Cloning, Characterization, and Targeted Disruption of *cpcat1*, Coding for an in Planta Secreted Catalase of *Claviceps purpurea*

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Accepted 27 April 1998.

Claviceps purpurea has been shown to secrete catalases in axenic and parasitic culture. In order to determine the importance of these enzymes in the host-parasite interaction, especially their role in overcoming oxidative stress imposed on the pathogen by the plant's defense system, the *catR* gene from *A. niger* was used to isolate a putative catalase gene from a genomic library of *C. purpurea*. *cpcat1* consists of an open reading frame of 2,148 bp that is interrupted by five introns. Its derived gene product shows significant homology to fungal catalases and contains a putative signal peptide of 19 amino acids and three putative N-glycosylation sites, which indicates that CPCAT1 is a secreted catalase. Disruption of the gene by a gene replacement approach resulted in the loss of two catalase isoforms, CATC and CATD, strongly suggesting that they are both encoded by *cpcat1*. CATD is the major secreted catalase of *C. purpurea* and is furthermore the only catalase present in the honeydew of infected rye ears. Deletion mutants of *cpcat1* were inoculated on rye plants and showed no significant reduction in virulence. Ovarian tissue and honeydew of plants inoculated with the mutants lacked CATD, confirming that this catalase is not essential for colonization of the host tissue by *C. purpurea*.

Additional keywords: active oxygen species.

An important factor in the capacity of a pathogen to infect a plant resides in the ability to either tolerate or eliminate constitutive (Osborn 1996) or induced (for review, see Hammond-Kosack and Jones 1996) toxic molecules produced by the plant cell. A further critical factor is the evasion of the pathogen detection system through the removal of molecules that could be detected by these systems, such as avirulence factors (Joosten et al. 1994). One of the first responses of a plant cell upon detection of a pathogen is the generation of reactive oxygen species, termed the oxidative burst (Doke 1983; Mehdy 1994; Low and Merida 1996). H₂O₂ produced during the oxidative burst is involved in several responses that

might contribute to the restriction of pathogen growth, such as cross-linking of cell wall structural proteins or phenolics (Bradley et al. 1992; Brisson et al. 1994; Otte and Barz 1996), triggering of rapid necrosis at infection sites, a phenomenon known as the hypersensitive response (HR) (for review, see Hammond-Kosack and Jones 1996), and could furthermore act directly as an antimicrobial agent (Peng and Kuc 1992). Despite these studies, mainly obtained from examination of plant cell cultures, there is only one case in the literature that shows convincing evidence that H₂O₂ is actually involved in conferring disease resistance on plants. In this study, the constitutive expression of an H₂O₂-generating glucose oxidase from *Aspergillus niger* in the apoplast of transgenic potato plants resulted in strong resistance to the bacterial soft rot pathogen *Erwinia carotovora* and in enhanced resistance to *Phytophthora infestans* (Wu et al. 1995). The resistance of the transgenic plants to soft rot was abolished when catalase was added simultaneously with the bacterium, consistent with the higher resistance being the consequence of H₂O₂ accumulation. However, the mechanism underlying this general resistance to these diverse pathogens is still unknown. If H₂O₂ plays a crucial role in plant defense, then the evolution of counteracting systems should provide pathogens with a major selective advantage in the colonization of plant tissue. The major importance of catalase is thought to be in its scavenging of toxic H₂O₂ by its conversion to oxygen gas and water (Natvig et al. 1996). Work with the phytopathogenic bacterium *Pseudomonas syringae* and the saprophytic bacterium *Pseudomonas putida* described the presence of several periplasmic catalases and a membrane-associated catalase, respectively (Klotz and Hutcheson 1992; Katsuwon and Anderson 1990). Recently, the secretion of catalase by *Claviceps purpurea* during the first stages of colonization of rye ovaries has been detected by means of biochemical and cytological techniques (Garre et al. 1998). Four catalases, differing in electrophoretic mobility, were identified in axenic cultures of *C. purpurea*. Catalase CATA, with an isoelectric point (IP) of about 5, could only be isolated from mycelia and is therefore probably an intracellular catalase. CATC and CATD (both with an IP of about 7), could both be isolated from mycelia and culture fluids, which indicates that these are extracellular catalases, whereas CATB (IP approximately 5.2) probably is cell wall associated (Garre et al. 1998). Of these, CATC was the major catalase in axenic culture fluids. Analysis of young infected ovaries and honeydew exuded from infected ovaries

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Nucleotide and/or amino acid sequence data are to be found at the EMBL data base as accession number AJ001386.

of rye revealed that CATD is the major secreted catalase in the first stages of infection, and could therefore be capable of nullifying the responses mediated by H_2O_2 . The cloning of the gene encoding CATD would provide an opportunity to generate CATD-deficient mutants and to study the role of this protein during the colonization of rye ovaries by *C. purpurea*.

This paper describes the cloning and characterization of a fungal catalase gene, *cpcat1* from *C. purpurea*, that encodes a catalase secreted during plant infection. Analysis of mutants in this gene, generated by gene replacement, confirm that it codes for CATD, the major catalase secreted by *C. purpurea* during rye colonization, thus allowing us to evaluate its role in pathogenicity on rye.

RESULTS

Cloning and characterization of *cpcat1*.

