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Aspergillus nidulans Catalase-Peroxidase Gene (*cpeA*) Is Transcriptionally Induced during Sexual Development through the Transcription Factor StuA

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Catalases, peroxidases, and catalase-peroxidases are important enzymes to cope with reactive oxygen species in pro- and eukaryotic cells. In the filamentous fungus *Aspergillus nidulans* three monofunctional catalases have been described, and a fourth catalase activity was observed in native polyacrylamide gels. The latter activity is probably due to the bifunctional enzyme catalase-peroxidase, which we characterized here. The gene, named *cpeA*, encodes an 81-kDa polypeptide with a conserved motif for heme coordination. The enzyme comprises of two similar domains, suggesting gene duplication and fusion during evolution. The first 439 amino acids share 22% identical residues with the C terminus. Homologous proteins are found in several prokaryotes, such as *Escherichia coli* and *Mycobacterium tuberculosis* (both with 61% identity). In fungi the enzyme has been noted in *Penicillium simplicissimum*, *Septoria tritici*, and *Neurospora crassa* (69% identical amino acids) but is absent from *Saccharomyces cerevisiae.* Expression analysis in *A. nidulans* revealed that the gene is transcriptionally induced upon carbon starvation and during sexual development, but starvation is not sufficient to reach high levels of the transcript during development. Besides transcriptional activation, we present evidence for post-transcriptional regulation. A green fluorescent protein fusion protein localized to the cytoplasm of Hülle cells. The Hülle cell-specific expression was dependent on the developmental regulator StuA, suggesting an activating function of this helix-loop-helix transcription factor.

Oxidative stress and the occurrence of reactive oxygen species is common to aerobically living organisms and might be deleterious for living cells (10, 18). Reactive oxygen species are generated during normal cell metabolism and comprise superoxide, hydroxyl radicals, hydrogen peroxide, and singlet oxygen. All aerobically living organisms employ one or several systems to cope with these toxic substances. Catalases and peroxidases are most commonly used to transform the harmful oxygen compound H_2O_2 into harmless products. Catalases are heme-containing enzymes, which convert H_2O_2 into oxygen and water. Peroxidases are heme-containing enzymes as well and inactivate H_2O_2 by reducing it to water. In addition to heme-containing catalases and peroxidases, nonheme varieties of these enzymes exist. Different cellular substrates can serve as electron donors for this reaction. Frequently, organisms use different isozymes, which are expressed simultaneously or under developmental-stage- and environment-specific conditions (26, 35).

One good example for the employment of several catalases and their differential regulation during the life cycle is the filamentous fungus *Aspergillus nidulans* (15). *A. nidulans* is able to grow as vegetative hyphae but then undergoes two developmental programs. After 20 h of vegetative growth it can enter an asexual reproductive pathway in which it generates thousands of single-cell, haploid conidiospores. In addition, it is able to reproduce itself with very durable sexually derived ascospores (1). Both spore types are produced at or in special morphological structures, called conidiophores or cleistothecia, respectively. The conidiophore consists of four different cell types—a stalk, metulae, phialides, and conidia—and grows away from the agar surface into the air. The asexual developmental pathway is very well characterized at the molecular level (1) and is triggered by a central cascade of transcriptional activators (3, 22). In an effort to characterize differentially expressed genes during asexual development, a catalase gene (*catA*) was discovered (20) that is transcriptionally and post-transcriptionally regulated, and the protein accumulated in conidiospores (19). Using the *catA* sequence, a second catalase, *catB*, was isolated (16). This gene is developmentally induced during conidiophore formation, but the transcript is almost absent in conidiospores. The *catB* expression, like that of *catA*, also responds to different stress conditions (6, 16). Finally, Kawasaki and Aguirre, taking advantage of the genomic sequencing project at Cereon Genomics LLC (Cambridge, Mass.), identified a third catalase gene, designated *catC* (15). The protein resides in peroxisomes and is constitutively expressed. Interestingly, mutations in a single gene or in all catalase genes did not have any detectable vegetative or developmental phenotype, suggesting the presence of more isozymes. Indeed, in native polyacryl amide gels a fourth catalase activity, *catD*, was observed (15). We have identified the corresponding gene and found that it is a catalase-peroxidase. The expression of the gene is transcriptionally and translationally regulated upon carbon starvation and during sexual development. One important regulator is the transcription factor StuA.

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                                                                                          K  G  L  T  N  G  L  E  A  A  R  S  T  A  A  K  V  A
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 S  G                    Intron                                   S  L  G  T  V  V  D  I  L  E  E  K  D  V  W  E  T  A  K
GCTGACGCTGCCGAGAAGCGCGTTGACAAGCTCCTTCTCCTCCGCAGTCACGTCCGCGCTGGCGAACAGGCCCGATGCACCCTCTCCGACAAGCGCAGGTGTGTTCGGCATGACGCGAAC 1080   pcrA
                                                                                                              prom2
 S  V  S  G  L  L  A  N  V  L  E  K  E  E  A  T  V  D  A  S  A  F  L  G  S  A  G  E  G  V  L  A  P  T  N  P  M  V  R  V
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                                 prom1
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                                                                                                 M  G  S  N  E  C  P  Y  S  R  Q  N  A
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 I  A  G  G  H  T  F  G  K  T  H  G  A  G  P  A  T  H  L  G  K  E  P  H  G  A  G  I  E  L  Q  G  L  G  W  E  S  G  F  E
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MATERIALS AND METHODS

***A. nidulans* strains and growth conditions.** Supplemented minimal and complete media for *A. nidulans* were prepared as described, and standard strain construction procedures were applied (14). Standard laboratory *Escherichia coli* strains (XL1-Blue and Top 10F′) were used. *A. nidulans* strains included FGSC4 (wild-type strain, ve$^+$; Fungal Genetics Stock Center), GR5 (*pyrG89 wA3 pyroA4 veA1*), RMSO11 (*pabaA1 yA2 ΔargB::trpCΔB veA1*), SMS1 (*pabaA1 yA2 ΔargB::trpCΔB veA1*; in addition a nondefined mutation in a gene that induces precocious sexual development) (24), TPM1 [*biA1 methG1 veA1 alcA(p)::abaA*], TTA292 [*biA1 methG1 veA1 alcA(p)::brlA*], UI132 [*yA2 wA3 argB2 ivoA1 sc12 methH2 galA1 alcA(p)::stuA integrated in argB locus*] (strains TPM1, TTA292, and UI132 were kindly provided by B. Miller, Moscow, Idaho), WIM126 (*pabaA1 yA2 ve$^+$*), SRF200 (*pyrG89 ΔargB::trpCΔB pyroA4 veA1*), SMS8 (a cross between strains WIM126 and SRF200; *pabaA1 yA2 ΔargB::trpCΔB pyroA4 ve$^+$*), SMS11 (SMS8 transformed with pMS42; integrated into the *cpeA* locus), SMS14 (SMS8 transformed with pMS41), SMS16 (SMS8 transformed with pMS42), GO256 (*biA1 stuA1 veA1*), and SMH1 and SMH4 (RMSO11 transformed with pHPR12; *pabaA1 yA2 ΔargB::trpCΔB ′3b ΔcpeA::argB veA1*).

