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Analysis of the *Aspergillus nidulans* thaumatin-like *cetA* gene and evidence for transcriptional repression of *pyr4* expression in the *cetA*-disrupted strain

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Abstract

The asexual spore or conidium plays a critical role in the life cycle of many filamentous fungi. However, the process of conidial germination remains surprisingly obscure. To better understand this process at the molecular level we characterized the *Aspergillus nidulans cetA* gene which is uniquely transcribed in conidiating cultures and whose transcript is significantly enriched in mature conidia. *CetA* is a member of a novel family of fungal genes of unknown function with homology to plant thaumatin-like (PR-5) defense proteins. We demonstrate by Northern analysis that *cetA* is a glucose-repressible gene. Transcriptional repression is dependent on the presence of protein kinase A. Western analysis indicates that the CETA protein is absent from conidia but is highly expressed during the first 6 h of germination and is secreted into the medium. Disruption of the *cetA* gene seemingly results in delayed germination, slow growth, abnormal hyphal branching, and cell-wall defects. However, further analysis shows that the mutant phenotype is the result of glucose-dependent transcriptional repression of the *pyr4* selectable marker used to disrupt the *cetA* gene. This is the first time that repression of a selectable marker ("position effect") has been reported in *A. nidulans*, a finding that may well be of significance in the analysis and interpretation of mutant phenotypes in this organism.

Keywords: *Aspergillus nidulans*; Conidial germination; Conidial enriched transcript; Cell wall; Secreted protein; Position effect; Transcriptional repression

1. Introduction

The asexual spore, or conidium, is vital in the life cycle of many fungi because it is the principal means for dispersion and serves as a 'safehouse' for the fungal genome in unfavorable environmental conditions (Osherov and May, 2001).

In *Aspergillus nidulans*, conidial germination is typically triggered by nutrients in a process whose molecular details have only recently emerged. In the presence of glucose, conidia swell rapidly and increase in adherence. Conidial swelling and adhesion occur within 1 h. DNA synthesis can be first detected after 2.5 h of incubation, germ tube emergence begins after 3.5 h, and the first nuclear division occurs after 4.5 h when cultured at 37 °C. The earliest measurable biochemical changes occurring within 20 min of germination are trehalose breakdown and protein synthesis (D'enfert, 1997).

At the molecular level, both the cAMP and ras regulatory circuits are involved in regulating the initial steps of germination in *A. nidulans*. Inactivation of the cAMP pathway by deletion of the adenylate cyclase *cyaA* or *pkaA* genes results in delayed trehalose breakdown and germination (Fillinger et al., 2002). Most importantly, stimulation of the cAMP pathway by expression of a constitutively active form of GanB, the Gα protein which activates cyaA, or through deletion of RgsA, the predicted GTPase activating protein which inactivates GanB, results in conidial germination in carbon-free minimal medium (Chang et al., 2004; Han et al., 2004; Lafon et al., 2005). Inhibition of the ras pathway by inducible expression of dominant-negative *A-ras* delays

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germination, while expression of dominant active *A-ras* in both *A. nidulans* and *Aspergillus fumigatus* results in conidial swelling and adhesion in the absence of a carbon source (Fortwendel et al., 2004; Osherov and May, 2000).

Both the receptors presumed to sense the carbon source and to activate the ras and cAMP pathways and their downstream targets remain unknown. Based on genetic and biochemical evidence, it has been suggested that dormant conidia of *A. nidulans*, *Neurospora crassa*, and *Fusarium solani* contain a pre-existing pool of mRNA and ribosomes, primed for rapid activation and translation (Loo, 1976; Osherov and May, 2000; Thau et al., 1970). In the presence of a carbon source, free ribosomes associate with mRNA within 15 min to form polysomes (Mirkes, 1974). The identity of these mRNAs could prove instructive, possibly revealing which proteins need to be translated at the very beginning of germination.

We recently used suppressive-subtraction hybridization to identify and clone 12 conidial enriched transcripts (*cetA–cetL*) that are present at relatively high levels in dormant conidia of *A. nidulans* (Osherov et al., 2002). Interestingly, *cetA* is similar to plant thaumatin-like PR5 genes encoding proteins with antifungal activity. These proteins are highly expressed in response to fungal infection (Anzlovar and Dermastia, 2003).

In this report, we undertook a detailed study of the *cetA* gene, analyzing its transcriptional regulation in wild-type and mutant strains of *A. nidulans*, as well as the expression pattern of the CETA protein. We identified homologs of CETA in both *A. nidulans* and *A. fumigatus* and analyzed their mRNA levels during conidial germination and growth. Our results indicate that *cetA* expression is regulated at both the transcriptional and translational levels and that the CETA protein is translated during early germination and secreted into the medium. Disruption of the *cetA* gene results in no obvious phenotype, indicating that the gene is not essential. Surprisingly, we found evidence for glucose-dependent transcriptional repression of the *pyr4* selectable marker that was inserted into the *cetA* gene. The implications of this finding are discussed.

