduces the level of H_2O_2 accumulated in the epidermal cells of rice.

To investigate the role of CPXB in pathogenicity, Δ CPXB, *cCPXB*, and the control strain were inoculated on leaf sheaths of rice. Appressoria on the epidermis of leaf sheaths were observed at 20, 29, and 48 hpi using microscopy and the extent of infection was scored as “multi-cells” and “one-cell”, corresponding to the infection site where infectious hyphae are observed in more than one cell or one cell, respectively, of the leaf sheath of rice (Fig. 5A). The percentage of One-cell infection by Δ CPXB strains at 20 hpi was significantly lower than that of the control strains (Fig. 5B). No Multi-cell infection sites were observed at this stage. At 29 hpi, the percentage of

the Multi-cell infection by the Δ CPXB strains significantly decreased compared with that in the control strain or *cCPXB*, and the difference in percentage disappeared at 48 hpi. At 6 dpi, inoculation of Δ CPXB, *cCPXB*, and the control strain formed lesions of similar sizes in leaf blades (Fig. 5C). These results suggest that CPXB supports the growth of infectious hyphae at the early stage of infection.

In the experiments of Figures 4 and 5, ectopic strains showed the same results as those of the control and *cCPXB* strains (data not shown).

Expression of CPXB during infection in leaf sheath of rice.

Skamnioti and associates (2007) examined the levels of mRNAs for *CATA*, *CATB*, *CPXA*, and *CPXB* after inoculation

Fig. 3. Activity of secreted catalase and sensitivity to H_2O_2 in Δ CPXB. **A**, Activity staining of secreted catalase in the potato dextrose agar (PDA). The strains of Δ CPXBs (14-1, 17-1, 22-3, 28-1, the 111-1), *cCPXB* (R12-3), and the control (WT; Ina86-137) were incubated on a nylon membrane laid on the PDA plate, which was stained for catalase activity. **B**, In vitro activity of catalase in the culture filtrate. Culture filtrates of strains as in A were gel filtered, concentrated, and incubated in the presence of H_2O_2 . Open and closed bars indicate the residual H_2O_2 before and after incubation, respectively. Values represent the averages of three measurements $\pm$ standard deviation (SD). CTL indicates distilled water. **C**, In vitro activity of catalase in the supernatant of conidial suspension (SCS). SCS prepared from strains as in A were subjected to catalase assay. Open and closed bars indicate the residual H_2O_2 before and after incubation, respectively. Values represent the averages of three measurements $\pm$ SD. CTL indicates distilled water. **D**, Hyphal growth of Δ CPXB on PDA plates containing H_2O_2 . Strains as in A were inoculated on plates including H_2O_2 at the concentrations indicated and incubated for 5 days. A representative photograph is shown. **E**, Quantitative measurement of D. Each strain was incubated as in D, and the colony diameters were measured at 5 days postinoculation. Open, hatched, and closed bars indicate the diameter of colonies on PDA agar plates containing H_2O_2 at 0, 1, and 5 mM H_2O_2 , respectively. Values represent the averages of five trials $\pm$ SD. **A**, through **E**, Experiments using the complemented line were separately conducted and shown in the right panels or rectangles.

of *M. oryzae* on leaves of barley for up to 20 h. Because the results in Figure 5B showed the delay in the infection rate of $\Delta CPXB$ at 20 to 29 hpi, we reexamined the expression level of *CPXB* in leaf sheath of rice (Fig. 6). The *CATB* mRNA drastically accumulated at 6 hpi (approximately 15-fold higher than that at 0 hpi), then decreased at 48 hpi, basically the same results as in the previous report (Skamnioti et al. 2007). For *CPXA* and *CPXB*, the level of mRNA was the highest at 6 and 3 hpi followed by decreases until 29 and 40 hpi, respectively. These results were different from those in barley leaves, where *CPXA* and *CPXB* showed a rapid suppression at 5 hpi followed by a transient activation at 12 hpi (Skamnioti et al. 2007).

DISCUSSION

Roles of fungal catalase in the pathogenicity.