Molecular biological methods, plasmids, and strain construction. Plasmid preparations and Southern and Northern blot analyses were performed according to standard protocols (23; see also the manuals of the suppliers of membranes). RNA, DNA, and protein isolation was described previously (25). Standard protocols were used to generate transgenic fungal strains (31). The plasmids used included pBluescript KS(−) (Stratagene, Heidelberg, Germany), pUC18 (MBI Fermentas, St. Leon-Rot, Germany), PCR2.1-TOPO (Invitrogen, Leek, The Netherlands), pMS49 (*cpeA* cosmid with *trpC* gene), pMS16 [a 3-kb *Pst*I subclone from cosmid pMS49 in pBluescript KS(−), with the *argB* gene as a *Not*I restriction fragment cloned into the *Not*I site], pMS47 (a 2.5-kb *Bam*HI subclone from cosmid pMS49 in pUC18), pMS46 [5.5-kb *Eco*RV subclone from cosmid pMS49 in pBluescript KS(−)], pHPR12 (cloning was done according to the scheme in Fig. 2), pMS18 (*gfp* as a *Cla*I restriction fragment cloned into *Cla*I site of pMS16), pMS41 (pMS18 with a 1.0-kb upstream region of ATG of *cpeA* and including the stunted response element [StRE]), and pMS42 (pMS18 with a 1-kb upstream region of ATG of *CpeA* but excluding the StRE). Primer extension was performed according to a protocol of MWG (Ebersberg, Germany; http://bio.locor.com/app_513/App513.htm) with an IRD 800 labeled primer (see Fig. 1), and 50 μg of total RNA as a template.

The oligonucleotides used were prom1 (5′-CAGATGCGTGGTCCTGC-3′), prom2 (5′-GTGTGTTCGGCATGACGC-3′), percDNA1 (5′-AGTTGAAGAC ACAAATGG-3′), and percDNA2 (5′-GTGACAGGTCAGTTGTCAGC-3′).

Protein purification, enzyme assays, and Western blot. Protein extracts were obtained by grinding mycelium in liquid nitrogen (24). For the induction of the sexual cycle, *A. nidulans* strains were inoculated onto cellophane membranes on complete medium agar plates. Development was monitored microscopically. Catalase-peroxidase was purified as described above, and Western blots were performed according to the manufacturer's protocols (24). The anti-green fluorescent protein (GFP) antibody was purchased from ABR (Golden, Colo.). Native polyacrylamide gels (Bio-Rad) were used for the activity staining of catalase and peroxidase as described previously (34).

Microscopy. To observe GFP-labeled samples, we used a Zeiss Axiophot microscope and a SeaScan camera system (INTAS, Göttingen, Germany) as described previously (28).

RESULTS

Cloning of the catalase-peroxidase gene, *cpeA*. In an attempt to isolate a gene that is induced during sexual development, we purified laccase II. This enzyme was described as being specifically expressed in sexual structures such as Hülle cells and primordia (13, 24). The protein was purified to apparent homogeneity, and three internal peptide sequences were determined, one of which showed sequence similarity to prokaryotic catalase-peroxidases. The internal peptide sequences were used to screen the partial genomic database at Cereon Genomics LLC, where we identified a DNA sequence (579 bp) whose translation product matched the peptides (Fig. 1). The DNA sequence information was used to isolate a corresponding cosmid from the pKBY library (FGSC). After subcloning of the *cpeA* gene as 3-kb *Pst*I, 2.5-kb *Bam*HI, and 5.5-kb *Eco*RV restriction fragmenta, we sequenced the locus on both strands (Fig. 1). cDNA was generated by reverse transcription-PCR with total RNA as a template for the reverse transcriptase reaction. Sequence comparison revealed no intron in the coding region. The sequence was translated into a 739-amino-acid (aa) polypeptide with a predicted molecular mass of 81.2 kDa. The polypeptide displayed, like one of the other peptides, a high degree of similarity to catalase-peroxidases of prokaryotic origin, with amino acid identities ranging from 54% to one of the archaebacterium, *Halobacterium salinarum*, to 64% of *Streptomyces reticuli* CpeB. CpeA is more distantly related to yeast cytochrome *c* peroxidase (Ccp1) and plant L-ascorbate peroxidases. The *A. nidulans* and *Neurospora crassa* (accession no. AF459787) sequences are the first two examples of complete sequences of catalase-peroxidases of eukaryotic origin (21). The two sequences share 69.3% identical amino acids. Alignment of CpeA (739 aa) with *Saccharomyces cerevisiae* Ccp1 (361 aa) showed that CpeA is similar to Ccp1 in the first and second halves of the protein (data not shown). This suggests a gene duplication event during evolution as suggested for the prokaryotic enzymes (32). Direct comparison of the first 439 aa of CpeA with the C terminus revealed 22% identical amino acids. If conserved amino acid changes were taken into consideration, the similarity went up to 40%. These values are comparable to 21% identical amino acids in the alignment of the two halves of the *E. coli* enzyme HPI.

Residues that have been shown to be essential for the catalytic activity of bacterial catalase-peroxidases and cytochrome *c* peroxidase are conserved in the *A. nidulans* enzyme. An amino acid triad, Arg-Trp-His, at the distal active site matches the positions R91, W94, and H95 in CpeA. A His proximal heme ligand aligns to H258, and a Trp residue shown to be important for peroxidase activity of Ccp1 is conserved in W309.