2. Materials and methods

2.1. Strains and culture conditions

The *A. nidulans* strains used in this study are described in Table 1. YAG medium (0.5% yeast extract (w/v), 1% glucose (w/v), 10 mM $MgCl_2$, and 1.5% agar (w/v) for solid plates), supplemented with trace elements and vitamins (Bainbridge, 1971), was used for growth. Ten millimolar uracil and 5 mM uridine were added to *pyrG* auxotrophic strains. Conidia were harvested in 0.2% Tween 80 (w/v), resuspended in double-distilled water (DDW), and counted in a hemacytometer. When specified, minimal medium (MM) was used. MM is composed of 70 mM $NaNO_3$, 2% glucose (w/v), 12 mM KPO_4, pH 6.8, 4 mM $MgSO_4$, 7 mM KCl, trace elements, and 1.5% agarose (for solid plates). When required, glucose was replaced by 2% sucrose, sorbitol, and glycerol or 0.2% (w/v) BSA (bovine serum albumin), casein, collagen, and glutamic acid. *A. nidulans* transformants were grown in YAS plates (containing 0.5% yeast extract (w/v), 1% glucose (w/v), 10 mM MgSO4, 0.2 M sucrose, 1.5% agar (w/v), trace elements, and vitamins; Bainbridge, 1971).

2.2. Nucleic acid preparation

RNA was prepared from freshly harvested *A. nidulans* strains and conidia germinated in YAG or MM liquid medium at 37 °C for the indicated time. Total RNA was prepared by the "hot SDS/phenol" method described previously (May and Morris, 1988), with the following modifications: after lyophilization, fungal material was ground with a sterile 1 ml tip in a 1.5 ml Eppendorf tube for 2 min, then ≈50 μl of the powder was mixed with an equal volume of glass beads and pulverized for an additional 5 min, prior to the addition of hot SDS/phenol as previously described. We found this additional grinding step essential to efficiently extract RNA from dormant conidia.

Aspergillus nidulans genomic DNA was prepared from freshly harvested flash-frozen mycelium using the hot SDS/phenol method as previously described (Jadoun et al., 2004).

2.3. Insertional inactivation of the A. nidulans cetA gene

Inactivation of *cetA* was performed with the *pyr4*-modified GPS-1 genome-priming system (New England Biolabs, Beverly, A.) as previously described (Jadoun et al., 2004). A 4192-bp DNA fragment flanking the *A. nidulans cetA* gene was generated by PCR, using the Expand Long Template PCR system (Roche Diagnostic, Penzberg, Germany) and the primers *cetA* K/O 5′ and *cetA* K/O 3′ (Table 2). These primers were designed to

Table 1
Aspergillus strains used in this study

Strain	Organism	Genotype	Source
R153	*A.nidulans*	*wA3;pyroA4*	FGSC[a]
GR5	*A.nidulans*	*wA3;pyrG89; pyroA4*	G.S. May
ΔpkaA (TKIS18.11)	*A.nidulans*	*pabaA,yA2,ΔpkaA:: argB;ΔargB:: trpC; trpC801,veA1*	N.P. Keller
cetA-K/O1	*A.nidulans*	*wA3; cetA-K/O:: pyr4;pyrG89;pyroA4*	This study
cetA-myc1	*A.nidulans*	*wA3; cetA-myc:: pyr4;pyrG89;pyroA4*	This study
AF293	*A.fumigatus*	Wild type	G.S. May

[a] Fungal Genetics Stock Center.

Table 2
Primers used in this study

Primer	Sequence	Restriction site
Primer N	5′-GTTTAAGACTTTATTGTCCG-3′	
Primer S	5′-GTTCCCAACATTTTGTCCG-3	
pyr4 5′	5′-CGACTCGCCACGTCTGGTCAA-3′	
pyr4 3′	3′-TGCCACACGCTCCCGGCGAT-3′	
AN gpdA 5′	5′-CCACCGGTGTCTTCACTACC-3′	
AN gpdA 3′	3′-CTTGACGGCATCCTTGATCT-3′	
cetA 5′	5′-ATGATGTTCACCAAGGCTCT CG-3′	
cetA 3′	3′-TTAAGCTTACGCGCCGAGA GTC-3′	
cetA prom 5′	5′-TCAGACAAGTCCATTTCC-3′	
cetA prom 3′	5′-TGCACTAGTTCGTGGTGGGT TGAGTATG-3′	*Spe*I
cetA term 5′	5′-GATCGCGGCCGCGCTTAACCC ATGACGGGGGGC-3′	*Not*I
cetA term 3′	5′-GATCGCATGCCAGTCGCTGCT GACACTCGGC-3′	*Sph*I
*cetA BamH*I 5′	5′-CGATAAGCTTGGATCCATG ATGTTCACCAAGGCT-3′	*Bam*HI
cetA c-myc 3′	5′-ACGATGCGGCCGCTTACAAGT CCTCTTCAGAAATGAGCTTT TGGCCGCCCAAGTCCTCTTCAGA AATGAGCTTTTGGCCGCC-3′	*Not*I
cetA K/O 5′	5′-ATCGGCGCGCCATCAGACCGA CGGATCCAC-3′	*Asc*I
cetA K/O 3′	5′-ATCGGCGCGCCCATTGGCT GTTCCACAACAC-3′	*Asc*I
AnCalA 5′	5′-ATGCTCTTCAACAAGATC ATCAG-3′	
AnCalA 3′	5′-TCATGAGTCGTAAGACC GCCTG-3′	
AfCalA 5′	5′-ATGTCTTTCTCCAAGTTCTT CAG-3′	
AfCalA 3′	5′TTAACCCAGGGTGAGGGTG AAG-3′	
AfCalB 5′	5′-ATGTTCACCAAGGCCTT CTTCG-3′	
AfCalB 3′	5′-CTAGTTGCCAATGTTCAC CACG-3′	
AfCalC 5′	5′-ATGTTCTTTTCCAGACTCCT TTTC-3′	
AfCalC 3′	3′-TCAGCCCAACGTAACCGT AATTCC-3′	

