CPTF1, a CREB-Like Transcription Factor, Is Involved in the Oxidative Stress Response in the Phytopathogen *Claviceps purpurea* and Modulates ROS Level in Its Host *Secale cereale*

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CPTF1, a transcription factor with significant homology to ATF/CREB bZIP factors, was identified during an expressed sequence tag (EST) analysis of in planta-expressed genes of the phytopathogen *Claviceps purpurea*. Using a gene-replacement approach, deletion mutants of *cptf1* were created. Expression studies in axenic culture showed that the H₂O₂-inducible gene *cpcat1* (encoding a secreted catalase) had a reduced basal expression level and no longer responded to oxidative stress in the *Δcptf1* mutant. Biochemical analyses indicated that CPTF1 is a general regulator of catalase activity. *Δcptf1* mutants showed significantly reduced virulence on rye. Electron microscopical in situ localization revealed significant amounts of H₂O₂ in *Δcptf1*-infected rye epidermal tissues, indicating that the plant tissue displayed an oxidative burst-like reaction, an event not detected in wild-type infections. These data indicate that CPTF1 is involved not only in oxidative stress response in the fungus but also in modulation of the plant's defense reactions.

Additional keywords: fungal transcription factor, host-pathogen interaction.

Claviceps purpurea is a ubiquitous phytopathogenic ascomycete with a wide host range including many grasses and cereals but with a high organ specificity; it infects only grass florets, following the path of the pollen tube to the basis of the ovary, where—after tapping the host's vascular tissue—it

establishes a balanced long-lasting host-pathogen interaction. The first visible sign of infection is formation of honeydew, a plant exudate enriched with fungal conidia. During the course of infection, the host's tissue is replaced by fungal sclerotial plectenchyma. Because of its strict dependence on the living host tissue to complete its lifecycle, *C. purpurea* is considered a biotroph despite its ability to grow in axenic culture. We have initiated a detailed analysis of several aspects of this specific interaction system (Oeser et al. 2002b). For example, we recently showed that polygalacturonases are essential for pathogenesis (Oeser et al. 2002a), and we identified two mitogen-activated protein (MAP) kinases involved in pathogenesis (Mey et al. 2002a and b). One focus of our research is the role of reactive oxygen species (ROS) in the interaction. An oxidative burst-like plant defense reaction has never been observed during the colonization of rye ovarian tissue by *Claviceps purpurea*; only at the borderline established during the formation of a stable interaction have ROS been detected (A. Hambrock and K. B. Tenberge, unpublished data). Nevertheless, we could demonstrate that *C. purpurea* secretes a set of ROS-scavenging enzymes in planta, the role of which is unclear. Functional analyses showed that deletion of the genes encoding the most abundant enzymes (catalases C/D and superoxide dismutase A) had no significant effect on virulence (Garre et al. 1998; Moore et al. 2002). A broader analysis of the role of ROS scavenging in the interaction became possible by an expressed sequence tag (EST) analysis of *C. purpurea* genes expressed in infected rye tissue (Oeser et al. 2002b), which revealed two potential transcription factors showing homology to bZIP factors involved in the environmental stress response of higher eukaryotes. As preliminary experiments indicated that the expression of one of these genes (named *cptf1*) could be influenced by exogenously applied H₂O₂ (Oeser et al. 2002b), we initiated a detailed functional analysis of this putative transcription factor.

RESULTS

cptf1 encodes a putative bZIP transcription factor.

The preliminary analysis of a small-scale EST library of *C. purpurea*-infected rye tissue had indicated that clone 302 could encode a bZIP transcription factor (Oeser et al. 2002b). An 0.8-kb *EcoRI* fragment of this clone was used as a probe to screen a genomic EMBL 3 library of *C. purpurea* by plaque filter hybridization. Restriction analysis showed that two hybridizing clones contained overlapping genomic regions. A

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Nucleotide sequence data reported for *cptf1* is available in the EMBL database under accession number AJ 428 492. Accession numbers of bZip factors *Neurospora crassa* atf1-like factor, CAC18235; *Aspergillus nidulans* AtfA, AAN75015; *Schizosaccharomyces pombe* atf1, CAC05510; *Homo sapiens* jun-B, CAA35738; *Xenopus laevis* cJun, CAB51637; *Gallus gallus* junD, CAC05510; *Homo sapiens* ATF2, NP_001871; *Rattus norvegicus* ATF2, Q00969; *Mus musculus* ATF2, B42026; *G. gallus* ATF2, 093602; *M. musculus* CRE-BP1, AAB21128; *M. musculus* ATF1, NP_031523; *Saccharomyces cerevisiae* Aca1p, NP_010964; *Saccharomyces cerevisiae* Sko1p, CAA48074; *Candida albicans* Cap1, AAD00802; *Saccharomyces cerevisiae* Yap1p, CAA41536; and *Schizosaccharomyces pombe* Pap1p, CAA40363.

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2.9-kb fragment was completely sequenced; it contains an open reading frame of 550 amino acids interrupted by two introns of 258 and 77 bp (confirmed by reverse transcription-polymerase chain reaction (RT-PCR); data not shown). The derived amino acid sequence shows significant homology to transcription factors of the CRE-BP/ATF/jun family in mammals and to related factors in yeasts and fungi (Fig. 1). These proteins contain bZIP DNA-binding motifs consisting of a conserved basic region directly involved in DNA binding and an adjacent leucine zipper that mediates dimerization. The different family members diverge considerably outside of the bZIP domain; the unique features contribute to specific regulatory properties. In mammalian systems, these factors are involved in the response to many different extracellular stimuli and stress factors (Hai and Hartmann 2001). Figure 2 shows an alignment between CPTF1 and two of its closest fungal homologues, AtfA from *Aspergillus nidulans* (without assigned function as of yet) and ATF1 from fission yeast (involved in sexual development and response to osmotic and chemical stress [Takeda et al. 1995]).