Upstream of the *cpeA* open reading frame we found a

FIG. 1. Sequence of the catalase-peroxidase (*cpeA*) and the pyrroline-5-carboxylate reductase (*pcrA*) locus. (A) A nucleotide sequence of the depicted area was obtained from both strands by using a 3-kb *Pst*I, a 2.5-kb *Bam*HI, and a 5.5-kb *Eco*RV restriction fragment as a template. The derived amino acid sequences, obtained after removal of one intron in the *pcrA* gene, are denoted below the DNA sequence in the single-letter code. Since the two genes are encoded on opposite strands of the DNA, we indicated the direction of translation at the start codons with arrows. The intron border sequences of *pcrA* are indicated. The amino acids of CpeA involved in heme coordination are boxed. The peptide sequences obtained previously from purified CpeA protein are shaded. The sequence used for primer extension is underlined (dotted line). The putative binding sites for the regulators StuA (StRE) and CreA are indicated in the *cpeA* promoter region by dark and light gray boxes, respectively. The striped boxes indicate the location of four putative AreA binding sites. The determined transcription start site is labeled with an asterisk above the sequence. The two primers (prom2 and prom1) used for the amplification of the promoter with or without the StuA binding site are indicated by a line above the DNA sequence. The displayed sequence of the *cpeA* and the *pcrA* genes are available under the accession numbers AJ305225 and AJ313094.

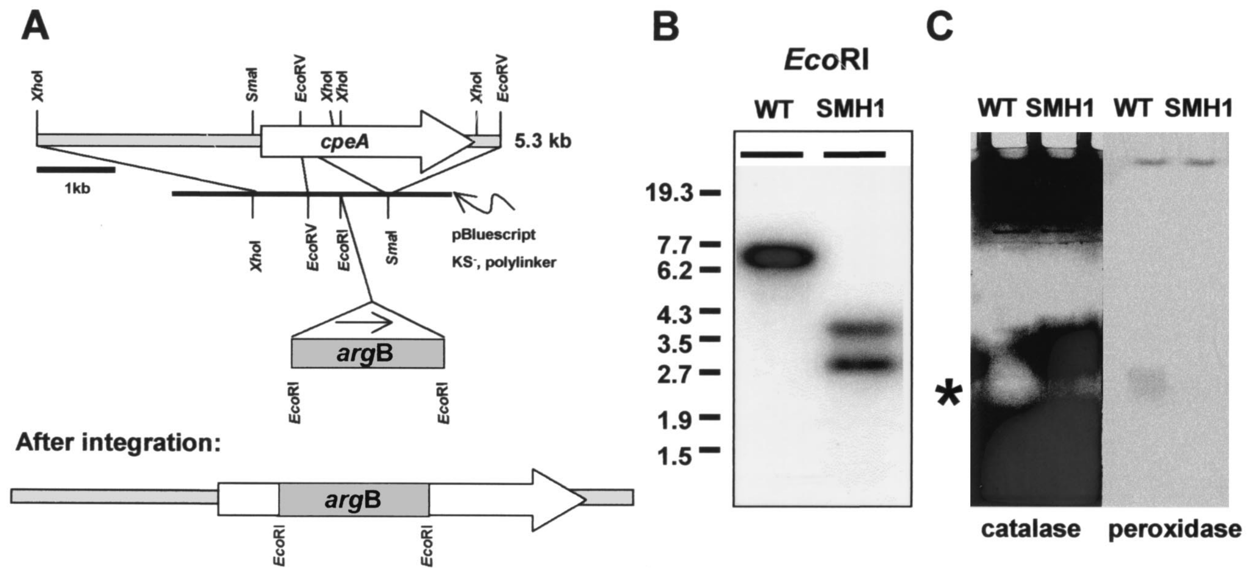


FIG. 2. Disruption of the *cpeA* gene. (A) Scheme of the construct used for homologous integration. A 5′ *Xho*I-*Eco*RV fragment and a 3′ *Eco*RV-*Eco*RV fragment were cloned into pBluescript, and the *argB* marker was inserted as an *Eco*RI restriction fragment in between them. (B) Southern blot analysis to confirm the integration event. Genomic DNA of a wild type (WT) and a disruptant strain (SMH1) was digested with *Eco*RI and hybridized to the 5.3-kb *Xho*I-*Eco*RV fragment depicted in panel A. (C) Activity stain of peroxidase and catalase in a native 10% polyacrylamide gel. The wild type and the disruptant strain (SMH1) were induced for sexual development, and mycelia were harvested after 72 h of growth. The total protein (50 μg) was separated in a native polyacrylamide gel, and activities were determined by staining as described earlier (34). The asterisk indicates the catalase activity not present in the diruptant strain. The other catalase activities visible above this band are found in both strains and indicate equal loading of protein.

polypeptide encoded on the other strand (Fig. 1). After the removal of one predicted intron from the sequence, we deduced a protein of 284 aa with a calculated molecular mass of 29.2 kDa. The protein displays high homology (45.7% identical amino acids) to pyrroline-5-carboxylate-reductase from *Pseudomonas aeruginosa* (accession no. AE004476) and thus was named *pcrA*. This enzyme is involved in proline biosynthesis.

Disruption of *cpeA*. Although CpeA was purified by using the laccase enzyme assay, the isolated gene encoded a catalase-peroxidase rather than a laccase. In order to answer the question whether the purified protein was a trifunctional enzyme or whether the two enzymes were copurified, we disrupted the *cpeA* open reading frame by using the nutritional marker gene *argB*. We transformed the wild-type strain RMSO11 with the linearized construct (pHPR12) and obtained three strains with the homologous integration event as shown in Fig. 2. Two strains (SMH1 and SMH4) were used for further analysis. To our surprise, protein extracts of the two disruptant strains still had laccase activity. To further characterize the strains, we analyzed catalase and peroxidase activities by using native polyacrylamide gel electrophoresis and subsequent activity stainings. We were able to detect one band in the peroxidase and the catalase assay, which were absent in the disruptant strain (Fig. 2). The other catalases—CatA, CatB, and CatC—were not resolved properly because of overloading of the gel. The catalase band due to CpeA had the highest mobility in the gel, a finding that is in agreement with the properties of CatD described before (15). These results strongly suggest that catalase-peroxidase copurified with laccase II. Interestingly, in *N. crassa* the purified fraction of catalase-peroxidase also contained laccase activity (W. Hansberg, unpublished data). This suggests that the purification protocol used in both filamentous fungi was not sufficient to separate the two proteins.