contain an *Asc*I restriction site at their 5′ end (underlined). The PCR-amplified 4,192-bp *cetA*-containing fragment was gel-purified by means of the Wizard SV gel and clean-up system (Promega), and cloned into the pGEM T/A cloning vector, as instructed by the manufacturer (Promega). This vector was then used as a target for the tp-1-*pyr4* transposon. Transposition was performed according to the manufacturer's instructions (New England Biolabs). pGEM T/A carrying the tp-1-*pyr4* transposon was cloned into the *Escherichia coli* DH10B strain. Clones carrying transposon-disrupted *cetA* plasmids were identified by PCR using *Taq* polymerase (Fermentas UAB, Vilnius, Lithuania), using *cetA*5′ forward primer and *cetA*3′ reverse primer (Table 2). These primers allow amplification of a 531-bp fragment containing the *A. nidulans cetA* gene. For verification of the transposon-disrupted *cetA* clones, the tp-1 inverted primer N and primer S supplied in the GPS-1 kit were used. This resulted in the identification of p*cetA*-K/O in which the transposon had inserted 196 bp downstream of the *cetA* ATG start codon. The tp-1-*pyr4* disrupted *cetA* fragment was released by cleavage with *Asc*I, purified with the Wizard SV gel and clean-up system and used for transformation of the *A. nidulans* GR5 strain (Table 1). Transformation was performed as described by Jadoun et al. (2004).

2.4. Nucleic acid manipulation

For Southern blot analysis, 10 μg of fungal genomic DNA was digested with *Xba*I and run on a 1% agarose gel. Transfer and hybridization conditions were performed as described previously (Jadoun et al., 2004). Probes were prepared by the random priming method, using [α-^{32}P]dCTP as described by Sambrook and Russell (2001). Templates for each of the probes were prepared by PCR amplification of genomic DNA using primer pairs *cetA* 5′ and *cetA* 3′ (for *cetA*); *AnCalA* 5′ and *AnCalA* 3′ (*AncalA*); *AfCalA* 5′ and *AfCalA* 3′ (*AfCalA*); *AfCalB* 5′ and *AfCalB* 3′ (*AfCalB*); and *AfCalC* 5′ and *AfCalC* 3′ (*AfCalC*) (Table 2). Northern analysis was performed as previously described (May and Morris, 1987). For detection of the *cetA* transcript, the same 537-bp *A. nidulans cetA* probe used for Southern analysis was used. For RT-PCR, total RNA was treated with DNase (DNA-free, Ambion, Austin, TX) according to the manufacturer's instructions. RNA concentration was assessed and 3 μg was taken for the RT reaction using PowerScript Reverse Transcriptase (Clontech, Palo Alto, CA, USA).

2.5. Construction of the pcetA-myc plasmid

The p*cetA-myc* plasmid containing the *cetA* promoter, *cetA* open reading frame containing two C-terminal c-*myc* tags (*cetA-myc*) and *cetA* terminator was constructed as follows: a 912 bp PCR fragment containing the *cetA* terminator was obtained by amplification of *A. nidulans* genomic DNA (strain R153) with the oligonucleotides *cetA* term 5′ and *cetA* term 3′ (Table 2). To create *cetA-myc*, a 630 bp PCR fragment containing *cetA-myc* was obtained by amplification of *A. nidulans* genomic DNA by the oligonucleotides *cetA Bam*HI 5′ and *cetA c-myc* 3′ (Table 2). To create the *cetA* promoter fragment, a 976 bp PCR fragment containing the *cetA* promoter was obtained by amplification of *A. nidulans* genomic DNA by the oligonucleotides *cetA* prom 5′ and *cetA* prom 3′ (Table 2). The three fragments described above were gel-purified, sub-cloned into the pGEM T/A cloning vector (Promega), and verified by sequencing. The three fragments, i.e., *cetA*-prom (*Xba*I/*Spe*I cut), *cetA-myc* (*BamH*I/*Not*I cut) and *cetA*-term (*Not*I /*Sph*I cut), were ligated

into the pRG4 plasmid (*Xba*I/*Sph*I cut), resulting in the plasmid p*cetA-myc*.