The *CatR* gene from *Aspergillus niger* was selected as a heterologous probe for use in the cloning of the *C. purpurea*

gene encoding CATD because it codes for a catalase that has typical attributes of a secreted protein (Fowler et al. 1993). An internal 1.75-kb *SalI-HindIII* fragment containing the most conserved part of the *A. niger CatR* coding region was used in Southern blot hybridization experiments at different temperatures. At 60°C, this probe strongly hybridized with few fragments generated by restriction of *C. purpurea* genomic DNA with several restriction enzymes (data not shown). The above conditions were used to screen approximately 50,000 plaques from a genomic EMBL3 library of *C. purpurea* strain T5 (Smit and Tudzynski 1992). Twenty of the strongest hybridizing plaques were selected for a second round of hybridization, which gave nine clear positive clones. The DNA extracted from these recombinant phages was restricted with *SalI* and hybridized with the *A. niger CatR* probe. Several fragments showed strong hybridization signals, with a 0.7-kb fragment present in all of the nine clones. DNA sequence analysis revealed that this fragment encoded for an amino acid sequence with high similarity to the conserved catalytic do-

Fig. 1. A, Restriction map of the 6.5-kb *KpnI* fragment of *Claviceps purpurea* genomic DNA cloned in plasmid pcp Δ cat1. Exons are indicated by black boxes; noncoding regions by a line; a putative signal peptide signal by a white box; and putative N-glycosylation sites by triangles. Target sequences for primer P1 and P2 are indicated by arrows. A solid bar indicates the 1.1-kb *SacI* fragment used as probe in Southern blot hybridizations in Figures 3 and 6. Restriction sites: Kp, *KpnI*; S, *SalI*; Sc, *SacI*; H, *HindIII*; P, *PstI*; **B**, Construction of the replacement vector pcp Δ cat1. The internal 0.3-kb *SalI-HindIII* fragment was removed and substituted by the phleomycin resistance cassette from pAN8-1 (Mattern et al. 1988). The final genomic structure at the *cpcat1* locus upon homologous recombination of the replacement fragment is shown.

main of known catalases. Exhaustive restriction analysis of phage DNA from these clones allowed the identification of a 6.5-kb *KpnI* fragment consisting of the whole gene, which was named *cpcat1*. This fragment was subcloned into pUC19, giving the plasmid pcpcat1; its restriction map is shown in Figure 1A. Sequence analysis of the 6.5-kb *KpnI* fragment showed the presence of an open reading frame (ORF) of 2,148 bp interrupted by five introns (Fig. 2), as predicted by the presence of intron consensus sequences (Ballance 1991) and lack of homology with other catalases. Northern (RNA) blot hybridization with RNA from axenic culture, with the 1.1-kb *SacI* fragment from the coding region as a probe, confirmed that this gene is transcribed as an mRNA of 2.4 kb, which is in accordance with the size deduced from the sequence (data not shown). The protein sequence derived from this ORF showed several features characteristic of secreted proteins, including (i) a typical signal peptide (von Heijne 1983) of 19 amino ac-

ids, the removal of which would produce a mature protein of 697 amino acids ($M_r = 76,957$), and (ii) three putative N-linked glycosylation sites (Gavel and von Heijne 1990). Together, these features and the isoelectric point (6.72), as estimated from the amino acid charge, suggested that *cpcat1* codes for either CATC or CATD.

The protein sequence deduced from *cpcat1* was compared with those from other catalase genes of filamentous fungi. The highest homology was found with *Aspergillus nidulans* CatB (61.6% identity) (Kawasaki et al. 1997), with the proteins deduced from three nearly identical *Aspergillus fumigatus* sequences (61.7%)—GenBank accession number Y07763 (T. Takasuka, M. Anderson, and D. W. Denning, *unpublished*), accession number U87850 (D. R. Wysong, R. D. Diamond, and P. W. Robbins, *unpublished*), and *Cat1* (Calera et al. 1997)—and with *A. niger* CatR (54.7%). Interestingly, the predicted peptides from these genes share some characteristics

Fig. 2. (continued from preceding page)

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2901 CCTAGCCTTTGGATTGCGAGTGCCTCGATCCTACCAAGATCATTCTGAGGAATATGCCCCTCTGATGCCGGTCCGGTGTGATGAGACTCGATGCCAATCCT
322 L A F G F D V L D P T K I I P E E Y A P L M P V G V M R L D A N P

3001 TCCAACTATTTTGCTGAGACTGAACAAGTCATGgtatgtctttcaatcctcgaccaagactgttgcgaagaaaagcttcagtttagggaaatccggtttg
355 S N Y F A E T E Q V M

3101 cttaccaacttcaattaacagTTCCAACCTGGCCATATTGTGCGAGGCGTGCAGTTTACGGAGGACCTCTGCTGCAGGGCCGCATCTTCTCGTACCTGG
366 F Q P G H I V R G V D F T E D P L L Q G R I F S Y L D

3201 ATACTCAAATTAACAGACACGGTGGCCCCAAGTTTGAGCAGCTTCCCATCAACAGGCTGTCTGCTTATTCACAACAACATCGCGATGGAGCTGGCCA
393 T Q I N R H G G P N F E Q L P I N R P V V P I H N N N R D G A G Q

3301 GAATTTTCATTATAAGAACATGCTCCTTgtaagcgcctcttgccctgttccaaccaaatacataattcgactcaatctaacaccttcttacaagATA
426 N F I H K N N A P Y T