The role of H_2O_2 has been demonstrated to be a molecular signal to a set of defense responses by the host plant infected with pathogens (Torres and Dangl 2005; Torres et al. 2006). It has been implicated as a chemical barrier against hemibiotrophic fungi. The rate of penetration by *Colletotrichum coccodes* was drastically enhanced by pretreatment of tomato leaves with catalase, concomitant with a reduction in the levels of H_2O_2 (Mellersh et al. 2002). Similarly, in wheat leaves inoculated with *Septoria tritici*, penetration rate and formation of lesions were enhanced or suppressed by pretreatment of leaves with catalase or H_2O_2 , respectively (Shetty et al. 2007). These results, in addition to the present study, support the idea that accumulation of H_2O_2 is a crucial defense mechanism against these pathogens via its toxic action. To our knowledge, functional analysis of catalase by generating knockout mutants has not been undertaken in hemibiotrophic fungi, apart from *Cat2* in *Cladosporium fulvum* (Bussink and Oliver 2001) and *CATB* of *M. oryzae* (Skamnioti et al. 2007).

In the biotrophic pathogen *Claviceps purpurea*, a transcriptional factor, *CPTF1*, regulates the expression of genes for catalase, and its deficient mutant exhibited reduced pathogenicity (Nathues et al. 2004). Similar results were obtained in *Ustilago maydis*, where a knockout mutant of *YAP1*, a regulator of the responses to oxidative stress, was shown to be drastically impaired in pathogenicity. Two genes putatively regulated by *YAP1*, including a gene for peroxidase, were identified, and the knockout mutants of both genes exhibited much reduced virulence, comparable with that of the *YAP1*-deficient mutant. Inoculation of the mutant deficient in *YAP1*-regulated peroxidase induced the accumulation of H_2O_2 in the vicinity of hyphae, as observed when inoculated with the *YAP1*-deficient mutant (Molina and Kahmann 2007). These observations indicate that ROS-degrading activity is linked to pathogenicity in these fungi.

In contrast, roles of ROS-degrading enzymes in the necrotrophic fungi examined so far seem not so essential to the pathogenicity. For example, in *Cochliobolus heterostrophus*, mutants of a gene for catalase, *CAT3*, and for an AP1-like transcriptional factor, *CHAP1*, were more sensitive to extracellular H_2O_2 without a reduction in pathogenicity (Lev et al. 2005; Robbertse et al. 2003). In *B. cinerea*, a mutant deficient in a gene for an extracellular catalase (*Bccat2*) was generated and subjected to the electron microscopic observation after treatment with cerium chloride that detects H_2O_2 , showing that the infected hyphae was directly exposed to H_2O_2 in planta. However, the mutant still exhibited full virulence (Schouten et al. 2002), indicating that *B. cinerea* has mechanisms other than extracellular catalase that confer tolerance to oxidative stress via H_2O_2 . One possible reason why these studies did not prove a positive role for extracellular catalase in pathogenicity is that virulence was tested by lesion formation, which is not sensi-

tive enough to detect subtle differences in the infection process, as already argued (Robbertse et al. 2003) and as demonstrated in this study (Fig. 5B and C).

Biochemical features of CPXB.

In this study, we partially purified the catalase activity from a culture filtrate of *M. oryzae*, obtained its internal amino acid sequence, and identified the gene as *CPXB*. The MW estimated by SDS-PAGE (Fig. 1) was larger than that calculated from the deduced amino acid sequence. At present, the reason for this inconsistency is not clear. It may be due to the post-translational modification of *CPXB*, such as glycosylation. In fact, the deduced amino acid sequence of *CPXB* includes seven potential *N*-glycosylation sites (Fig. 1B), implying that the inconsistency in the MW might be due to glycosylation of the mature protein. Glycosylation in the fungal extracellular peroxidase has been reported or suggested (Garre et al. 1998; Nie et al. 1999; Schreiber et al. 2008; Sugano et al. 1999) and is considered to increase thermostability (Nie et al. 1999). The catalase activity of *CPXB* was stable at room temperature (data not shown) and in the presence of SDS (Fig. 1); therefore, the glycosylation of *CPXB* might contribute to the stable conformation in these conditions.