2.6. Antibodies and Western blot analysis

Aspergillus nidulans conidia were allowed to germinate in YAG liquid medium for the indicated time. Fungal biomass was collected onto Miracloth (Calbiochem, Germany), washed once in distilled water, and dried. Ungerminated, freshly harvested conidia were used for the 0 h time point. Fungal biomass was then frozen in liquid nitrogen and lyophilized overnight. The lyophilized pellets were ground with a sterile 1 ml tip in a 1.5 ml microcentrifuge tube for 2 min, weighed at 10 mg dry weight per extraction reaction, and an equal volume of glass beads was added. The following extraction procedure was subsequently performed: 0.2–0.4 ml of sample buffer containing 1% SDS, 9 M urea, 25 mM Tris–HCl, pH 6.8, 1 mM EDTA, and 0.7 M β-mercaptoethanol was vigorously mixed with the lyophilized powder, vortexed for 30 s, boiled for 5 min, cooled on ice for 5 min, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12.5% gel (Osherov and May, 1998). Protein was transferred to a nitrocellulose membrane BA 85 (Schleicher & Schuell, Germany) in a Mini-PROTEAN three cell blotting apparatus according to the manufacturer's instructions (Bio-Rad, USA). Blots were blocked by incubation with TBST (TBS containing 0.1% Tween 20) containing 5% skimmed milk for 1 h at room temperature. CETA protein was detected following incubation of the blot with undiluted anti c-*myc* monoclonal antibody hybridoma supernatant (clone 9E10) for 1 h followed by two washes in TBST for 15 min. The tagged protein was visualized by ECL (enhanced chemiluminescence) using a horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, USA) at 1:10,000 dilution.

2.7. Calcofluor staining and microscopy

CetA-K/O1 and R153 control *A. nidulans* conidia were incubated at concentrations of 10^4 conidia/ml for the indicated time at 37 °C on glass coverslips in 24-well plates (Nunclon surface; Nunc, Roskilde, Denmark). Coverslips were placed on 1% calcofluor white (fluorescent brightener 28, Sigma) and directly analyzed. The dye fluoresces when bound to chitin, and thus primarily stains the cell-wall. Images were obtained by fluorescence microscopy on an Olympus BX40 microscope equipped with a digital camera (Olympus DP70) and viewed at a total magnification of 200 and 400×.

3. Results

3.1. Identification of a family of fungal cetA-related genes

We analyzed 38 fungal genomes (http://www.ncbi.nlm.nih.gov) for the presence of *cetA*-like genes. Hits from BLASTP or TBLASTN searches with an *E* value of $<10E^{-4}$ were considered as putative CETA protein homologs and named Cal (*cetA*-like) proteins with the prefix of the species name added to prevent confusion. One homolog was found in *A. nidulans* (AnCALA) (48% amino-acid identity to CETA), three in *A. fumigatus* (AfCalA-C), (66, 52, and 49% amino-acid identity to CETA, respectively), and one in *Magnaporthe grisea* (MgCalA) (27% amino-acid identity), *N. crassa* (NcCalA) (30% amino-acid identity), and *Coccidioides immitis* (CiCalA) (41% amino-acid identity), respectively. As previously described (Osherov et al., 2002), the CETA protein shows low but significant homology with plant thaumatin-like (TL) proteins (*E* value $= 10^{-3}$; 30% amino-acid identity). Sequence alignment of the CETA, Cal, and plant TL proteins revealed the presence of conserved amino-acid sequences (Fig. 1A). A dendrogram of these proteins by the ClustalX neighbor-joining algorithm for cluster analysis shows that the CETA protein is most similar to AfCalA, while all *A. nidulans* and *A. fumigatus* CETA and Cal proteins are closely related and occupy a distinct branch (Fig. 1B).

3.2. Northern analysis of the A. nidulans and A. fumigatus cetA and cal mRNAs

Our previous work demonstrated that *cetA* mRNA is highly abundant in dormant conidia and rapidly disappears during the first few hours of germination (Osherov et al., 2002). We, therefore compared the mRNA levels of *cetA* with those of the *A. nidulans* and *A fumigatus cal* genes. Total RNA was extracted from dormant and germinating *A. nidulans* and *A. fumigatus* conidia grown on rich YAG medium at different time points and analyzed by Northern blot. Interestingly, unlike *cetA*, *AnCalA* mRNA is not detectable in dormant *A. nidulans* conidia, but is expressed during the first 4 h of germination (Fig. 2A). Similarly, in *A. fumigatus*, both *AfCalA* and *AfCalB* are expressed only during the first 1–2 h of germination (Fig. 2B). *AfCalC* was not expressed under these conditions (data not shown).