3401 CCCCCAACACTCTGAACAAGGGATTTCCCAAGCAAGCCAACAGGAAGGAAGGCAAGGGCTTTTTCACCGCTCCCGACGCAAGGTCTCTGGCTCCCTGCA
437 P N T L N K G F P K Q A N Q K E G K G F F T A P G R K V S G S L Q

3501 ACGGCGTCTCAGCAACACGTTCTCTGATCACTGGACCCAGCCCCGCTATTCTTCAACTCCATCAGCCTATCGAGCAGCAGTTCTTGATTAATGCGATC
470 R R L S N T F L D H W T Q P R L F F N S I T P I E Q Q F L I N A I

3601 CGCTTCGAAACCTCGCACCTCAGCCAGGTGTGCGAGAAGACGTCCTCGCGCAGCTCAACAAGATCAGCCACGATGTTGCCGTCGCGTCGGCAAGGCTC
503 R F E T S H L S Q V V Q K N V L A Q L N K I S H D V A V R V G K A L

3681 TTGGCCTGGACGCACCCCCCGCAGATGACAAGTACTACCACAACAACAGACCAAGGGCCTGTCCATCTTTGGCGAGGAGCTGCCACCATTGCTACCTC
537 G L D A P P A D D K Y Y H N N Q T K G L S I F G E E L P T I A T L

3801 CATCGTCGGCGTCTTGGCCTCTACTGCATCAAAAACTCGCTGGCCAGGCCAGGCCCTCAAGGACGCCCTTCAAAACAGGGAGGTCAACGTCATCGTG
570 I V G V L A S T A S K N S L A Q A Q A L K D A F K T R E V N V I V

3901 GTGGCTGAATCCCTCGCCACCGGTGTGGACAGACATACGACGCCGCCGACGCGAGTCAGCTTCGACGGCATCGTTGTGCGCGAGGAGTCAGAGCCCTCT
603 V A E S L A T G V D Q T Y D A A D A V S F D G I V V A D G V E A L F

4001 TTGACAACACCAAGAAATCCTCGCTCTTCCAGCCTGGTCTGCTAGCCAAATCGTAAACGACGGATACAACTGGGGCAAACCCATGGGATTCTCGGCAG
637 D N T K K S S L F Q P G R P S Q I V N D G Y N W G K P M G F L G S

4101 CGCCAAGAGGGCGGTTAAGGCGACTTCTGTGGTGGAGGGTCCGGTGTGTACATGAGAAACAGCACGGCTACTCTGGTGGGAGACTTCCAGAAGGGGCTT
671 A K R A V K A T S V V E G A G V Y M R N S T A T L V G D F Q K G L

4201 GCCAAGTTCAAGTTTACGGACCGGTTCCCTATGGACACTAAATAAGTGGGAGTGTGATTTAGAGCAGTGAAGTGGTGTGCGGCTTTTGTCTTTCTTT
703 A K F K F T D R F P M D T K ***

4301 TCTTTTCTTTTCTTTTCTAGTCTATTCCTACCTGCCTGTAATCTCCCATGCCATTTTCTTTTCTTTTGTACATCACACCATACCATTCATAG

4401 CCATATAATGCTGGATAGCCATGACATTGTGTGTCTGGTCTTTGGATTACGCTACAGATTGCAAGATGCGAGATGTGAGATTCAAGGCGCGAGATGCGA
PolyA

4501 GGCTGCGAGGCTGCTGCGAGGGTGAATGGCGGTTTGTGAGTAAATGACTTGGACCAAAACGAATGCTTCATCTTGAGATCCAGGCGTAATTTTACAC

4601 TGCAGTTCTTACTCAA

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with the protein deduced from *cpcat1*, such as a putative signal sequence and N-linked glycosylation sites at the same positions in two of the three sites found in the *C. purpurea* protein. Moreover, the positions of the first and third intron in *cpcat1* are precisely conserved in all of the genes referred to above, despite the presence of additional introns in every case. In contrast, *A. nidulans* CatA (Navarro et al. 1996), a second *A. fumigatus* catalase deduced from sequence AFU87630 (GenBank accession number U87630; D. R. Wysong, R. D. Diamond, and P. W. Robbins, unpublished) and *Botrytis cinerea* CatA (Van der Vlugt-Bergmans et al. 1997) showed only low homology (less than 38% identity) with the sequence deduced from the *cpcat1* gene. In addition, they do not have a putative signal sequence. This comparison supports the idea that the protein encoded by *cpcat1* is a secreted catalase.

Analysis of the upstream noncoding sequence of *cpcat1* (Fig. 2) showed typical fungal promoter elements, such as a TATA box and several CAAT sequences, as well as putative binding sites for regulatory proteins whose role in the expression of *cpcat1* is unknown. An abbreviated form (5'-ATAAA-3') of the consensus polyadenylation signal in higher eukaryotic organisms was found 157 bp downstream of the stop codon.

Fig. 3. Determination of the copy number of *cpcat1* in strain T5 of *Claviceps purpurea*. A blot containing lambda DNA digested with *HindIII* (λ) as DNA size marker and genomic DNA of strain T5 digested with *BamHI* (B), *EcoRI* (E), or *HindIII* (H) was simultaneously hybridized with a 32 P-labeled, 1.1-kb *SacI* fragment containing part of the *cpcat1* coding region (Fig. 1A) and 32 P-labeled lambda DNA. Sizes of the lambda *HindIII* fragments are indicated on left side of figure.