Fig. 4. Accumulation of H_2O_2 in the epidermal cells of leaf sheaths inoculated with $\Delta CPXB$. **A**, Representative 3',3'-diaminobenzidine tetrahydrochloride (DAB)-staining patterns in the epidermal cell of the leaf sheath of rice cv. Nipponbare inoculated with the strain of control (WT; Ina86-137) or $\Delta CPXB$ (22-3) at 24 h postinoculation (hpi). Arrowheads and arrows indicate appressoria and infectious hyphae, respectively. A bar indicates 30 μ m. **B**, Number of appressoria that induced DAB-positive signals in the epidermal cells of leaf sheaths. The strains of $\Delta CPXB$ (Δ ; 17-1, 22-3, 28-1), *cCPXB* (c; R12-3), and control (WT; Ina86-137) were inoculated to the leaf sheath of rice cv. Nipponbare. More than 100 appressoria per leaf sheath were observed at 24 hpi, and the number of appressoria with DAB signals directly beneath them was counted. Asterisks indicate a significant difference from Ina86-137 based on Dunnett's test at $P < 0.05$. Values represent the averages of five measurements $\pm$ standard deviation. An experiment using the complemented line was separately conducted and shown in the right rectangles in each column.

Roles of catalase in the pathogenicity of *M. oryzae*.

Among four catalase genes of *M. oryzae*, *CATB* was shown to contribute to the integrated architecture of the cell wall over the life cycle of the fungus, indicating that the regulation of the fungus-derived ROS is essential to the pathogenicity (Skamnioti et al. 2007). The hypothesis is supported by the observation that ROS production is required for the morphogenesis of appressoria and penetration through the host cuticle in *M. oryzae* (Egan et al. 2007). In a hemibiotroph, *Colletotrichum acutatum*, increase of the protein level of a catalase-peroxidase was detected at the stage of appressorium formation, which was tightly linked to the level of intracellular ROS, indicating that the gene is involved in pathogenicity through the regulation of ROS produced by the fungus (Brown et al. 2008).

An *M. oryzae* mutant, *des1*, was shown to be deficient in pathogenicity, which was restored by treatment of the rice leaf sheath with diphenylene iodinium, an inhibitor of NADPH-

oxidase involved in the generation of ROS in plants (Chi et al. 2009). Mutant *des1* was highly sensitive to H₂O₂ in correlation with the lack of extracellular activity of peroxidase and laccase, and the expression of *CPXB* was downregulated in *des1*, although the extracellular activity of catalase was not tested (Chi et al. 2009). Taking the results in Figure 3 into consideration, it cannot be excluded that *DES1* confers resistance to extracellular H₂O₂ through *CPXB*. *DES1* is a vacuole-localized protein, and its other biochemical properties are not clear, including how *DES1* regulates these genes (Chi et al. 2009).

A detailed histological study showed that the primary invasion by *M. oryzae* is biotrophic and invaded cells are dead after the hypha move into the adjacent cells (Kankanala et al. 2007). Therefore, the necrotrophic phase is considered to start when Multi-cell infection is observed, whereas One-cell infection indicates that the infection stays at biotrophic phase. In this context, the results in Figure 5, showing that the effect of disruption of *CPXB* on the infection was restricted before 29 hpi,

Fig. 5. Infection by $\Delta CPXB$ of the epidermal cells of the leaf sheath. **A**, Representative photographs of “multi-cells” infection and “one-cell” infection. Arrowheads and arrows indicate appressoria and infectious hyphae, respectively. A bar indicates 30 μ m. **B**, Percentage of multi-cells and one-cell infection by $\Delta CPXB$. Conidial suspensions of $\Delta CPXB$ (Δ ; 17-1, 22-3, 28-1), *cCPXB* (c; R12-3), and a control strain (WT; Ina86-137) were inoculated to five-leaf sheaths, and the infection status of more than 100 appressoria per leaf sheath was scored at 20, 29, and 48 h postinoculation. Asterisks in each panel indicate a significant difference from Ina86-137 based on Dunnett’s test at $P < 0.05$. Values represent the averages of five measurements $\pm$ standard deviation. Experiments using the complemented line were separately conducted and shown in the right rectangles in each column. **C**, Lesions on a leaf blade sprayed with conidial suspensions of $\Delta CPXB$ (17-1, 22-3, 28-1), *cCPXB* (R12-3), and a control strain (WT; Ina86-137) at 7 days postinoculation.