3.3. The cetA gene is under complex transcriptional control

Sequence analysis of the promoter region of *cetA* identified several conserved putative *creA* (glucose repressor) binding sites (SYGGRG) (Felenbok et al., 2001), suggesting that *cetA* mRNA expression may be repressed by glucose. We, therefore tested the effect of glucose on *cetA* transcription. Initially, we compared *cetA* mRNA levels in the presence of MM containing glucose or albumin as alternate sole carbon sources. While *cetA* is briefly transcribed during the first hours of germination on MM-glucose (<6 h), its transcription is constitutive on MM-albumin (Fig. 2C). To test the effect of glucose addition on *cetA* transcription, *A. nidulans* conidia were allowed to germinate for 6 h in the presence of albumin as the sole energy source. Under these conditions, *cetA* levels remain constant. Addition of 2% glucose after 6 h resulted in a rapid reduction of *cetA* mRNA levels as

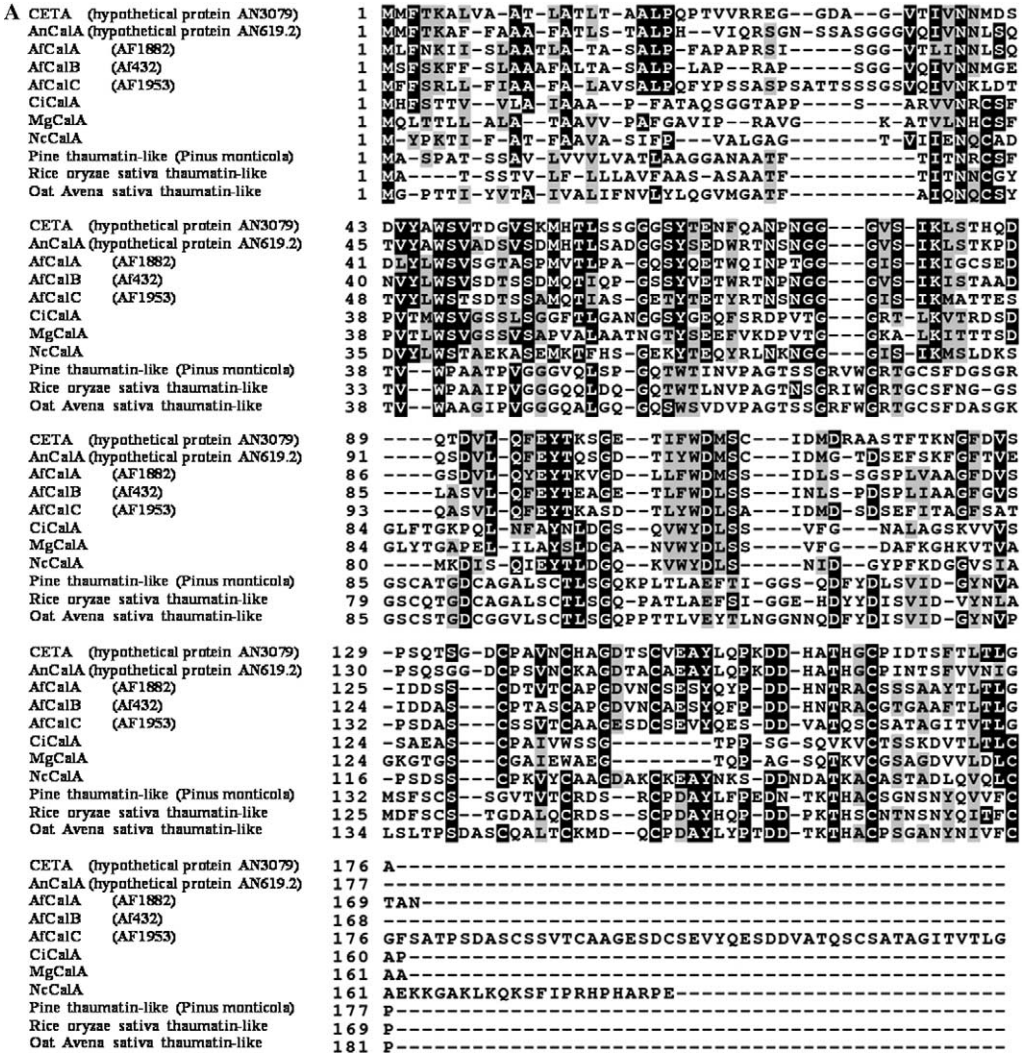

Fig. 1. (A) Amino-acid comparison of *A. nidulans* CETA protein (GenBank Accession No. AN3079.2) with *A. nidulans* AnCALA (AN7619.2), *A. fumigatus* AfCalA-C (Afu3g09690, Afu8g01710, Afu3g00510, respectively; ftp.bioinf.man.ac.uk), *M. grisea* MgCalA (hypothetical protein MG06538.4), *N. crassa* NcCalA (hypothetical protein CAD70756), *C. immitis* CiCalA (hypothetical protein contig AAEC01000025), rice thaumatin-like (CAA48278), oat thaumatin-like (P50695) and pine thaumatin-like proteins (AAS85755). The Clustal W 1.8 program was used for the alignments. A dash indicates a gap in the sequence. A black background indicates that the sequence is identical among at least six of the aligned species, gray indicates a conserved sequence. (B) Dendogram showing relatedness between the gene products described above. The tree was generated with the ClustalX program using the neighbor-joining algorithm for cluster analysis. The numbers on the branches indicate the number of amino-acid substitutions per site.

compared to the control. These results indicate that *cetA* transcription is repressed by glucose (Fig. 2C).