To determine the copy number of *cpcat1* in the genome of *C. purpurea*, a genomic Southern blot containing T5 DNA digested with *BamHI*, *EcoRI*, and *HindIII* was hybridized with a probe consisting of a central part of the coding region of *cpcat1* (Fig. 1A). Only one strong hybridizing fragment appeared in each lane, indicating that there is only one copy of *cpcat1* in the genome of *C. purpurea* (Fig. 3). Weaker fragments appeared in every lane, suggesting that there are additional catalase genes with some homology to the *cpcat1* gene.

Construction and characterization of deletion mutants.

In order to determine if *cpcat1* codes for the secreted catalase CATD, *cpcat1* was knocked out in strain 20.1, a haploid derivative of the field isolate T5 (Müller 1997). For construction of the replacement vector *pcp* Δ *cat1*, a 0.3-kb *SalI-HindIII* fragment from the central part of *cpcat1* (in plasmid *pcp**cat1*) was replaced by a phleomycin resistance cassette from plasmid pAN8-1 (Mattern et al. 1988) to produce a nonfunctional *cpcat1* allele (Fig. 1B). A 9.4-kb *KpnI* fragment containing the *cpcat1::Phleo^r* construct was used to transform strain 20.1. In order to detect homologous integration of the replacement fragment, 112 phleomycin-resistant transformants were selected and analyzed by Southern blot hybridization. Figure 1B shows the event expected upon recombination between the 9.4-kb *KpnI* fragment and the wild-type allele in the genome. After restriction of genomic DNA from the transformants with *KpnI* and hybridization with the 9.4-kb *KpnI* fragment, four transformants, MS54, MS63, MS80, and MS99, showed only the expected 9.4-kb *KpnI* fragment (Fig. 4), indicating that the wild-type allele of *cpcat1* was replaced by the nonfunctional allele used in the transformation. The viability of these transformants showed that the *cpcat1* gene is nonessential for growth of *C. purpurea* in axenic culture. The remaining transformants showed the wild-type 6.5-kb *KpnI* fragment and an additional fragment corresponding to an ectopic integration of

Fig. 4. Southern blot hybridization of transformants of strain 20.1 with *pcp* Δ *cat1* containing the desired integration event. A blot containing genomic DNA, digested with *KpnI*, from the strains indicated in the figure and a control lane with 1.6 ng of purified, 9.4-kb *KpnI* fragment (Fig. 1B) was hybridized with the same 32 P-labeled fragment.

the replacement fragment in the genome of *C. purpurea*. Two of these transformants, MS55 and MS72, having an ectopic integration of the replacement fragment and showing a standard growth rate, were selected for further experiments. To characterize the catalases of strains MS54, MS63, MS80, and MS99, they were grown under conditions in which the wild type produces low amounts of CATD (Mantle medium with reduced sugar content; Garre et al. 1998). The ectopic transformants MS55 and MS72 and the haploidized wild type 20.1 were cultivated identically as controls. Protein extracts from the culture medium of strains carrying the nonfunctional allele of the *cpcat1* gene showed a dramatic reduction (on average around 85-fold) of the total catalase activity (Table 1). A reduction of the catalase activity was also visible in mycelial protein preparations, although less pronounced (around five-fold); the residual activity is probably due to the presence of the intracellular catalase CATA (Table 1). The levels of catalase activity in protein extracts from all strains having a functional *cpcat1* gene showed no drastic differences. Isoelectric focusing (IEF) gels of mycelial proteins and subsequent staining for catalase activity revealed that the activity band corresponding to both CATD and CATC was absent in protein preparations from strains MS54, MS63, MS80, and MS99, whereas protein preparations from strains having the wild-type *cpcat1* allele exhibited this activity band (Fig. 5). Due to the difficulty of distinguishing between CATC and CATD in IEF gels, culture medium proteins from all strains were analyzed with cationic native polyacrylamide gel electrophoresis (PAGE), which enhances the mobility differences between CATC and CATD (Garre et al. 1998). Although the protein preparations from strains carrying the wild-type *cpcat1* allele showed both CATC and CATD, indicating that the growth conditions were adequate for the synthesis of these proteins, similar analysis of strains carrying the nonfunctional allele showed neither CATC nor CATD (data not shown). Activity staining of protein extracts from the strains MS54, MS63, MS80, and MS99 showed normal levels of CATA and CATB (Fig. 5). These data confirm that these mutants lack CATD and CATC as a consequence of the disruption of *cpcat1*, strongly suggesting that both proteins are encoded by *cpcat1*. The different mobility of two catalase isoforms encoded by *cpcat1* in cationic native PAGE could indicate variations in posttranslational processing.

Expression of *cpcat1* in planta.

Due to the difficulty in extracting high amounts of mRNA from infected rye ovaries, the presence of *cpcat1* mRNA during infection was studied by means of reverse transcription-

polymerase chain reaction (RT-PCR). A coding region of *cpcat1* containing two introns was selected for amplification with primers P1 and P2 (Fig. 1A). The presence of intron sequences between the primer target sequences enabled the RNA-derived PCR products to be distinguished from the larger, intron-containing PCR products arising from DNA contamination. Rye florets were inoculated with conidia of *C. purpurea* wild-type strain T5. RNA from infected ovaries at previously defined stages of infection constituting the whole infection process (Tenberge et al. 1996) was amplified with the primers P1 and P2. A PCR product of the expected size (474 bp) was found in all stages (Fig. 6A), indicating that this gene is transcribed throughout the entire infection. That the low level of amplification in the sample from stage 1 is probably due to the low amount of fungal RNA in the sample is shown by the similar results of a control PCR, with primers specific for the *gpd1* gene (Jungehülsing et al. 1994; data not shown). The identity of the RT-PCR product was confirmed by Southern blot analysis with a 1.1-kb *SacI* fragment from the coding region of *cpcat1* as a probe (Fig. 6B).