It is obvious that the three fungal factors presented in Figure 2 have conserved areas outside of the highly conserved bZIP domain, which does not necessarily imply related functions. Interestingly, all three lack the second conserved leucine residue (Fig. 2, black arrowhead), which is present in most mammalian and yeast bZIP factors. Figure 3 gives a schematic comparison of these three proteins, of the closely related *Neurospora crassa* factor (not yet characterized; a single copy gene like *cptf1*), and two related mammalian factors. CPTF1 has a comparable overall structure; its bZip domain is located at the carboxy-terminus, there is a Q-rich domain in the central part of the protein, and there are several putative MAPK phosphorylation sites. In contrast to most of the other factors, there is only one putative PKA phosphorylation site in CPTF1 at the very carboxy end of the protein.

cptf1 is expressed in planta.

Since cDNA clone 302 was obtained from mRNA from infected rye tissue, expression of *cptf1* in planta was anticipated. An in planta expression analysis was performed by RT-PCR experiments, using RNA from infected tissue 5 days after infection (dpi) (5 dpi = stage I (S I)), 10 dpi = S II, 15 dpi = S

III, and 20 dpi = S IV). To be able to distinguish between RT-PCR products obtained from mRNA and those from contaminating genomic DNA, we used primers RTP1/RTP2 (spanning intron 2; Fig. 4) for amplification, yielding a 304-bp fragment with genomic DNA template, whereas the RT-PCR samples from stages II through IV yielded smaller (227 bp) fragments, as expected if the mature messenger of *cptf1* is the template. A Southern hybridization using the 0.8-kb *EcoRI* fragment of the *cptf1*-cDNA clone as probe confirmed the identity of the RT-PCR fragments (data not shown). Since the RT-PCR is not quantitative, these results only confirm that *cptf1* is transcribed during the infection process.

Generation of *cptf1* deletion mutants.

In order to study the role of *cptf1* in the pathogenicity of *C. purpurea* and to identify genes regulated by this putative transcription factor, we used a gene-replacement approach to create deletion mutants of *cptf1*. The strategy is shown in Figure 4. Using primer pairs FP1/RP1 and FP2/RP2, respectively, a 5' and a 3' fragment of the gene were amplified and ligated to the phleomycin-resistance cassette from vector pAN8-1. The construct was excised using the (vector-derived) *DraI/NdeI* sites and was used to transform protoplasts of strain 20.1. Phleomycin-resistant transformants were screened for the presence of a "diagnostic" PCR fragment of 1.5 kb generated by primers HOM1 and PAN-pro, which can arise only if the desired replacement event has occurred (Fig. 4). Positive primary transformants were subjected to genetic purification by single-spore isolation; presence of the wild-type gene copy was detected by PCR with the primer pair HOM1/HOM2 (2.1 kb). Three homokaryotic replacement mutants were obtained (*Δcptf1-1*, *Δcptf1-2*, and *Δcptf1-3*), showing the diagnostic fragment and lacking the wild-type fragment. The recipient strain 20.1 shows only the wild-type fragment, as does transformant T9, which probably is an ectopic transformant (Fig. 5). The correct replacement of the locus in the mutants was confirmed using primer pairs pAN-T and HOM2, spanning the right flank of the construct (Fig. 4 and data not shown). To confirm the PCR analysis, Southern experiments were performed using genomic DNA of the transformants (and the wild-type strain) digested with *KpnI* and the replacement fragment (Fig. 4) as probe. The three mutants were shown to lack

Fig. 1. Phylogenetic tree of bZip proteins including the *cptf1*-derived sequence, CPTF1.

the wild-type gene fragment and, thus, represent true deletion mutants. Transformant T9 contains the wild-type fragment and two additional fragments of higher molecular weight, confirming an ectopic integration of the vector (data not shown).

CPTF1 controls catalase activity.

The availability of *Δcptf1* mutants allows the identification of genes controlled by this factor. The ATF-CREB proteins, to which CPTF1 shows significant homology, bind specifically to the cAMP response element (CRE consensus sequence: TGACGTCA) (Deutsch et al. 1988; Fujii et al. 2000). In silico

analysis of promoters of ROS-related genes from *C. purpurea* revealed the presence of a CRE consequence sequence in the 5' upstream sequence of the catalase gene *cpct1* (Garre et al. 1998). In Figure 6, a Northern experiment is shown, using RNA from mycelia of the wild-type strain and the *Δcptf1-1* mutant, with (+) and without (–) H₂O₂ addition. Hybridization of the same filter with a gene being constitutively expressed under these conditions (*cpmco1*, encoding a putative iron transporter [Oeser et al. 2002b]) and ribosomal RNA served as controls. It is obvious that the basal expression level of *cpct1* is drastically reduced and the H₂O₂ induction is minimized in

Fig. 2. Alignment of amino acid sequences of fungal bZip transcription factors. The alignment was done using CLUSTALW (Higgins et al. 1994). Identical residues in two or three sequences are boxed, identical residues in all three sequences are marked by an asterisk (*), and semiconserved substitutions by a dot. The bZip domain is indicated by grey shading; the leucine residues of the leucine zipper domain are indicated by arrow heads, the "missing" leucine residue (compared with the canonical sequence) is indicated by a black arrow head.

the $\Delta cptf1$ -mutant, strongly suggesting that the expression of *cpcat1* is regulated by CPTF1.