The PKA (protein kinase A) pathway is involved in conidial germination and glucose sensing in *A. nidulans* and *Saccharomyces cerevisiae*, respectively (Fillinger et al., 2002; Gelade et al., 2003). We, therefore measured *cetA* transcription in an *A. nidulans* mutant lacking the catalytic subunit of PKA (strain *ΔpkaA*) and a mutant in which the GanB GTPase activating protein RgsA is inactive (strain *ΔRgsA*), leading to constitutive activation of the PKA pathway (Lafon et al., 2005). Notably, we found that the level of *cetA* mRNA in the *ΔRgsA* strain grown on MM-glucose is reduced by 2 h post-germination, compared to 6 h in the control wild-type strain. In contrast, *cetA* is constitutively expressed for up to 24 h in the *ΔpkaA* strain (Fig. 2D).

3.4. The pyr4 selectable marker used to disrupt cetA undergoes glucose-dependent transcriptional repression

To investigate the effect of loss of function of the *cetA* gene in *A. nidulans*, a disruption plasmid (p*cetA-K*/O) was constructed by transposon mutagenesis as described in the Materials and methods (Fig. 3A). After transformation of p*cetA-K*/O into GR5, 20 *pyrG*+ transformants were purified and screened with PCR for insertion into the *cetA* locus. Three putative mutants were identified and further characterized by Southern blot analysis (Fig. 3A). Based on the Southern blot analysis all three transformants were disrupted in the *cetA* gene, and all exhibited the same phenotypes described below. The growth phenotype of the *cetA-K*/*O1* strain was compared to the wild-type strain by

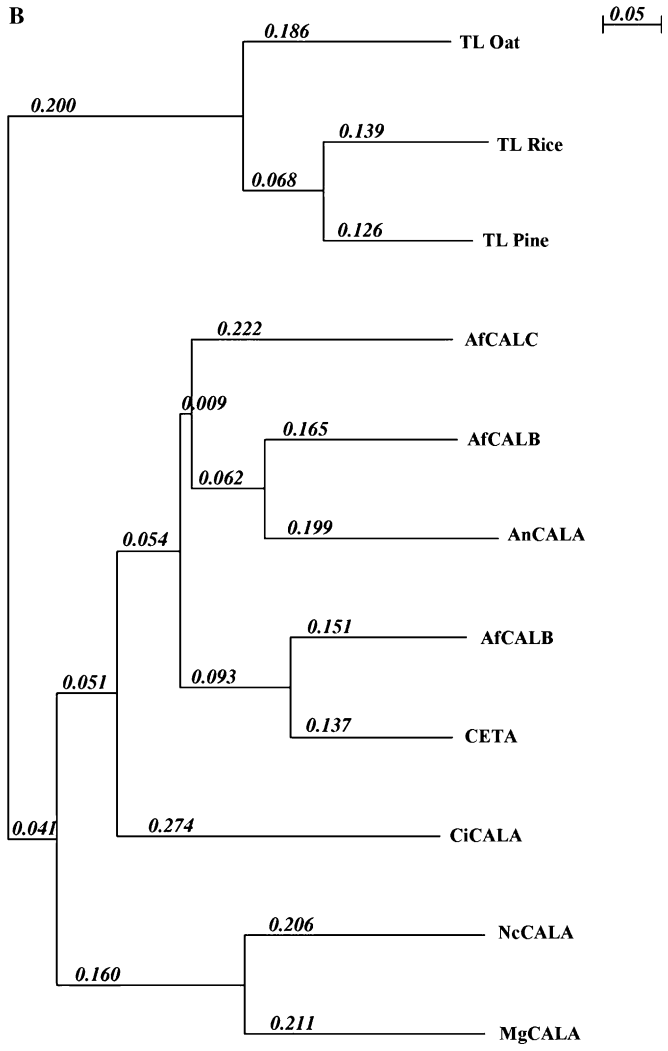

Fig. 1 (*continued*)

streaking (Fig. 4A, top panel) on YAG agar plates. Colonies of the *cetA-K/O1* strain appeared to be smaller, more compact and elevated in comparison to the wild-type strain. Microscopic examination of the *cetA-K/O1* strain grown on liquid YAG medium revealed defects in hyphal ultrastructure and branching (Fig. 3B, top panel). In addition, whereas calcofluor-staining of the wild-type strain is concentrated in growing hyphal tips and septae, in the mutant, large portions of the hyphae are intensely stained, suggestive of a defect in the localization of chitin (Fig. 3B, lower panel).