Protein preparations from all stages of infection were analyzed with IEF and subsequent catalase staining. The activity band corresponding to CATC/CATD was present from the start of the infection throughout the entire infection (Fig. 7). The protein preparation from stage 1 (5 days after inoculation) also showed rye catalase; this indicates that either both catalases are present in the same ovary or that the sample contained noninfected ovaries. In order to distinguish between the isozymes CATC and CATD, the protein preparations were analyzed with cationic native PAGE; only CATD was present (data not shown). This presence of CATD during the early stages of infection would be in accordance with a role in the destruction of H₂O₂ generated during the plant defense response.

Table 1. Catalase-specific activity of transformants^a

Strain	Culture medium	Mycelium	Honeydew	Stage 2
20.1	7.71	0.38	1.87	2.44
MS55	8.19	0.50	3.23	1.60
MS72	10.41	0.41	3.37	NM ^b
MS54	0.13	0.06	ND ^c	NM
MS63	0.13	0.11	ND	0.44
MS80	0.09	0.07	ND	NM
MS99	0.06	0.09	ND	0.14

^a Specific activity = nkat · µg⁻¹ protein

^b Not measured.

^c Not detectable (less than 0.008 nkat · µg⁻¹ protein).

Fig. 5. Analysis of catalase isoforms present in the mycelium of transformants. Protein extracts were prepared from 5-day-old mycelia grown in Mantle medium with low glucose content (Brockmann et al. 1992). In every lane of an isoelectric focusing (IEF) gel with a pH range from 5 (bottom) to 8 (top), 2.5 µg of protein was loaded. After electrophoresis, the gel was stained for catalase activity.

Pathogenicity of *cpcat1* mutants.

The pathogenicity of the four independently obtained *cpcat1* mutants was compared with that of strains MS55 and MS72 and the wild-type strain 20.1, which was used as a recipient in the transformation. Ears from healthy plants were inoculated with conidia from all strains, and parameters defining the extent of the infection were analyzed (Table 2). Ears inoculated with both the conidia of mutants and those of wild-type strains showed a similar level of infection. There were no significant differences in the time it took to produce honeydew, the amount of honeydew produced, or the formation of sclerotia. Southern analysis of *KpnI*-digested genomic DNA isolated from germinated conidia of honeydew confirmed that the infection was caused by *cpcat1* mutants (data not shown).

To characterize the infection carried out by the *cpcat1* mutants, the catalase activity in honeydew was measured (Table 1). As expected, no catalase activity was found, confirming that the catalase secreted during the infection is the protein CATD encoded by the *cpcat1* gene of *C. purpurea* and therefore is not of plant origin. A decrease of the catalase activity in ovaries infected with the *cpcat1* mutants MS63 and MS99 was also evident, although less pronounced, probably due to the presence of CATA and CATB (see below). In ovaries infected with MS63 and MS69 (harvested at stages 1 and 2 of infection) only the fungal catalases CATA and CATB could be detected, whereas ovaries infected with the wild-type strain showed also CATD (see Figure 8). These data confirm that the major catalase of ovaries infected with wild-type strains is indeed CATD and that this catalase is not essential for the virulence of *C. purpurea*.

DISCUSSION

Using the *catR* gene of *A. niger* as a probe, we have cloned a gene, *cpcat1*, that shows homology to bacterial and fungal catalases. Using a gene replacement approach, we have cre-

ated deletion mutants of *cpcat1*. Analysis of protein extracts of the mutants grown in axenic culture indicated that CATC and CATD are both encoded by *cpcat1*; they probably vary in their degree and/or kind of posttranscriptional modification. Given that the protein sequence has three putative N-glycosylation sites, it would be possible to attribute the isoforms to differing glycosylation levels. Glycosylation in catalases has been previously reported for a secreted catalase of *Penicillium chrysogenum* (Chaga et al. 1992), for CAT1 from *A. fumigatus* (López-Medrano et al. 1995), for a *Penicillium vitale* catalase (Gudkova et al. 1985), and for catalases from *A. niger* (Wasserman and Hultin 1981; Kikuchi-Torii et al. 1982). In the latter case, the presence of covalently bound

Fig. 6. Expression analysis of *cpcat1*. **A**, Reverse-transcription-polymerase chain reaction (RT-PCR) with RNA from different stages of infection (S.I: stage 1; S.II: stage 2; S.III: stage 3; and S.IV: stage 4) with primers P1 and P2 (Fig. 1A). As a control, genomic DNA was amplified by PCR with the same primers. Only size markers of 2.32, 2.03, and 0.56 kb (from top to bottom) from lambda DNA digested with *HindIII* (lane λ) are shown. **B**, Gel from **A** was blotted and hybridized with the 1.1-kb *SacI* fragment as probe (Fig. 1A). Lane S.I was exposed three times longer than the other lanes.