Since the analysis of the *cptf1* sequence had identified potential MAPK phosphorylation sites (Fig. 3), we tested if two recently functionally characterized MAPKs of *C. purpurea* (Mey et al. 2002a and b) modulate the activity of CPTF1. As shown in Figure 6, the H_2O_2 induction of the putative target gene of CPTF1, *cpcat1*, is not effected by the inactivation of either *cpmk1* or *cpmk2*; this strongly suggests that CPTF1 is not regulated by one of these two MAPK cascades.

To confirm the influence of CPTF1 on expression of *cpcat1* on enzyme activity level, we analyzed protein extracts from cultures grown under conditions used for the biotechnological production of ergot alkaloids (discussed below), which have been shown to induce high catalase activities (S. Moore, E. Nathues, and P. Tudzynski, unpublished data). As shown in Figure 7A, a catalase activity gel of protein extracts from the wild-type strain under these conditions exhibits at least five different catalase isozymes (Garre et al. 1998): CATA (prob-

bly an intracellular form), CATB (probably cell wall associated), CATC and CATD (secreted, both forms encoded by *cpcat1*), CATE (induced only by these special conditions and in sclerotial tissue in planta [S. Moore, E. Nathues, and P. Tudzynski, unpublished data]). Interestingly, in the two $\Delta cptf1$ mutants tested, not only CATC and CATD are reduced as expected, but all catalase isoforms have a significantly reduced activity, indicating that CPTF1 may affect the expression of all these catalase genes in *C. purpurea*. Using the same protein extracts, a superoxide dismutase (SOD) activity gel was performed (Fig. 7B), showing the normal CPSODA activity band in the mutants and confirming that the lack of catalase activity is not due to the quality of the extracts and that *cpsod1* (encoding CPSODA [Moore et al. 2002]) is not controlled by CPTF1.

CPTF1 is important for pathogenesis.

Deletion of *cptf1* has no effect on growth rate and sporulation of axenic cultures (data not shown). In order to investigate the importance of CPTF1 during parasitic growth of *C. pur-*

Fig. 3. Schematic maps of bZip factors showing homology to CPTF1. Conserved domains are boxed. Oblique stripes = Q-rich domain; horizontal stripes = L-zipper; dotted = basic region; squared = zinc finger; arrow heads = PKA sites; vertical bars = MAPK sites; brackets in *Rattus norvegicus* ATF2 = splice variant region.

Fig. 4. Schematic map of *cptf1* and disruption strategy. Top: genomic copy of *cptf1*; open and black boxes indicate exons and introns, respectively. The 5' and 3' nontranscribed regions are indicated by black bars. The restriction enzymes indicated are: Hc, *HincII*; Sc, *SacII*; P, *PstI*; S, *Sall*; and H, *HindIII*. Primers RTP1 and RTP2 were used for reverse transcription-polymerase chain reaction (PCR). Primers FP1 and RP1 and FP2 and RP2 were used to amplify the 5' and 3' parts of the gene for construction of the replacement vector. Center: the replacement fragment containing 5' and 3' parts of *cptf1* flanking a phleomycin resistance cassette: P-gpdA, promoter of *gpdA* from *Aspergillus nidulans*; Phleo^r, phleomycin resistance gene; and T-trpC, terminator of *trpC* from *A. nidulans*. Sites for restriction enzymes *DraI* and *NdeI* used to excise the fragment are indicated. Bottom: disrupted *cptf1* after homologous integration of the replacement fragment. The primer pairs HOM1 and PAN-Pro and HOM2 and PAN-T, used for diagnostic PCR of the replacement fragment, are indicated.

purea, young florets of rye were inoculated with conidial suspensions of wild-type strain 20.1, the ectopic transformant T-9, and the three deletion mutants $\Delta cptf1-1$, $\Delta cptf1-2$, and $\Delta cptf1-3$. Virulence was assessed by comparing both time of honeydew appearance and amount of honeydew exuded by the ears infected with the wild-type strain and the mutants. As compiled in Table 1, in different sets of experiments including comparable groups of rye ears (each with 30 to 40 infected florets), both the wild-type strain and the ectopic transformant induced massive appearance of honeydew at about 7 dpi, whereas ears infected by all three deletion mutants gave rise to significantly reduced amounts of honeydew, which was exuded 2 to 4 days later. After about 45 days, the development of sclerotia was monitored. Wild-type and ectopic transformants produced comparable numbers of fully developed sclerotia, whereas the deletion mutants produced reduced numbers of sclerotia, a relatively high proportion of which did not fully develop (data not shown). Thus CPTF1 clearly has impact on virulence of *C. purpurea* on rye.