However, when the *cetA-K/O* strains were outcrossed to a tester strain (A52, Table 1), the mutant phenotype segregated in only 15% of the progeny ($n = 150$), instead of at the expected 1:1 ratio. In addition, the mutant phenotype disappeared when the *cetA-K/O1* strain was grown on YAG or MM plates in the presence of uracil/uridine (YAGUU, MMUU) (Fig. 4A, middle panel). When individual *cetA-K/O1* colonies grown on YAGUU plates were re-streaked on YAG plates, the mutant phenotype reappeared (Fig. 4A, lower panel), suggesting that loss of the mutant phenotype on YAGUU plates was not a result of recombination of the *cetA* gene. Northern analysis of conidial RNA demonstrated that *cetA-K/O1* colonies did not express *cetA* mRNA when grown on either YAG or YAGUU plates (Fig. 4B, lanes b and c). This indicates that the mutant phenotype seen on YAG plates was not a result of *cetA* disruption.

The dependence of the mutant phenotype on the presence of uracil/uridine in the growth medium led us to hypothesize that transcriptional repression of the *pyr4* marker used to disrupt *cetA* may be responsible for the observed effects. To test this hypothesis, we measured by RT-PCR the levels of *pyr4* mRNA in three *cetA*-disrupted mutants (*cetA-K/O1-3*) (Fig. 5 top panel, lanes a–c) in comparison to three isolates in which the p*cetA*-K/O plasmid had randomly integrated in the genome, outside of the *cetA* locus (Fig. 5 top panel, d–f). Our results indicate that *pyr4* undergoes partial transcriptional repression when inserted into the *cetA* locus. Interestingly, the level of *pyr4* mRNA increased to almost wild-type levels when the *cetA-K/O* strains were grown in the absence of glucose on MM and albumin as a sole carbon source (Fig. 5, lower panel, lanes a–c). Interestingly the increase in *pyr4* mRNA levels was accompanied by a remediation of the mutant phenotype (Table 3). This suggests that the increase in *pyr4* expression in the *cetA-K/O* strains grown in the presence of MM and albumin is sufficient for normal growth to occur. Together these results imply that the *pyr4* marker in the *cetA-K/O* strains undergoes glucose-dependent transcriptional repression, possibly through the glucose-repressible *cetA* promoter region.

3.5. The CETA protein is transiently expressed during germination and is secreted into the culture medium

To investigate the temporal expression pattern and localization of the CETA protein, an integrative plasmid, p*cetA-myc*, containing the *cetA* gene tagged with two C-terminal *c-myc* epitopes (Fig. 6A), was constructed as described in Materials and methods. After transformation into GR5, *pyrG*+ transformants were examined by Southern blot analysis. One of the transformants, *cetA-myc1* in which the plasmid had integrated in a single copy at the *cetA* locus was selected for further study. We examined the expression pattern of the tagged CETA protein by Western blot analysis. Total protein was extracted from dormant and germinating *cetA-myc1* conidia at different time points and analyzed by Western blot using *c-myc*-specific monoclonal antibodies. CETA- *myc* protein is expressed as a ~23 kDa band that is not detectable in untransformed control R153 cells (Fig. 6B). The *cetA-myc1* strain germinated and developed normally on MMUU, suggesting that expression of the myc-tagged CETA protein had no deleterious effects on morphogenesis (not shown). Interestingly, CETA protein is not detectable in dormant *A. nidulans* conidia, but is expressed at increasing levels during the first 6 h of germination (Fig. 6B, top panel). Analysis of culture medium for the presence of extracellular CETA protein