Fig. 7. Detection of catalases during parasitic growth of *Claviceps purpurea* on rye. Proteins prepared from ovaries infected with the wild-type strain T5 (stages 1 to 4), from exuded honeydew, noninfected ovaries (lane ovaries), from mycelium grown for 5 days in Mantle medium with reduced sugar content (lane mycelium), and from the corresponding culture medium (culture fluid) were applied to an isoelectric focusing (IEF) gel with a pH range from 5 (bottom) to 8 (top). After electrophoresis, the gel was stained for catalase activity. Approximately the same catalase activity (≈ 20 nkat) was applied to every lane, with the exception of stage 1 (< 10 nkat), which contains only very limited fungal biomass.

Table 2. Infection parameters

Strain	Germination rate (%)	Ears (no.)	Start of Honeydew (dpi ^a)	Amount of Honeydew	Sclerotium formation (%)
20.1	94.5	3	6.0	+++	68
MS55	43.5	4	6.0	+++	59
MS72	48.0	4	6.0	+++	ND ^b
MS54	56.5	5	6.6	+++	64
MS63	61.0	6	6.2	+++	63
MS80	53.0	5	6.2	+++	ND
MS99	82.5	5	5.6	+++	71

^a Days post infection.

^b Not determined.

sugars enhances the stability of *A. niger* catalase (Wasserman and Hultin 1981). Furthermore, the *A. niger* catalase appears in three isoforms. Two of them are characterized by the presence of extra sugars bound noncovalently to the smallest form (Kikuchi-Torii et al. 1982).

The protein encoded by *cpcat1*, CPCAT1 (= CATC/D), belongs to a class of catalases with characteristics of secreted proteins, which are grouped together in a tree analysis (Fig. 9), indicating a common ancestor. The subcellular localization of proteins belonging to this group has been preliminarily described for CAT1 from *A. fumigatus* and *A. niger* CatR. CAT1 seems to be extracellular (Calera et al. 1997) and CatR is probably a cell-wall-associated protein; under the growth conditions used by Fowler et al. (1993), more than 90% of catalase activity is cell associated. This assumption is further supported by the identification of cell-wall-associated catalases in *A. niger* (Witteveen et al. 1992). This could mean that extracellular catalases in *A. niger* are normally cell wall associated and under certain conditions can be released into the medium. Our earlier observations (Garre et al. 1998) indicate that CATB of *C. purpurea* could be such a cell-wall-associated enzyme, since, in contrast to CATC/D, the enzyme is only rarely found in the culture medium. The different catalase isoforms are differentially regulated in some fungi, e.g., in *A. nidulans*, and are thought to belong to the cellular defense mechanism against general oxidative stress and during differ-

entiation (conidiation, etc.). Preliminary Northern experiments indicate that expression of *cpcat1* in axenic culture is induced by stress, e.g., any medium transfer leads to expression of *cpcat1* within 1 h (data not shown). As shown in Figure 2, the promoter of *cpcat1* contains a stress-response element (STRE) that plays a crucial role in the stress control of yeast genes, including *CTT1*, which encodes a cytosolic catalase (Schüller et al. 1994).

For a pathogenic fungus such as *C. purpurea*, the colonization of plant tissue must surely be accompanied by stress, especially if part of the plant defense response includes production of active oxygen species (AOS). Therefore, fungal catalases could play a role in self-defense of the fungus; secreted catalases could even interfere with the plant's defense mechanism. In accordance with this idea, all major fungal catalases (CATA, B, D) are found in infected tissue. Furthermore, CATD is even found in honeydew of infected plants, and *cpcat1* is expressed throughout the entire infection cycle. Nevertheless, deletion of *cpcat1*, i.e., loss of the major secreted catalase form, does not impair the parasitic properties of *C. purpurea*, as judged from timing and amount of honeydew and sclerotia production. Several explanations are possible. First, either H_2O_2 is not required for triggering the plant defense response in rye, or it is not produced during the infection. Oxalate crystals have been found at the ergot-rye interface (Tudzynski et al. 1995; A. Hambrock, K. B. Tenberge,

Fig. 8. Detection of catalases in ovaries infected by *cpcat1* mutants. Protein prepared from ovaries infected with the wild-type strain 20.1, MS55 and mutants MS63 and MS99 at stages 1 and 2 was applied to an isoelectric focusing (IEF) gel with a pH range from 5 (bottom) to 8 (top). As controls, a lane containing noninfected ovary proteins (lane ovaries) and a lane containing proteins from 5-day-old mycelium grown in Mantle medium with reduced sugar content (lane mycelium) were included. Approximately the same protein amount was applied in the samples from stage 1 (24 μ g), and stage 2 (12 μ g), except in the control lane with mycelial proteins (76.8 μ g) and noninfected ovary proteins (22 μ g).

and P. Tudzynski, *unpublished*). Oxalate may act as a substrate for an assumed oxalate oxidase in rye, thus contributing to the generation of H_2O_2 (Dumas et al. 1995; Zhang et al. 1995). H_2O_2 production by rye is further supported by the detection of peroxidase activity, accumulation of polyphenolics, and lignification at the persistent colonization barrier of ergot in the rye rachilla (Hambrock 1996). Second, an additional catalase (e.g., CATB) could compensate for the loss of CATD in the deletion mutants. Although *cpcat1* mutants lack CATD and CATC, they have normal levels of CATB in axenic culture; in addition, CATB is found in rye tissue infected with *cpcat1* mutants, though in stage 1 in traces only (Fig. 8). Third, the pathogenicity test may not be sensitive enough. If it has a role in detoxifying AOS, the function of a catalase should be of highest importance during the initial stage of infection, and an artificial inoculation could produce a cooperative infection that, especially in the first stages, differs from the natural infection process (e.g., by high spore titers).