To analyze the biological function of the bifunctional enzyme, we compared a wild-type strain with SMH1 and SMH4 and found no significant differences with respect to hyphal growth, asexual spore formation, sexual spore production, or viability of the spores. However, we found an interesting regulation of gene and protein expression.

CpeA is expressed during early sexual development and upon carbon starvation. After the determination and analysis of the primary structure of the CpeA protein, we wanted to analyze the temporal expression pattern of the gene. We inoculated strain FGSCA4 onto agar plates, incubated the plates at 37°C for several days, and harvested the cultures after defined time periods. RNA was extracted and subjected to a Northern blot analysis (Fig. 3). Although no transcript was detectable in hyphae (vegetative), the signal increased when cells entered the sexual cycle (after 40 h).

Because catalases and peroxidases are required to cope with reactive oxygen species, we tested whether other growth conditions, in addition to sexual development, would induce transcription of *cpeA*. Mycelia were grown in liquid culture for 20 h at 37°C and then either exposed to an agar surface to induce development, further grown in submerged culture with or without glucose or nitrogen, grown in submerged culture with alternative carbon sources, or grown under oxidative stress conditions, under osmotic stress, or at high temperature (Fig. 3). After 3 h of growth under these conditions the mycelia were

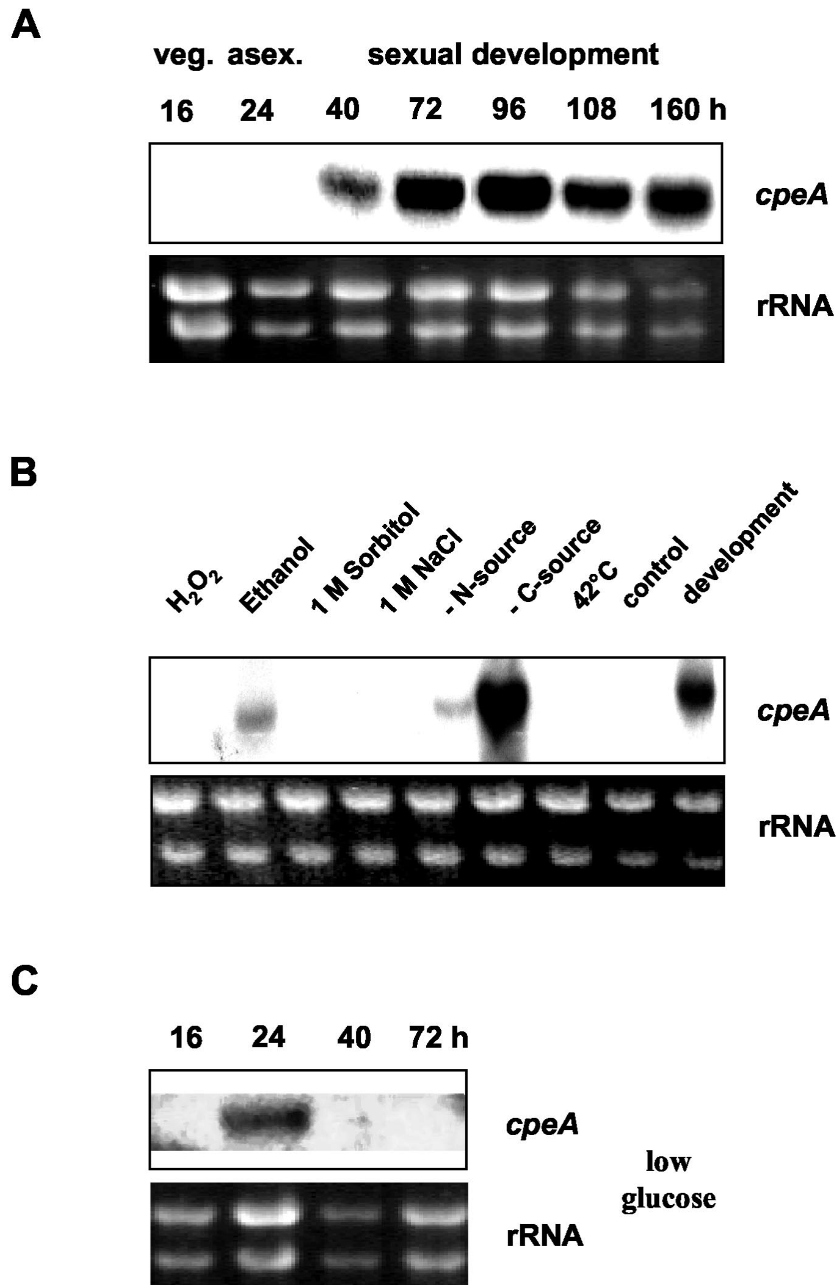


FIG. 3. Transcriptional regulation of *cpeA*. (A) Northern blot analysis of RNA isolated at different time points during the life cycle of *A. nidulans.* A total of 10^5 conidiospores were inoculated onto membranes and exposed onto a minimal medium agar plate. The plates were incubated at 37°C, and mycelia were harvested after 16 h (veg.), 24 h when asexual development was completed (asex.), and during sexual development at the time points indicated. RNA was isolated from the mycelia and hybridized to a *cpeA*-specific probe (upper panel). Then, 2 μg of the same RNA preparation was loaded onto a gel and stained with ethidium bromide as loading controls for the gel. (B) Northern blot analysis of RNA isolated from mycelia after different treatments. Conidia of FGSCA4 were inoculated in complete liquid medium and incubated at 37°C for 20 h. The mycelium was harvested and divided into nine equal portions. One fraction was resuspended in minimal medium and incubated for 3 h before RNA isolation (lane "control"). One fraction was induced for development (lane "development") and then used for RNA isolation. The other seven samples were resuspended in minimal medium containing 0.5 mM H_2O_2–4% ethanol instead of 2% glucose–100 mM glucose plus 1 M sorbitol or 1 M NaCl into medium lacking any nitrogen or carbon source. Heat shock treatment was performed at 42°C. The treatments are indicated above the lanes. RNA was processed as described in panel A. (C) Northern blot analysis of RNA from mycelia induced for development and grown at a low glucose concentration (0.8%) in which no sexual development occurred. The time points of harvesting are indicated above the lanes.