To test if the observed reduced virulence of $\Delta cptf1$ mutants on rye, especially the retarded production of honeydew, is due to a reduction of “fitness” of the fungus leading to a reduced growth rate or if the mutation leads to increased defense reactions of the plant, we performed a detailed electron microscopy (EM) analysis of infected ovarian tissue. No obvious morphological differences between comparable infection stages of the wild-type strain and the mutant were observed (data not shown). However, an in situ H_2O_2 detection analysis using the $CeCl_2$ reaction (discussed below) detected a surprising difference (Fig. 8); in wild-type infected tissue, no significant amount of electron-dense cerium perhydroxide, known to specifically precipitate in the presence of H_2O_2 (Briggs et al. 1975; Thomas and Trelease 1981), can be detected in the infected area, neither around the hyphae nor in more distant areas. This indicated both that the production of H_2O_2 by the fungus in planta is very low and that there is no obvious defense reaction of the plant leading to an oxidative burst (Fig. 8I). In noninfected healthy rye tissue, H_2O_2 only was detected in developing xylem elements. However, in the $\Delta cptf1-1$ -infected tissue, there is a massive cerium perhydroxide pellet, indicating significant H_2O_2 production, not only around the hyphae but also in tissue areas more distant from hyphae (Fig. 8E and H). The in situ analysis revealed that this mutant strain-induced reaction is host tissue specific. Precipitate hardly was found in the ovary wall mesophyll (Fig. 8B) but was detected almost exclusively in all epidermal layers of the rye ovary: in the outer epidermis of the ovary penetrated by the pathogen (Fig. 8A), in the inner epidermis and in the

directly adjacent epidermal tissue that surrounds the single ovule, in the two integuments (Fig. 8D through H, orientation is shown in C). This strongly suggests that the rye tissue reacts via an oxidative burst, which is further supported by the localization of the H_2O_2 -indicating precipitate at the host plasma membrane surface (Fig. 8H). This is especially significant, since such a reaction had never been observed with any wild-type strain or any other mutant of *C. purpurea*. It might be of special importance in this interaction system that the strongest induced reaction was found in the two inner epidermal layers that, together with the subepidermal transmitting tissue, constitute the “host pollen tube path,” which the fungus routinely follows during infection.

DISCUSSION

We have identified and characterized a new transcription factor in *C. purpurea*, CPTF1, which is homologous to bZip transcription factors from yeasts and mammals. These factors, which have been intensively studied in higher eukaryotes, are involved in the cellular response to environmental stimuli. A recent review (Hai and Hartman 2001) lists more than 20 different mammalian factors belonging to the ATF-CREB subfamily of bZIP transcription factors, the complexity of which is increased by the ability to form heterodimers among themselves or with related factors from other families. Thus they represent a highly complex regulatory network, which allows the cell to respond to a huge variety of environmental stresses in a flexible and specific way. Until now, there are only limited reports on bZIP transcription factors in filamentous fungi, although several have been classified in yeast (especially the AP-family [Toone and Jones 1999]).

A typical bZip domain shows a “basic region” immediately adjacent to the “leucine zipper,” which contains leucine residues at every seventh position (Landshulz et al. 1988). Sequence analysis shows that, while CPTF1 has a typical basic region, the leucine zipper lacks one of the leucine residues (Fig. 2, black arrowhead). Mutations of one or more leucine residues in the leucine zipper of mammalian factors are known to interfere with dimer formation, resulting in lower DNA binding ability (Abel and Maniatis 1989). The homology analysis presented in Figure 2 shows that the closest relatives to CPTF1 (ATFA of *A. nidulans* and ATF1 of fission yeast) both lack the same leucine residue. It is interesting to note that CPTF1 and its closest fungal homologues show a mutated leucine zipper; since the functionality of ATF1 has been proven, this modification does not seem to be deleterious. Investigations on the yeast GCN4 factor support this idea (van Heeckeren et al. 1992).

ATF1 in fission yeast (like AP-1 in yeast) is part of the environmental stress response system mediated by the MAP kinase Sty1. ATF1 is mainly responsible for the regulation of meiosis-

Fig. 5. Molecular characterization of $\Delta cptf1$ mutants. Polymerase chain reaction with primers HOM1 and PAN-Pro (diagnostic, a) and HOM1 and HOM2 (wild-type, b), using genomic DNA from wild-type strain 20.1, three $\Delta cptf1$ transformants, and an ectopic transformant (T-9) as template. Sizes of the wild-type (2.1) and diagnostic fragment (1.5) are indicated.

Fig. 6. Expression analysis of *cpcat1*, a potential target gene of CPTF1 RNA from mycelia of strains 20.1 (WT), $\Delta cptf1-1$, $\Delta cpmk1-1$, and $\Delta cpmk2-1$ with or without H_2O_2 treatment (+/-) was separated in an agarose gel, was blotted on nylon membrane, and **A**, hybridized to an internal 1.6-kb polymerase chain reaction fragment of *cpcat1* (Garre et al. 1998). **B**, The same filter was rehybridized with an 850-bp internal *EcoRI* fragment of *cpmcol* (Oeser et al. 2002b), and subsequently **C**, with a rDNA probe as control.

specific and osmotic stress-responsive genes, and together with a related bZIP factor, Pap1, controls genes involved in response to oxidative stress and to xenobiotics (Nguyen et al. 2000; Quinn et al. 2002; Shiozaki and Russell 1996; Toone et al. 1998). Although CPTF1 shows higher homology to ATF1 than to Pap1, it seems to be more functionally related to Pap1, since it mediates response to low H₂O₂ concentrations. It will be interesting to see if—as in fission yeast—the *C. purpurea* oxidative stress response also is mediated by two transcription factors.

ATF/CREB factors bind specifically to the palindromic ATF/CRE recognition sequence (Deutsch et al. 1988; Fujii et al. 2000). The specific response of *cpcat1* to CPTF1 correlates with the presence of a complete CRE consensus sequence in the promoter of *cpcat1* (Moore et al. 2002) but not, e.g., in *cpsod1* (Moore et al. 2002) or *cpmco1* (used as a control in Fig. 6) (Oeser et al. 2002b), supporting the view that CPTF1 could bind to this recognition sequence. However, there are additional potential CRE-like sequences not fully corresponding to the canonical consensus site in *cpcat1* (and also in *cpsod1*). Therefore, a more detailed analysis of CPTF1 interaction with the CRE-like site in the *cpcat1* promoter will be necessary, including heterologous expression of the protein, band shift assays, and mutational analysis of the putative CRE sites.