CPCAT1 is the first catalase of a plant-pathogenic fungus that has been shown to be secreted in planta. The only other example of a functional analysis of a secreted fungal catalase comes from a human pathogen, *Aspergillus fumigatus*. As in plants, an oxidative burst is involved in human and animal pathogen defense systems (Low and Merida 1996), and a secreted catalase of *A. fumigatus* has been suggested to be of importance in pathogenesis. In this system as well, knocking out of one of the two catalase genes in *A. fumigatus* did not have any influence on pathogenicity in a model test system (Calera et al. 1997). Obviously, the complexity of AOS-detoxifying systems is such that deletion of one component does not result in drastic changes. A detailed cytological analysis of plant tissue infected with the *cpcat1* mutants will clarify if there is still catalase activity around the hyphae, as indicated by the biochemical analyses (see Figure 8). If this is the case, then another cell-wall-associated (or secreted) catalase could be involved; as outlined above, the best candidate

Fig. 9. Tree analysis of fungal catalases based on derived amino acid sequences from *Candida albicans cta1*: accession number AB006327 (Nakagawa et al. 1995); *Candida tropicalis pox9*: Okada et al. (1987); *Hansenula polymorpha cat*: Didion and Roggenkamp (1992); *Saccharomyces cerevisiae CTA1*: Cohen et al. (1988); *Saccharomyces cerevisiae CTT1*: Hartig and Ruis (1986); *Schizosaccharomyces pombe cat*: Nakagawa et al. (1995); *Botrytis cinerea catA*: Van der Vlugt-Bergmans et al. (1997); *Aspergillus nidulans catA*: Navarro et al. (1996); *Aspergillus nidulans catB*: Kawasaki et al. (1997); *Aspergillus fumigatus cat1*: Calera et al. (1997); *Aspergillus niger catR*: Fowler et al. (1993). Secreted/cell-wall-associated catalases are marked by an asterisk.

for such a protein is CATB. Therefore, the next step in analyzing the importance of AOS detoxification in the interaction of *C. purpurea* and rye will be the cloning and functional analysis of the gene coding for CATB. Furthermore, investigation of other enzymes involved in the detoxification of AOS, such as peroxidases and superoxide dismutases, may be crucial for a good understanding of this subject.

MATERIALS AND METHODS

Strains and culture conditions.

C. purpurea strains used in this work were strain T5, a standard field isolate of *C. purpurea* (Fr.:Fr.) Tul., isolated from rye (*Secale cereale* L.; Hohenheim, Germany), and strain 20.1, a putative haploid derivative from strain T5 obtained by treatment with benomyl (Müller 1997). Mycelia were routinely grown at 28°C on complete medium (BII) (Esser and Tudzynski 1978) for maintenance and DNA isolation, or Mantle medium for conidia harvesting (Mantle and Nisbet 1976). For protein and RNA isolation, three colonies grown on solid Mantle medium were cut into small pieces, and then cultivated in 100 ml (Erlenmeyer flasks of 300-ml capacity) of Mantle medium with reduced sugar content (1% glucose) on a G25 rotary shaker (New Brunswick Scientific, Edison, NJ) at 150 rpm for 5 days at 28°C.

Escherichia coli strain DH5 α (Hanahan 1983) was used for all the subcloning experiments, and strain K803 (Wood 1996) was employed for propagation of *C. purpurea* genomic lambda clones.

Parasitic culture on rye plants.

Rye plants were cultivated in growth chambers as described by Smit and Tudzynski (1992), and inoculated with 10 μ l of solution containing 10⁴ conidia collected from Mantle plates as described elsewhere (Tenberge et al. 1996).

Preparation of protein extracts.

Liquid cultures were filtered (filter type 595; Schleicher & Schuell, Dassel, Germany) to separate mycelium from culture medium. Mycelial proteins were prepared from frozen mycelium ground with a mortar and pestle. Ground mycelium was suspended in double-distilled water and used directly. Culture fluid and honeydew were dialyzed against double-distilled water and used directly or after concentration by lyophilization. Proteins from infected and noninfected rye ovaries were prepared as previously described for rye leaf tissues (Feierabend et al. 1992).

Catalase activity assay and protein concentration determination.

Catalase (EC 1.11.1.6) activity was determined spectrophotometrically by measuring the decomposition of H₂O₂ at 230 nm (Eising and Gehardt 1986). Protein concentration was determined according to Bradford (1976).

Gel electrophoresis and catalase activity staining.

Native IEF was performed in a 1.5-mm-thick vertical slab minigel system as previously described (Robertson et al. 1987) with carrier ampholytes from pH 8 to 5. Samples containing 30 mM dithioerythritol (DTE), 30% (vol/vol) glycerol, and 2% (vol/vol) ampholytes pH 3 to 10 were applied to the

gel and electrophoresed for 90 min at 200 V and 150 min at 400 V. Native cationic PAGE was performed in a minigel system containing 7.5% (wt/vol) acrylamide, as previously described (Brockmann et al. 1992). After electrophoresis, gels were stained for catalase activity (Clare et al. 1984).