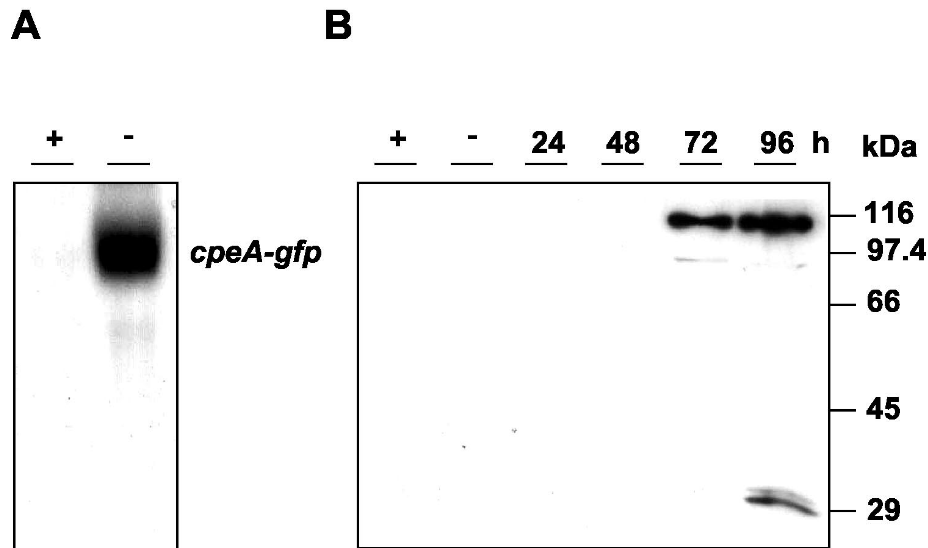


FIG. 4. Posttranscriptional regulation of CpeA. To test for CpeA-GFP expression upon carbon starvation and during development, mycelia of SMS14 (CpeA-GFP) was grown for 20 h at 37°C and then shifted for 5 h to medium containing glucose (+) or without glucose (−) or induced for development. (A) RNA accumulation upon starvation was analyzed with a *gfp*-specific probe. (B) To detect the fusion protein in a Western blot, 40 μg of protein was analyzed with the anti-GFP antibody. Under starvation conditions, no protein band is visible (left two lanes). During development, the protein appears after 72 h, and the small form is detectable after 96 h.

harvested, and the *cpeA* transcript levels were determined by Northern blot analysis. Besides the developmental induction after 40 h, we observed a strong signal when the mycelia were starved for carbon and a less-intense signal when the mycelia were starved for nitrogen. The repression of the gene under high-glucose conditions could be mediated through the carbon catabolite repressor CreA, for which we found several putative binding sites in the promoter region (Fig. 1). Induction upon nitrogen limitation could be achieved by the transcription factor AreA (Fig. 1). A slight induction of the expression of CpeA was also observed when ethanol was used as sole carbon source. A similar expression pattern was found for CatD (15).

To determine whether transcriptional activation during sexual development is due to carbon starvation of the mycelium, we grew *A. nidulans* in the presence of 0.8% glucose instead of 2% glucose. Under these conditions vegetative and asexual development occurs normally, but sexual development is not initiated (33). The *cpeA* transcript only accumulated transiently to a low level but did not reach the transcript levels observed during sexual development (Fig. 3).

Posttranscriptional regulation. After the analysis of gene expression at the transcriptional level we wanted to study the timing of protein appearance. To detect the polypeptide, we constructed a translational fusion with the GFP (SMS14) and analyzed the fusion protein in Western blot analyses with anti-GFP antibodies (Fig. 4). No specific band was detected in protein extracts obtained from starved hyphae, although *cpeA* transcript was induced to a high level. The results were confirmed when we microscopically inspected the hyphae after 3 and 6 h. Green fluorescence of the hyphae only appeared after 24 h of starvation (results not shown). This suggests a long delay between transcription and translation. The mechanism for this posttranscriptional control remains to be elucidated.

In analyzing the CpeA-GFP fusion protein, we observed that the protein is C terminally cleaved (Fig. 4). We observed, in addition to the protein band of ca. 100 kDa, a band of ca. 30 kDa. Since the CpeA protein was tagged at the C terminus, the small band must represent a small portion from the C terminus. The N-terminal part is not detectable in this strain. The small form was especially enriched in older cultures induced for sexual development. When Hülle cells were 1 day old (72 h of growth), only the high-molecular-mass band (100 kDa) appeared, whereas when the enzyme was detected in extracts from 2-day-old Hülle cells the smaller (30-kDa) product was observed. Whether this phenomenon can be explained by partial proteolysis or whether it is of functional importance cannot be decided yet. Interestingly, a processing of catalase-peroxidase was reported recently in *Streptomyces reticuli*, where the C-terminal part of the protein is necessary for manganese peroxidase activity (34).

CpeA is a cytoplasmic enzyme and localizes in Hülle cells. To study the spatial distribution of the enzyme, we fused sGFP translationally to the carboxyl terminus of CpeA and expressed the fusion construct under the control of the natural *cpeA* promoter. We transformed pMS42 into SMS8 and performed Southern blot analysis to analyze the integration pattern of the construct. In two of the transformants the construct was integrated homologously at the *cpeA* locus, resulting in a gene duplication with the transgenic copy under the control of the natural promoter (results not shown). Since this situation represents the natural regulation status and should not cause misscheduled expression because of genomic integration someplace in the genome, we selected one of these strains (SMS11) for further analysis. Microscopic inspection under fluorescence conditions revealed that Hülle cells were brightly stained (Fig. 5), whereas primordia were weakly stained and