The protein analyses confirm and extend the Northern data. The overall reduction of activity of all catalase isozymes in axenic culture indicates that CPTF1 controls not only expression of *cpcat1* but of all the respective catalase genes; on the other hand, CPSODA activity is not affected by the *cptf1* knock out.

In addition to the target genes, the identification of upstream signaling components controlling CPTF1 activity would be highly interesting. The presence of putative MAPK phosphorylation sites in the CPTF1 sequence indicates that a MAP kinase cascade is involved. The recently identified MAP kinases of *C. purpurea* CMPK1 and CMPK2, which are essential for pathogenicity (Mey et al. 2002a and b), obviously are not involved in control of CPTF1, since the H₂O₂ induction of the potential target gene *cpcat1* is not impaired in the respective deletion mutants (Fig. 6). CPMK1 and CPMK2 are functional homologues of the *Magnaporthe grisea* genes *pmk1* (corresponding to FUS3 of *S. cerevisiae*) and *Mps1* (corresponding to Slr2), respectively. In *Schizosaccharomyces pombe*, ATF1 and Pap1 are controlled by a third type of MAPK, Sty1 (= Hog1 of *S. cerevisiae*); a homologue of this stress-responsive MAPK recently has been identified in *C. purpurea* (E. Nathues and P. Tudzynski, unpublished data), it could very well be the upstream component of CPTF1. The presence of a single PKA phosphorylation site could indicate

Fig. 7. Activity staining of protein extracts separated on an isoelectric focusing gel, pH 3 to 10. **A**, Catalase activity. **B**, Superoxide dismutase activity. 1 = ectopic transformant T9; 2 and 3 = *Δcptf1-1* and *Δcptf1-2*; and 4 = wild-type strain 20.1.

Table 1. Pathogenicity tests of *Δcptf1* mutants on rye plants^a

Strain	Experiment 1			Experiment 2		
	Ears infected	Honeydew production ^b	Amount of honeydew	Ears infected	Honeydew production ^b	Amount of honeydew
20.1	7	7.7 ± 0.5	++++	5	7.6 ± 0.6	++++
T-9	9	7.6 ± 0.5	++++	5	6.4 ± 0.6	++++
ΔTF1	12	11.8 ± 1.6 ^c	+	9 ^d	10.0 ± 0.8 ^c	+
ΔTF2	12	12.0 ± 2.1 ^c	+	7 ^d	9.8 ± 0.8 ^c	+
ΔTF3	14 ^c		+	3	9.6 ± 0.6 ^c	+

^a Rye florets were inoculated with conidia from wild-type strain 20.1, ectopic transformant T-9, and mutants *Δcptf1-1*, 2, 3 (ΔTF1-3). Appearance of visible honeydew was monitored daily, amount of honeydew was determined 3 to 4 days after first appearance.

^b Days postinfection.

^c Delay in start of honeydew is significant as compared with start of honeydew in wild-type strain 20.1, determined using student's *t*-test.

^d Two ears did not show visible honeydew production.

^e Three ears did not show visible honeydew production.

Fig. 8. Cerium chloride-mediated in situ localization of H_2O_2 visualized by transmission electron microscopy in rye ovaries infected by $\Delta cptf1$ mutants of *Claviceps purpurea* (A, B, and D through H) and by the wild-type strain 20.1 (I) at 7 days postinoculation. **A**, Hyphae (F) living on the outer epidermal wall (oew) below the ovary cuticular membrane (cm) and inside the outer ovary epidermis (e) of the host (H). **B**, Hyphae of the mutant strain (F) growing intercellularly in the ovary wall between two mesophyll parenchyma cells (H) in the intercellular space (is). No precipitate is detectable. **C**, Illustration of the spatial sites of the depicted areas, indicated by rectangles, in relation to infection routes of *C. purpurea* in a rye pistil. indicate route corresponding to the pollen tube path; + + + and - - - indicate alternative routes, double-arrow indicates filament base, long arrows indicate different penetration sites; m = micropylar region; ov = ovule; ow = ovary wall; ra = rachilla; si = stigma; sy = style; **D** through **G**, Hyphae (F) growing in the integuments of the host (H) towards the ovary base. **E**, Strong electron-dense precipitate is present in the cell wall apoplast next to and distant from hyphae growing between living cells of the integuments. Inner epidermal layers are recognizable by their cuticular membranes (cm); **F**, Strong electron-dense precipitate present in the zone of apparently dead host cells penetrated by a filamentous part of the CPTF1-deficient strain. **G**, Interface of pathogen and host at higher magnification showing precipitate present in host cells (H), in the intercellular space (is), and in addition, in the fungal cell wall. **H**, An apparently noncolonized host area next to the transmitting tissue showing cerium perhydroxide precipitate in the periplasmic space and in the cellular junction zone. **I**, Hypha (F) growing below the cuticular membrane (cm) in the integument zone. No precipitate is visible in this wild-type control. Arrowheads indicate precipitate at the host plasma membrane, and a star indicates electron-dense cerium perhydroxide precipitate. Scale bars represent 1 μm .

also that the cAMP pathway influences CPTF1 activity; this dual control has been shown, e.g., for the *U. maydis* transcription factor Prf1 (Müller et al. 1999). Preliminary data indicate that indeed cAMP has some influence on the expression of *cpcat1* (B. Oeser, *unpublished data*); but a detailed analysis is necessary.