indicate that *CPXB* promotes infection in the biotrophic phase. The expression of *CPXB* in the leaf sheath of rice was transiently upregulated at 3 hpi (Fig. 6), preceding the accumulation of H_2O_2 in the epidermal cells of the rice leaf sheath at 6 hpi (Tanabe et al. 2009). Based on these observations, it is hypothesized that *CPXB* plays antioxidative roles against the host-produced H_2O_2 at the biotrophic phase, and the sensitivity to H_2O_2 is reduced as infection proceeds to the necrotrophic phase, which is independent of *CPXB*. This hypothesis accounts for the unaltered pathogenicity of $\Delta CPXB$ in lesion formation at 6 dpi (Fig. 5C). In the previous study, addition of exogenous catalase to the washed conidia of rice blast fungus enhanced the size of lesion in leaf blade (Tanabe et al. 2009). The observation might indicate that, at the late stage of infection, removal of H_2O_2 still supports fungal infection; however, *M. oryzae* acquires tolerance to H_2O_2 by other means than catalase.

MATERIALS AND METHODS

Fungal materials and growth conditions.

M. oryzae wild-type isolates Ina86-137 (race; 007.0, MAFF number 101511) and P91-15B (race; 001.0) were grown on oatmeal agar plates at 26°C. A conidial suspension was prepared as described previously (Koga 1994). The washed conidia were obtained from the conidial suspension by centrifugation (twice) and were resuspended in distilled and sterile water (washed conidial suspension) as described previously (Tanabe et al. 2008). To test for sensitivity to H_2O_2 , the fungi were cultured for 5 days at 26°C on PDA plates (Sigma-Aldrich Inc., St Louis) containing H_2O_2 . For detection of catalase activity on agar plates, the fungi were grown for 5 days at 26°C on a nylon membrane laid on the surface of the PDA plate. After removal of the membrane, the agar plate was stained for catalase activity as described below. To analyze the secreted catalase, *M. oryzae* was cultured in YG medium (2% [wt/vol] yeast extract and 0.5% [wt/vol] glucose) at 25°C with rotation at 120 rpm.

Plant material, inoculation, and in situ detection of H_2O_2 .

Rice seedlings (*Oryza sativa* cv. Nipponbare) were grown in hydroponic culture for 3 weeks as described previously (Tanabe et al. 2006), sprayed with a conidial suspension (1×10^5 conidia/ml), and incubated at 20°C in a moist chamber for 24 h, then transferred to a greenhouse at 25°C for 7 days. For the leaf sheath assay, excised leaf sheaths were covered with the washed conidial suspension (1×10^5 conidia/ml) and incubated at 25°C for the times indicated (Koga 1994). For the in situ detection of H_2O_2 , the leaf sheaths were stained with a solution of DAB at 1 mg/ml for 12 h (Thordal-Christensen et al. 1997). For evaluation of hyphal infection in epidermal cells of the leaf sheaths, the inoculated leaf sheaths were fixed in 45% (vol/vol) ethanol, 5% (vol/vol) acetic acid, and 1.85% (vol/vol) formaldehyde; intact appressoria were counted under a microscope; and the extent of infection was scored as multi-cells or one-cell by counting the number of appressorium beneath which infectious hyphae were observed in more than one cell or one cell, respectively.

Partial purification of catalase from the culture fluid.

P91-15B was cultured in YG medium at 25°C for 15 days, and the medium was filtered through Miracloth (Calbiochem, La Jolla, CA, U.S.A.). Total soluble and insoluble protein at 50 and 70% saturation with $(NH_4)_2SO_4$, respectively, was recovered by centrifugation at $10,000 \times g$ for 30 min at 4°C. The resultant pellet was dissolved in water and desalted with Sephadex G-75 column chromatography, equilibrated with 10 mM Tris-HCl (pH 7.5). The fractions having catalase activity

were collected, applied to a DEAE cellulose column equilibrated with 10 mM Tris-HCl (pH 7.5), washed with 10 mM Tris-HCl containing 50 mM NaCl (pH 7.5), and eluted with 10 mM Tris-HCl containing 100 mM NaCl (pH 7.5). Fractions were combined, concentrated in vacuo, and dissolved in water, followed by desalting with Sephadex G75 column chromatography equilibrated with water. After concentration in vacuo, the proteins were separated by SDS-PAGE on 5 to 20% polyacrylamide gel, and the band stained with Coomassie brilliant blue R-250 with the same mobility as that stained for catalase activity was excised. Peptide fragments obtained by digestion with lysyl endopeptidase were subjected to the Edman degradation method.