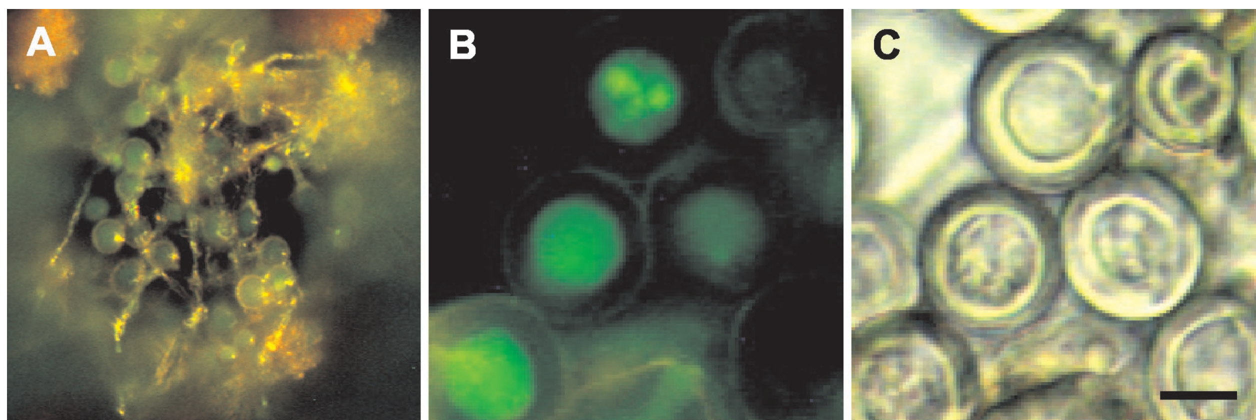


FIG. 5. Localization of CpeA-GFP during the course of sexual development. Strain SMS11 (CpeA-GFP) was induced for development and mature cleistothecia (A) with attached Hülle cells, and individual Hülle cells were analyzed under fluorescence conditions (B) or by phase-contrast microscopy (C). The scale bar represents 40 μm in panel A and 10 μm in panels B and C.

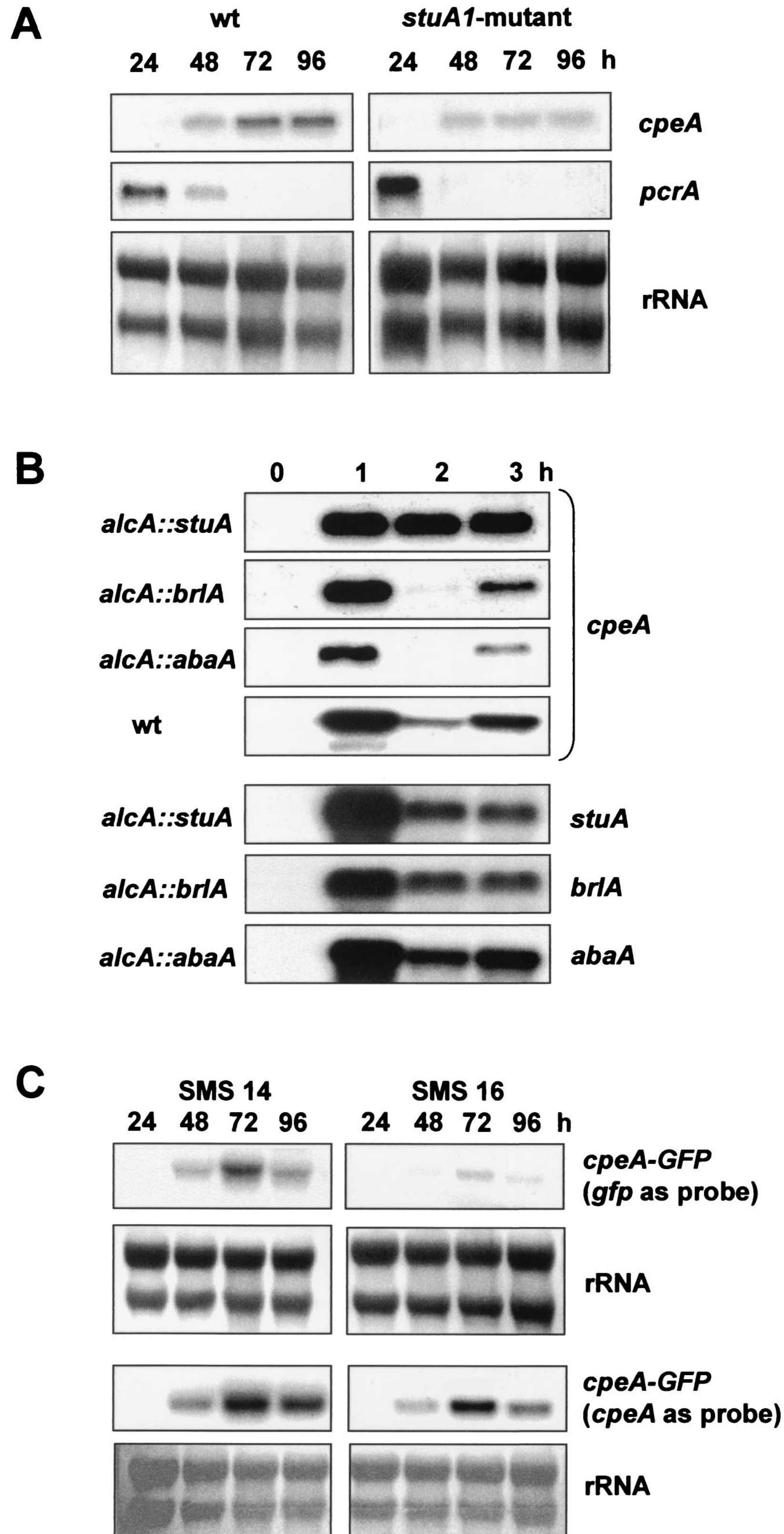
hyphae and conidiophores showed no fluorescence (not shown). The fluorescence occurred in the cytoplasm and a further subcellular localization could not be achieved because of the intense GFP signal. Fraaije et al. applied immunogold labeling and found catalase-peroxidase in the cytoplasm and in addition in peroxisomes (9).

cpeA **expression is activated through** ***stuA*.** In order to study the regulatory circuits leading to a developmental upregulation of *cpeA* transcription, we tested whether known regulators affect the expression of the gene. First, the start of transcription was determined by primer extension 52 bp upstream from the predicted translational start (Fig. 1). Analysis of the DNA sequence upstream of the transcription start site revealed binding sites for the regulators BrlA, AbaA, and StuA (the StuA binding site is indicated in Fig. 1). Therefore, we have chosen strains with mutations in *brlA*, *abaA*, or *stuA*; analyzed the induction of *cpeA* during sexual differentiation; and found that only in a *stuA* mutant was developmental induction greatly reduced (Fig. 6). Since *cpeA* and *pcrA* share the promoter region and the StuA binding site is located within the open reading frame of *pcrA*, we analyzed the transcription of the upstream gene as well. In the wild type, *pcrA* is downregulated at the time when *cpeA* becomes induced. The downregulation of *pcrA* was unaffected in the *stuA1* mutant (Fig. 6). To further prove that StuA is directly responsible for the observed induction of *cpeA*, we used a strain in which the promoter of *stuA* was replaced by the *alcA* promoter. The latter promoter is repressed in the presence of glucose and highly induced by ethanol or threonine. This allows high expression of StuA in the absence of developmental processes, namely, in submerged culture. This time and spatially mis-scheduled expression of *stuA* should lead to an induction of target genes if no other development-specific transcription factor is required (the *alcA* fusion strains were kindly provided by B. Miller, Moscow, Idaho). In addition, we tested for the stimulation of *cpeA* expression upon induction of BrlA or AbaA by using the same experimental approach. We grew the corresponding strains in the presence of glucose and shifted the medium after 20 h to medium containing ethanol for 1, 2, and 3 h. The mycelia were harvested and processed for Northern blot analysis. As already observed during expression analyses, *cpeA* was induced in wild-type cells due to ethanol as carbon source (Fig. 3). In these shift experiments, we observed that transcription was induced after 1 h; the transcript level then decreased (by 2 h), and after 3 h it increased again. The same pattern of transcription was observed in strains that expressed *brlA* or *abaA* under the control of the *alcA* promoter, indicating that these two regulators are not involved in *cpeA* activation. However, in the strain with the *alcA*::*stuA* construct, the transcript level remained high at all time points after induction of the *alcA* promoter. This suggests that StuA induces the expression of *cpeA* (Fig. 6).