An interesting aspect of CPTF1 is its effect on virulence, supporting the theory that oxidative stress is an important factor in the host–pathogen interaction. This effect was unexpected, since deletion of neither *cpcat1* nor *cpsod1*, both encoding major ROS-scavenging enzymes, had any effect on virulence (Garre et al. 1998; Moore et al. 2002; S. Joshi, S. Moore, P. Tudzynski, *unpublished data*). In *M. grisea*, inactivation of the stress-response MAPK OSM1 (a homologue of Hog1p) had no effect on pathogenicity (Dixon et al. 1999). In *Cochliobolus heterostrophus*, deletion of the three catalase genes identified in the genomic sequence had no effect on pathogenicity (Robbertse et al. 2002), and deletion of a gene encoding an extracellular catalase in *Botrytis cinerea* did not effect virulence (Schouten et al. 2002). The development of the $\Delta cptf1$ -mutants in planta—as taken from the time of honeydew appearance—is retarded, and the development of sclerotia, the important dormant structures of the fungus, seems to be significantly impaired, though a statistically reliable evaluation on the degree of ripeness of sclerotia is impossible. Since the formation of durable sclerotia is essential for survival of the fungus over the “host-free” winter period, this deficiency in sclerotia formation and maturation would probably have a drastic effect on the survival of the fungus in nature. Since the role of the oxidative status in the development of sclerotia in the pathogenic fungus *Sclerotia sclerotiorum* has recently been demonstrated (Georgiou et al. 2001), this obvious maturation defect in $\Delta cptf1$ mutants adds further evidence to the impact of CPTF1 on the oxidative status of the mycelium or the surrounding host tissue.

A real surprise was the detection of a bona fide oxidative burst in infected rye tissue, which we had not observed before.

This could indicate that the plant suddenly recognizes the pathogen, i.e., CPTF1 controls one or more factors that either induce or suppress the host reaction. A key to the understanding of these observations could be the increased H_2O_2 presence around the hyphae in host epidermal tissue. Preliminary tests using the chloronaphthol staining technology (discussed below) had shown that *C. purpurea* 20.1 (and the $\Delta cptf1$ mutants as well) secretes (low amounts of) H_2O_2 in axenic culture (data not shown). Lack of detectable H_2O_2 in plant tissue could be due to secreted catalase activity. The occurrence of high H_2O_2 levels around the $\Delta cptf1$ hyphae in plant tissue could be explained by the lack of catalase activity, i.e., by reduced degradation of H_2O_2 . However, deletion of *cpcat1*, leading to loss of the major secreted catalase isozyme CATC and CATD has no effect on virulence (Garre et al. 1998). Thus, increased production of fungal endogenous ROS would be a better explanation. Based on the present data, we suggest the model presented in Figure 9. CPTF1 regulates the ROS level in a dual mode; it acts as repressor for a ROS-generating system, e.g., an NADPH oxidase gene (named here *cpnoxA*) and as a positive regulator for the catalase genes. In *A. nidulans*, it has been shown recently that the HOG homologue SakA negatively controls the NADPH oxidase gene *noxA* (Kawasaki et al. 2002; Lara-Ortiz et al. In press). Since control of CPTF1 by a *C. purpurea* HOG homologue is likely, these data support our hypothesis. Since inhibition of SOD activity and deletion of *cpsod1* seem to reduce H_2O_2 secretion in axenic culture (data not shown), we postulate that the cell wall–associated CPSODA mediates the formation of H_2O_2 by dismutation of the O_2^- formed by CPNOXA. The whole system could be designed to keep the H_2O_2 level (or generally the ROS level) in planta low, since a significant level of ROS could induce the plant response (Pellinen et al. 2002; Langebartels et al. 2002; Wohlgenuth et al. 2002), causing an oxidative burst-like reaction, which is indeed observed in $\Delta cptf1$ -infected rye tissue. It is now generally accepted that the oxidative burst is especially effective against biotrophic pathogens, whereas its role in ne-

Fig. 9. Model for the role of reactive oxygen species (ROS) in the *Claviceps purpurea*-rye interaction. Schematic drawing of a hyphal tip of **A**, *C. purpurea* wild type and **B**, a $\Delta cptf1$ mutant growing intercellularly in rye ovarian tissue. Both strains secrete H_2O_2 , probably produced mainly from internal superoxide by a cell wall associated Cu,Zn superoxide dismutase (SOD). The superoxide is generated by an NADPH oxidase (nox), which might be controlled by CPTF1. In the wild type, CPTF1 controls catalase activity, ensuring a “shield” of catalases around the hyphae, which decomposes any (fungal or plant) H_2O_2 . In the $\Delta cptf1$ mutant, the catalase genes are not induced (and the *nox* gene might be derepressed), leading to a high H_2O_2 level around the hyphae. This increased ROS level might induce plant defense reactions, e.g., an “oxidative burst.”

crotrophic systems is not so clear (Hückelhoven and Kogel 2003). Thus it appears that the biotroph *C. purpurea* has developed a strategy for its successful colonization of living host tissue (reduction of ROS level) that is completely different from that of a necrotrophic pathogen like *Botrytis cinerea*. That broad host range pathogen increases ROS levels in the interaction significantly, thereby causing hypersensitive response reactions and death of host cells, which it can then easily colonize (Govrin and Levine 2000; Prins et al. 2000; Schouten et al. 2002; Tenberge et al. 2002). The question remains why the fungus produces O_2^- or H_2O_2 at all, i.e., which is the function of the putative NADPH oxidase. Since the interesting work of J. Aguirre and W. Hansberg and their collaborators has presented evidence for a role of ROS in fungal vegetative and sexual development (Hansberg and Aguirre 1990; Kawasaki et al. 2002; Peraza and Handsberg 2002; Lara-Ortiz et al. In press), fungal development in general could depend on the ROS status. The role of NADPH oxidases in the development of higher eukaryotes is well-established (Arnold et al. 2001; Irani et al. 1997; Suh et al. 1999). Thus, the role of CPTF1 may go beyond scavenging deleterious ROS during the fungus-plant interaction and involve basic development processes.