Disruption of *cpcat1*.

As a prerequisite for the inactivation of *cpcat1*, the gene was disrupted in vitro by substitution of an internal 0.3-kb *HindIII*-*SalI* fragment with a phleomycin resistance cassette. The replacement fragment for *cpcat1* was constructed in two steps, employing two plasmids derived from phages containing the *cpcat1* gene (Fig. 1B). The 3.2-kb *EcoRI*-*XbaI* fragment bearing the phleomycin resistance cassette was isolated from plasmid pAN8-1 and introduced into *EcoRI*-*XbaI* sites of the pUC19 polycloning site of a plasmid carrying a 5-kb *SalI* fragment that contained the 3' region of *cpcat1* beyond the *KpnI* site (see Figure 1A). In the next step, the 5' part of the gene, extending from the *HindIII* site (at the middle of the gene) to a *BamHI* site upstream of the *KpnI* site, was purified from a second plasmid by restriction with *BamHI* and *HindIII*, and afterwards cloned, in the correct orientation, into the *BglII* site of the phleomycin resistance cassette (Fig. 1B), yielding the plasmid pcp Δ cat1. The replacement fragment was released by restricting pcp Δ cat1 with *KpnI* and purified by agarose gel electrophoresis. After staining with ethidium bromide, the corresponding 9.4-kb *KpnI* fragment was excised and then eluted with the Biotrab-Electroreparation system according to the supplier's instructions (Schleicher & Schuell, Dassel, Germany). Protoplasts from strain 20.1, generated with Novozym 234 (Novo, Bagsvaerd, Denmark), were transformed with 10 μ g of the 9.4-kb *KpnI* fragment as described by Jungehülsing et al. (1994).

Nucleic acid manipulation and analysis.

Standard recombinant DNA methods were performed according to Sambrook et al. (1989) and Ausubel et al. (1987). Genomic DNA of *C. purpurea* was prepared from lyophilized mycelium according to Cenis (1992). RNA for expression analysis during the parasitic cycle of ergot was prepared according to Chambers and Russo (1986) as described previously (Müller et al. 1997). RNA from axenic culture was isolated from lyophilized mycelium with Trizol reagent according to the supplier (Gibco, Eggenstein, Germany).

For Southern blot analysis, restricted chromosomal DNA (5 to 10 μ g) or PCR products were electrophoresed in 1 to 2% agarose gels with Tris-acetate-EDTA buffer (Sambrook et al. 1989), blotted onto positively charged Nylon filters (Hybond-N⁺, Amersham, Braunschweig, Germany), and hybridized to radioactively labeled DNA probes (Feinberg and Vogelstein 1983) at 65°C (homologous probes) or 60°C (heterologous probe) in Denhardt's hybridization solution (Sambrook et al. 1989). For homologous hybridization experiments, filters were washed twice in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1 % sodium dodecyl sulfate (SDS) at room temperature for 5 min and twice in 0.1 $\times$ SSC, 0.1% SDS at 65°C for 15 min. In heterologous hybridization experiments, with the 1.75-kb *SalI* fragment from the *A. niger* *CatR* gene as a probe, filters were washed twice in 2 $\times$ SSC, 0.1% SDS at room temperature for 5 min and then twice at 60°C for 15 min. Northern blot hybridization experiments

were carried out as previously described (Garre et al. 1994), with 25 to 50 µg of total RNA per sample.

For molecular cloning of *cpcat1*, a genomic EMBL3 library of strain T5 (Smit and Tudzynski 1992) was screened by plaque filter hybridization according to Sambrook et al. (1989) with the 1.75-kb *SaI*I fragment from the *A. niger* *CatR* gene as a probe and employing the hybridization conditions described above.

DNA sequencing was carried out with a DNA sequencer from LICOR (model 4000) and the thermo sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, Braunschweig, Germany) together with IRD41-labeled primer (MWG, Ebersberg, Germany). Computer analysis of the sequence was performed with HUSAR (EMBL, Heidelberg, Germany). Multiple alignment was carried out with PILEUP (Feng and Doolittle 1987).

For RT-PCR for the *cpcat1* gene, oligonucleotides P1: 5'-GTG ACG TGC ACG GAT TTG-3', and P2: 5'-CGT TGA TGA TTT GCA GGT TG-3', whose target sequences are indicated in Figure 1A, were employed. RT-PCR was carried out as described previously (Müller et al. 1997) with 1/10 part of the RT-reaction. The PCR run started with a first cycle of 4 min at 94°C, followed by 30 cycles of 1 min at 61°C, 30 s at 72°C, and 1 min at 94°C. The PCR was ended with an extension step of 5 min at 72°C. RT-PCR for the *GPD 1* was done as described by Müller et al. (1997).

ACKNOWLEDGMENTS

We wish to thank T. Fowler (Genencor International, San Francisco) for providing us with the *CatR* gene from *A. niger*; K. B. Tenberge for helpful discussions, B. Oeser for supplying us with RNA from parasitic cultures of *C. purpurea*, S. Moore for critical reading, and A. Kammerahl for typing the manuscript. V. G. gratefully acknowledges a fellowship provided by the European Community within the Human Capital and Mobility network "Molecular Phytopathology" and a short-term fellowship (ASTF 8769) provided by EMBO.

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