In the *cpeA* promoter region, several potential StuA binding sites with the core sequence CGCG were detected. One of them (indicated in Fig. 1) matches the full consensus of the StRE [(A/T)CGCG(T/A)N(A/C)] (8). To further prove the effect of StuA on *cpeA* expression, we deleted this putative StuA binding site from the *cpeA* promoter in the *cpeA::gfp* fusion construct and monitored the expression by Northern blot and fluorescence microscopy (Fig. 6 and 7). When we used the construct with the StuA binding site (SMS14, with a single integration of pMS41), we detected an upregulation of the transcript during development and upon starvation. Hülle cells showed strong fluorescence in their cytoplasm, and we observed weak fluorescence in the dikaryotic mycelium of the fruiting bodies. In contrast, the promoter without the StuA binding site (SMS16, with a single integration of pMS42) did not support developmental induction, whereas the response to carbon starvation was unaltered. The CpeA-GFP fusion protein was no longer detectable in Hülle cells, but the expression in the dikaryotic mycelium remained. This shows that StuA is responsible for the tissue-specific induction of CpeA.

DISCUSSION

In this study we have analyzed catalase-peroxidase of *A. nidulans* and found that the gene is expressed upon carbon starvation and is highly induced during sexual development in Hülle cells. We tested several potential regulators for an effect on *cpeA* expression. Among those were *nsdD* and *veA*, with a


A
wt
stuA1-mutant
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cpeA
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alcA::stuA
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alcA::abaA
wt
cpeA
alcA::stuA
stuA
alcA::brlA
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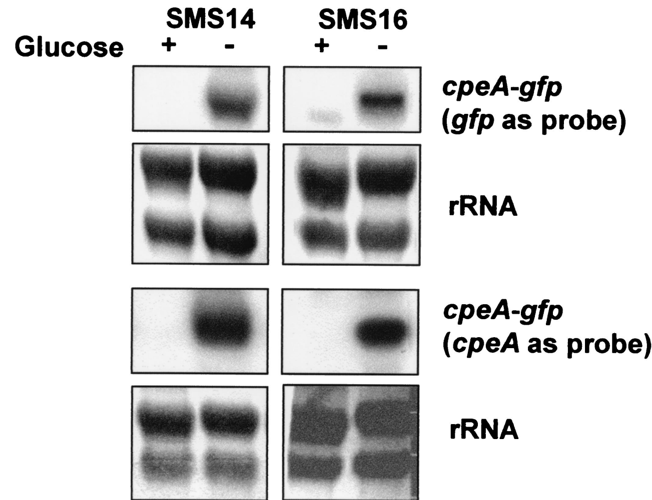

FIG. 6. Transcriptional activation of *cpeA* is regulated through StuA during sexual development. (A) RNA was isolated from FGSCA4 and a *stuA1* mutant strain (GO256) at the time points indicated above the lanes. RNA was hybridized to *cpeA* (upper panel)- and *pcrA* (middle panel)-specific probes. rRNA was stained on the membrane with methylene blue as a loading control (lower panel). (B) Forced expression of *stuA*, *brlA*, and *abaA* was achieved by growth of the strains for 20 h on glucose medium (repressing conditions) and then shifted to ethanol-containing medium for the times indicated above the panels. RNA isolated from wild-type strain FGSCA4 and the three transgenic strains UI132 (*alcA::stuA*), TPM1 (*alcA::abaA*), TTA292 (*alcA::brlA*) was processed for a Northern blot. The membrane was hybridized to a *cpeA* specific probe (upper 4 panels) and to a *stuA*, *brlA* and an *abaA* specific probe (lower 3 panels). (C) The transgenic strain SMS14 (*cpeA::gfp*) and SMS16 [*cpeA(ΔStuA binding sites)::gfp*] were analyzed for *cpeA-gfp* expression after the induction of development. As a probe for the Northern blot, we used *gfp* (upper panels) or *cpeA* (lower panels). rRNA staining was used as loading control. (D) Strains SMS14 and SMS16 were also analyzed for CpeA-GFP expression upon carbon starvation (lower panels). *gfp* or *cpeA* were used as probes as indicated.

putative role in the early stages of sexual development (7; K.-S. Chae, unpublished data; also, results not shown), and *brlA* and *abaA*, with a function in asexual development. For none of these factors could we demonstrate an involvement in *cpeA* regulation. In addition, we tested *stuA.* Although StuA was described before as a repressor, we present evidence that it has an activating function with regard to *cpeA.*