To test this model, we have initiated a functional analysis of one or more NADPH oxidases in *C. purpurea*, using a PCR approach based on fungal *nox* sequences. We have so far identified two putative *cpnox* genes, corresponding to the fungal *noxA* and *noxB* subfamilies defined by Lara-Ortiz and associates (In press) (S. Giesbert, P. Tudzynski, unpublished data).

In addition, we have initiated an expression profile approach based on an array of in planta expressed genes of *C. purpurea* (Oeser et al. 2002b), in order to identify additional CPTF1-controlled genes and to allow a more detailed analysis of the impact of ROS control systems on virulence in this system.

It should be noted that, until now, only a few transcription factors are known to be of importance for the virulence of phytopathogenic fungi. To our knowledge, there are only *prf1* in *U. maydis* (Hartmann et al. 1996), which is an HMG domain factor involved in mating and pathogenicity, and *toxE* in *Cochliobolus carbonum*, which is a novel factor containing a basic region and ankyrin repeats while lacking a leucine zipper domain and which influences pathogenicity by regulating biosynthesis of the toxin (Ahn and Walton 1998). A comparable factor recently has been identified in the tomato pathogen *Cladosporium fulvum* (Bussink et al. 2001), so far without known function. In the chestnut blight fungus *Cryphonectria parasitica*, so far the only "normal" bZIP factor with impact on virulence has been described; it is involved in general amino acid control (Wang et al. 1998).

Thus, to our knowledge, CPTF1 represents the first stress-responsive fungal bZIP factor with impact on virulence; more important, it is the first fungal transcription factor shown to be involved in modulation of the host's defense reactions. It is interesting to note that during the EST analysis that led to the identification of *cptf1*, a second putative bZIP factor, *cptf2*, was found. Functional analysis of this factor is under way (Oeser et al. 2002b).

MATERIALS AND METHODS

Strains and culture conditions.

The wild-type *C. purpurea* strain used in these experiments was 20.1, a putative haploid derivative of standard field isolate T5 (Fr.:Fr. Tul., isolated from rye (*Secale cereale* L.; Hohenheim, Germany) and obtained by haploidization-inducing benomyl treatment (Hüsken et al. 1999). For conidia harvest-

ing and DNA isolation, mycelia were cultivated on Mantle agar (16 g of agar per liter) with 100 g of sucrose per liter (Mantle and Nisbet 1976) at 28°C for 12 to 14 days.

Escherichia coli TOP10F' (Invitrogen, San Diego, CA, U.S.A.) was used for all the subcloning experiments. *E. coli* LE392 and XL1Blue (Stratagene, La Jolla, CA, U.S.A.) were employed for propagation of *C. purpurea* genomic and cDNA lambda clones, respectively.

Protein analysis.

Extraction of proteins and native isoelectric focusing (IEF) were performed as described by Moore and associates (2002); the method of Beauchamp and Fridovich (1971) was used for SOD activity staining, and catalase activity staining was done according to Clare and associates (1984).

Nucleic acid extraction and analysis.

Standard recombinant DNA methods were performed according to Ausubel and associates (2001). Fungal genomic DNA was prepared from lyophilized mycelium, as described by Cenis (1992). Isolation of total RNA for mRNA isolation, Northern analysis, and RT-PCR was done using the RNeasy total RNA isolation system from Promega (Madison, WI, U.S.A.). Heterologous and homologous Southern hybridization experiments were done as previously described (Garre et al. 1998). Northern blot hybridization experiments were done according to Church and Gilbert (1984). ^{32}P -labeled probes were prepared by random oligo priming (Ausubel et al. 2001). The PCR was done as described by Saiki and associates (1988), using the red Taq polymerase (Sigma, St. Louis); products were cloned using the PCR 2.1 TOPO-cloning kit from Invitrogen. DNA sequencing was carried out as described by Moore and associates (2002). Protein and DNA sequence alignment, editing, and construction of phylogenetic trees was done with the help of DNA Star (Madison, WI, U.S.A.) and CLUSTALW (Higgins et al. 1994). Homology searches were done using Blast at NCBI (Altschul et al. 1990); prediction of protein domains was performed using PROSCAN (Combet et al. 2000); putative MAPK phosphorylation sites were screened directly (Clark-Lewis et al. 1991; Müller et al. 1999).

Expression studies.

For Northern analysis of *cpca1* expression in axenic culture, mycelia were grown for 5 days in Mantle medium (discussed above); H_2O_2 (0.5 mM final concentration) was added as indicated for 1 h. Mycelia were harvested, and RNA was purified (discussed above). For expression analysis of *cptf1* during the parasitic cycle of ergot, total RNA from rye ovaries infected for 5, 10, 15, and 20 days with *C. purpurea* wild-type strain and from noninfected ovaries was prepared according to Chambers and Russo (1986). RT-PCR was carried out as described by Mey and associates (2002a), using primers RTP1 and RTP2 (Fig. 4). PCR-amplified products of cDNA were cloned in the PCR 2.1 cloning vector and were sequenced.