Generation of a *CPXB*-deficient mutant and its complement.

Generation of a *CPXB*-deficient mutant was carried out as previously described (Saitoh et al. 2008). The 5'-end fragment that starts with the putative initiation codon (1,330 bp) and the 3'-end fragment that ends with the putative termination codon (1,124 bp) of *CPXB* were amplified by polymerase chain reac-

Fig. 6. Changes in the levels of mRNAs from *CPXA*, *CPXB*, and *CATB* of Ina86-137 during infection in the rice leaf sheath. Total RNAs were extracted from the leaf sheath of rice inoculated with Ina86-137 at the time indicated, and the relative level of mRNA for **A**, *CPXB*; **B**, *CPXA*; and **C**, *CATB* was measured by quantitative reverse-transcription polymerase chain reaction and normalized using the expression levels of 28S rRNA of *Magnaporthe oryzae*.

tion (PCR) using primer combinations F1/R1 and F2/R2, respectively. The nucleotide sequences of the primers were F1, 5'-CCAAGCTTATGCACGCATCACTCTCTCAAG-3'; R1, 5'-CCCAAGCTTCCCATGAGATCTTGTGTTGCCA-3'; F2, 5'-CAAGTTCGCAAGGCTACCA-3'; and R2, 5'-TTATTTCTTGA CTTTGAGTCTGA-3'. After verifying the amplified sequences, both fragments were cloned into the entry vector pETH to construct pETH-CPXB-KO. pCAMBIA-CPXB-KO was generated by the LR reaction of pETH-CPXB-KO with pCAMBIA-Bar-RfA and introduced into *M. oryzae* isolate Ina86-137 via *Agrobacterium tumefaciens* EH105, followed by selection as previously described (Saitoh et al. 2008).

For complementation of Δ CPXB, the genomic DNA fragment containing the protein coding region (2,006 bp) and 5'-flanking region upstream of putative initiation codon (219 bp) was amplified by PCR. After verifying by sequencing, the fragment was ligated to *PtpC* promoter and inserted upstream of *Tnos* in pCAMBIA-0380 with the *PtpC-Bar* fragment. The resultant binary vector, pCAMBIA-cCPXB, was used for transformation of Δ CPXB.

To verify the genomic recombination, Southern blot analysis was carried out using a 32 P-labeled genomic fragment (559 to 1,330) as a probe, which was amplified by PCR using primers F3 (5'-ATGCACGCATCACTCTCAAG-3') and R3 (5'-CCCA TGAGATCTTGTGTTGCCA-3').

Catalase assay.

An in-gel assay of catalase activity was performed as described by Woodbury and associates (1971). For measurement of catalase activity, 1 ml of 200 μ M H₂O₂ was incubated with the culture filtrate or SCS containing 5 μ g of protein at 25°C for 15 min, and the residual H₂O₂ was determined by the luminol method (Schwacke and Hager 1992). The protein concentration of the culture filtrates and SCS were determined using a Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, U.S.A.). SCS was prepared from the conidial suspension as described previously (Tanabe et al. 2008).

Analysis of expression of fungal genes in planta.

The leaf sheath of Nipponbare was inoculated with a conidial suspension (1×10^5 conidia/ml) of Ina86-137 and total RNA was isolated using the RNeasy Plant Kit (Qiagen, Valencia, CA, U.S.A.). The expression of fungal genes was investigated by quantitative reverse-transcription PCR as described previously (Kato et al. 2009). The gene expression levels were normalized using 28S ribosomal RNA gene (GenBank accession number AF362554). The nucleotide sequences of the primers were F, 5'-GGGGCGATTTTATAGCCTTCA-3'; and R, 5'-ATTCGAAGGCCACGTTCAA-3'. The primers for the detection of the mRNAs for CPXA, CPXB and CATB of rice blast fungus were designed according to Skamnioti and associates (2007).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Broad Institute *Magnaporthe oryzae* genome database:
www.broad.mit.edu/annotation/fungi/magnaporthe
 PSORT protein prediction server: psort.hgc.jp
 NIAS Genebank database:
www.gene.aifrc.go.jp/databases-micro_search_en.php