Transcription factor StuA activates *cpeA* expression. Three lines of evidence suggest that StuA regulates the transcriptional activation of the *cpeA* gene. (i) In *stuA* mutants *cpeA* expression levels are reduced. (ii) Overexpression of StuA in submerged cultures causes induction of *cpeA*. (iii) Deletion of the StuA binding site prohibits transcriptional upregulation of *cpeA* in Hülle cells. The *stuA* gene was isolated by complementation of a developmental mutant that is blocked in asexual development, unable to enter the sexual cycle, and does not even produce Hülle cells (17). StuA belongs to a group of regulators of fungal morphology sharing a common DNA-binding motif named the APSES domain, the term being derived from the currently known members of the group: ASM-1, Phd1, StuA, Efg1, and Sok2 (4). The APSES motif has structural similarity to the DNA-binding domain of eukaryotic basic helix-loop-helix (bHLH) proteins (8), which form homo- and heterodimers mediated by the HLH domain (2). The StuA protein binds to promoter elements with the consensus sequence (A/T)CGCG(T/A)N(A/C) and has been shown to act as a repressor of the central regulators of asexual development *abaA* and *brlA* to allow their correct temporal and spatial expression (8, 30). Since we found that StuA enhances the expression of *cpeA* during sexual development, it can be concluded that the regulator is able to mediate both negative and positive transcriptional control. One possible explanation for this dual function is to assume interaction of StuA with different partners via HLH domains to form distinct heterodimers during asexual and sexual development, which then could act as repressors or activators of transcription, respectively. Similarly, a dual function as repressor and activator of transcription has been proposed for the *Candida albicans* homologue of StuA, Efg1, which is essential for hyphal development in the fungal pathogen (27). Formation of multiple dimer combinations of bHLH proteins is a well-known mechanism for regulation of differential gene expression in mammals (2). Additional research is needed to identify potential dimerization partners of StuA and to unravel their different contribution to the regulation of the morphogenetic pathways in *A. nidulans.*

Role of catalase-peroxidase during sexual development. Catalases and peroxidases are involved in the detoxification of harmful oxygen species. The complex regulation of the expression of the three catalases in *A. nidulans* suggests distinct enzymatic properties that are required at specific stages of the life cycle. Whereas CatA is mainly expressed in asexual and sexual spores, CatB is found in the hyphae and is induced during asexual sporulation but is absent from conidia and CatC is expressed in hyphae (15). Most likely, catalase-peroxidase serves a similar function in coping with deleterious oxygen molecules, as do the other catalases. Since the initial stages of

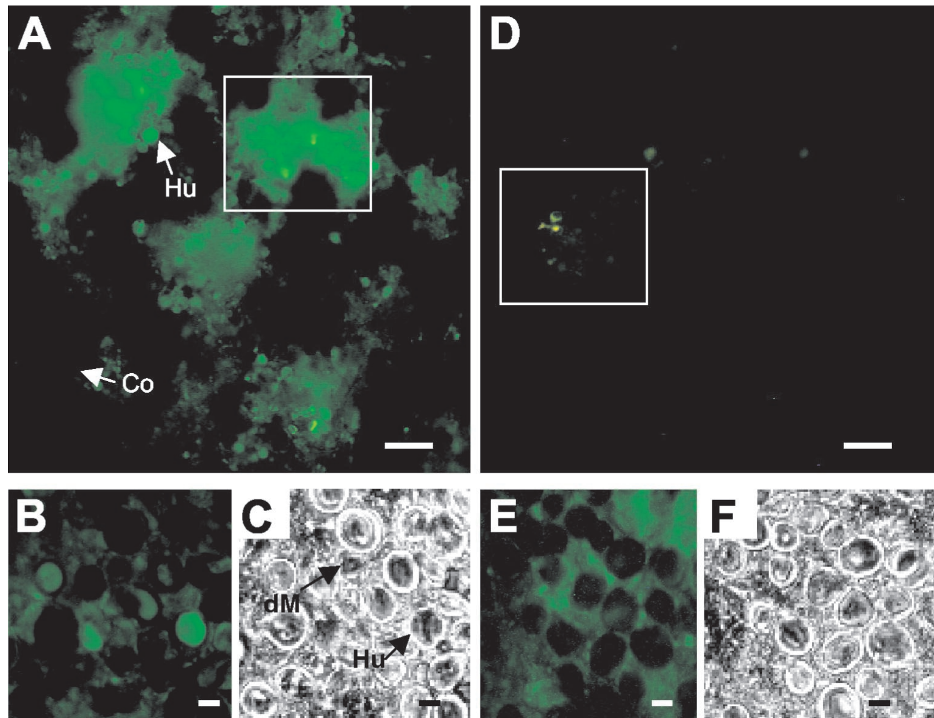

FIG. 7. Visualization of CpeA-GFP expression in SMS14 (*cpeA::gfp*) (A, B, and C) and SMS16 [*cpeA(ΔStuA binding sites)::gfp*] (D, E, and F). (A) View of an agar plate grown with SMS14 with induced sexual development. Hülle cells (Hu) are brightly stained, conidiophores (Co) are not stained. The boxed area shows a cleistothecium. Exposure time, 0.8 s. (B and C) A cleistothecium was broken and observed under a coverslip under fluorescence (2-s exposure) or phase-contrast illumination. Hu, Hülle cells; dM, dikaryotic mycelium. (D) View onto an agar plate grown with SMS16 with induced sexual development. Almost no fluorescence is detectable (0.8-s exposure). (E and F) Observation of Hülle cells and dikaryotic mycelium under fluorescence or phase-contrast illumination. Hülle cells were not stained, but the dikaryotic mycelium shows fluorescence with a similar intensity to that seen in panel B. Exposure time, 4 s. The scale bars represent 100 μm in panels A and D and 10 μm in panels B, C, E, and F.

fruiting body development largely occur underneath a layer of Hülle cells, one could speculate that the Hülle cells serve a protective function of the dikaryotic mycelium.

Another attractive possibility for a role of catalase-peroxidase during development comes from the observation that in *N. crassa* a hyperoxidant state was detected at the start of different morphogenic transitions (12, 29). Therefore, CpeA could have an important function in coping with this dangerous state. It has even been postulated that this hyperoxidant state triggers differentiation (11). Likewise, in *N. crassa*, catalase-peroxidase was isolated from conidiating cultures and has been detected in cells in the stationary phase when they showed increased vacuolization and begin lysing (21).

More evidence for an association of sexual development with oxidative stress comes from studies in *Podospora anserina*. It was observed that the number of peroxisomes, a cellular compartment, which harbors enzymes to cope with oxidative stress, increases dramatically during ascus formation (5). Mutation of a gene, *car1*, involved in the biogenesis of these organelles resulted in a karyogamy defect and thus a block in sexual development. Since *Podospora anserina* and some *Aspergillus* species produce fruiting bodies but lack Hülle cells, the Hülle cells in *A. nidulans* could only be one strategy to cope with oxidative stress. This is supported by the fact that loss-of-function of the two enzyme activities did not lead to any strong developmental phenotype. These results suggest that a concerted action of different enzymes is employed to detoxify reactive oxygen species in different cell types and that successful protection can be achieved by different and overlapping functions.

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