Construction of the Δ *cptf1* replacement vector.

To inactivate *cptf1*, the gene was disrupted by replacement of a 70-bp internal fragment with the phleomycin resistance cassette (Mattern et al. 1988). The replacement vector was constructed by cloning 5' and 3' fragments of *cptf1* upstream and downstream, respectively, from the phleomycin resistance cassette. A 5' fragment was amplified using primer FP1, containing a *DraI* adapter sequence at the 5' end and primer RP1 (Fig. 4). A 3' fragment was similarly amplified using primers FP2 and RP2, both containing an *XbaI* adapter sequence. PCR amplified fragments were cloned in the vector PCR 2.1. The 3' fragment was cloned into the *XbaI* site in pAN8.1, and subse-

quently, the 5' fragment was cloned in the *Eco*RI site. A clone having both 5' and 3' fragments correctly oriented with respect to each other was selected as replacement vector pAcp^{tf}1. A 5.3-kb replacement fragment was excised from pAcp^{tf}1 with *Dra*I and *Nde*I and was purified by agarose gel electrophoresis. The fragment was isolated using Biotrap membrane (Schleicher and Schuell, Keene, NH, U.S.A.) and was used for transformation.

Transformation and analysis of fungal transformants.

Transformation of *C. purpurea* was performed according to Smit and Tudzynski (1992) with the following modification: recipient strain 20.1 was grown for three days on cellophane sheets on Mantle agar (instead of liquid culture). Protoplasts were generated in 20 ml of lytic solution containing 20 mg of Driselase (Sigma) and 50 mg of Trichoderma lytic enzyme (Sigma) and were transformed with 15 µg of replacement fragment.

For the selection of phleomycin resistant transformants, transformed protoplasts were mixed with modified BII agar medium (Esser and Tudzynski 1978; BII/8, pH 8, lacking Fe and containing 20% sucrose and 12 g of agar per liter) containing 33 µg of phleomycin per ml (Cayla, Toulouse, France) and were distributed in five plates. The protoplasts were allowed to regenerate at 28°C, and after 3 to 5 days, phleomycin-resistant colonies were transferred to BII/8 plates containing 100 µg of phleomycin per ml and were subjected to at least one round of single spore isolation to obtain homokaryotic transformants.

The phleomycin-resistant transformants carrying a homologous integration of the replacement construct were identified by PCR, using primers HOM1 and PAN-Pro or PAN-T and HOM2 (Fig. 4) and 10 to 100 ng, respectively, of genomic DNA. Amplified PCR fragments were cloned in vector PCR 2.1 and were sequenced. The presence of the wild-type gene copy in the *Acp^{tf}1* mutants was checked using primers HOM1 and HOM2, which give rise to a 2,100-bp fragment with the wild-type strain.

C. purpurea pathogenicity assays.

Rye plants were cultivated in growth chambers as described by Smit and Tudzynski (1992). Florets of blooming ears (60 to 80 florets per ear; altogether 40 ears in two independent experiments) were inoculated with conidial suspensions as described by Tenberge and associates (1996). In order to avoid cross contamination, the ears were covered with paper bags equipped with cellophane windows immediately after inoculation.

Cytological methods.

At the electron microscopic level, H₂O₂ was in situ-localized by the cerium chloride technique according to Briggs and associates (1975), Thomas and Trelease (1981) and Bestwick and associates (1997). The H₂O₂ reacts directly with cerium chloride to form an electron-dense and stable precipitate of cerium perhydroxide. As described above, inoculated ovaries were cut in half longitudinally and were incubated in a freshly prepared solution of 5 mM CeCl₃ in 50 mM Tris-maleate buffer, pH 7.5, for 1.5 h. As control treatment, the cerium treatment was omitted. The samples were washed in 25 mM sodium phosphate buffer (NaPB), at first, at pH 6 for 30 min and, then, at pH 7 for 10 to 15 min. Thereafter, the samples were fixed in 3% (vol/vol) glutaraldehyde in NaPB, pH 7, for 4 h, were postfixed with 2% OsO₄ in NaPB, and were further processed via ethanol dehydration into epoxy resin, as described in detail by Tenberge and associates (1999). Ultrathin sections were cut with diamond knives and were examined

with and without conventional contrasting, using a Zeiss EM 900 electron microscope at 80 kV. Hundreds of sections prepared from four different blocks of the mutant at different timepoints early during infection were tested in comparison with the wild-type-induced pathogenesis.

PCR and RT-PCR primers.

(Adapter sequences are underlined.)

RTP1: 5' GAA GAT GAC GGA TGA AGA GAA ACG 3'
 RTP2: 5' GTT GGG TAA CCG GAC AAT CTT TG 3'
 FP1: 5' TTT AAA CTC AGT CTG GAG CCA AAT C 3'
 RP1: 5' CTC GAA ACA GCT TTG CCA C3'
 FP2: 5' TCT AGA GGT AAG AAG ACT GCA GCT ATC 3'
 RP2: 5' TCT AGA CGT CGT TCC ACG ATA CCG 3'
 HOM1: 5' CCT CGA CCA CAT CAA CAA C 3'
 PAN-Pro: 5' TGC TAC CTC CAA TTC CTC ATC 3'
 HOM2: 5' CCC ACG ATA AAC CCT TGT CC 3'
 PAN-T: 5' CCC AGA ATG CAC AGG TAC AC 3'

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