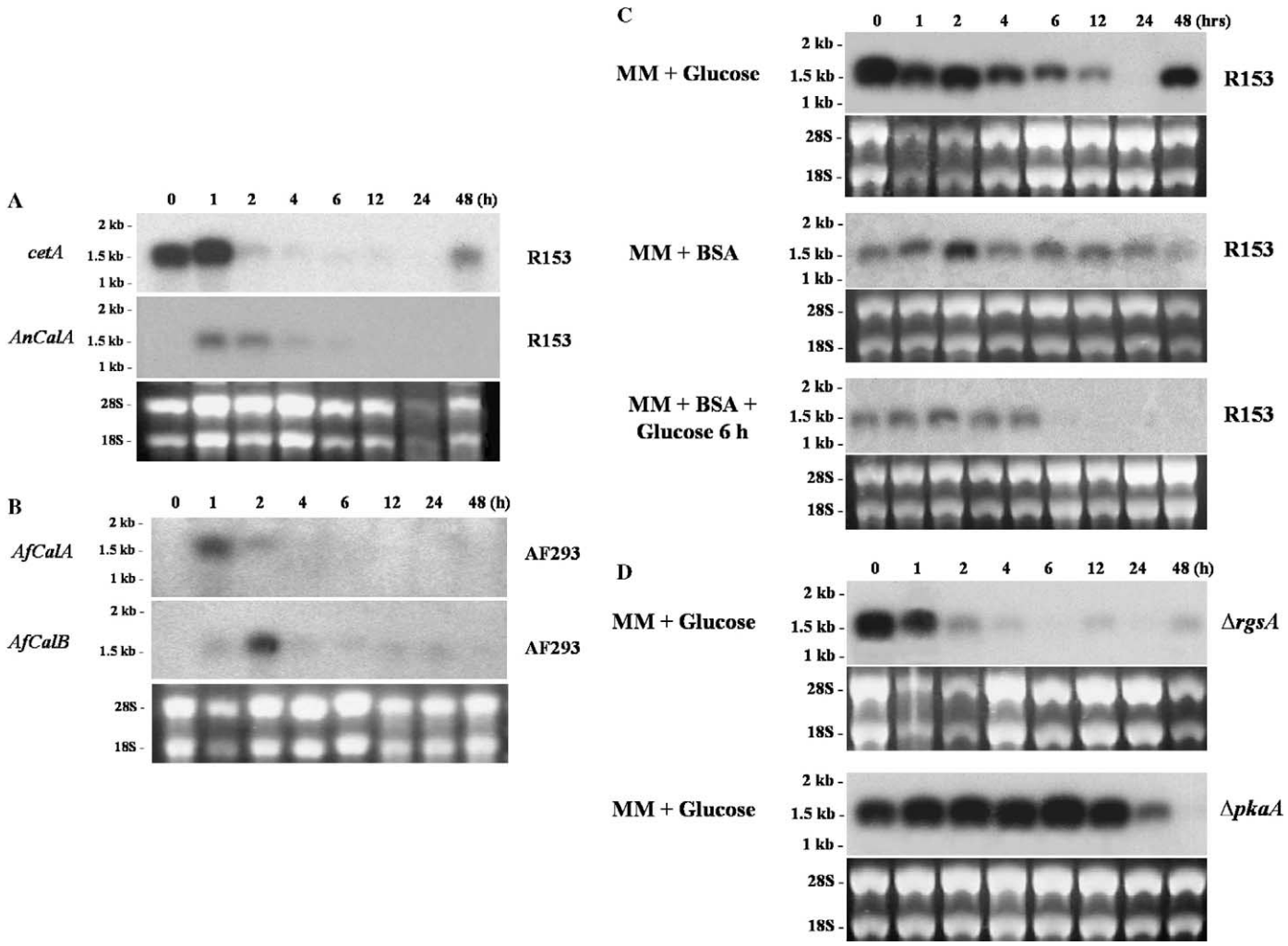

Fig. 2. Expression of *cetA* and *cal* mRNA in *A. nidulans* and *A. fumigatus*. Northern blot analysis of (A) *A. nidulans cetA* and related *calA*, (B) *A. fumigatus AfcalA* and *AfcalB* mRNA expression in YAG rich medium, (C) *cetA* expression on MM containing either glucose (top panel), BSA (middle panel) or following glucose addition to BSA (lower panel). (D) *cetA* expression in *ΔRgsA* and *ΔpkaA* mutant strains on MM containing glucose. Each lane contains 5 μg of total RNA from ungerminated conidia (0 h), swollen germinating conidia (1, 2, and 4 h), germlings (6 h), early and late mycelium (12 and 24 h), and conidiating mycelium (48 h). Each blot was hybridized with the corresponding ^{32}P-labeled DNA probe. Ethidium–bromide staining of rRNA in the agarose gel was performed to control the loading (lower panels).

indicates that it is indeed a secreted protein and that it accumulates mainly during the first 6 h of germination (Fig. 6B, lower panel). When the culture medium was replaced with fresh medium after 12 or 24 h and the culture grown for an additional 12 h, CETA protein was not detected in the culture medium (Fig. 6B, lanes 12S, and 24S). This indicates that the CETA protein is not being secreted later in culture, and that its presence is the result of earlier accumulation.

4. Discussion

Conidial germination in *Aspergillus* spp. is a complex process involving multiple levels of control, including sensing of a suitable trigger (a carbon source), signal transduction (via the PKA and ras pathways and possibly additional unknown pathways), and the transcription and translation of specific genes (Osherov and May, 2001). Previously, we used suppressive-subtraction hybridization (SSH) to identify conidial enriched transcripts (*cet* genes) (Osherov et al., 2002). Of these, the *cetA* gene was particularly intriguing, in that it encodes for a protein with similarity to plant thaumatin-like proteins which have antifungal activity. In the study described in this report, we have started the characterization of the transcriptional regulation of this gene and its function.

We searched for CETA protein homologs with NCBI tBlastN and Fungal Blast which contains 38 sequenced fungal genomes. CETA-like proteins were only found in the ascocarp-producing ascomycetes (Pezizomycotina) such as *C. immitis*, *M. grisea*, and *N. crassa*. Interestingly, there are no CETA homologs in non-ascocarp-producing ascomycete yeast such as *S. cerevisiae* or *Schizosaccharomyces pombe* or in basidiomycetes such as *Cryptococcus neoformans* and *Ustilago maydis*. Significant homology was also found to plant thaumatin-like PR5 proteins. PR5 proteins

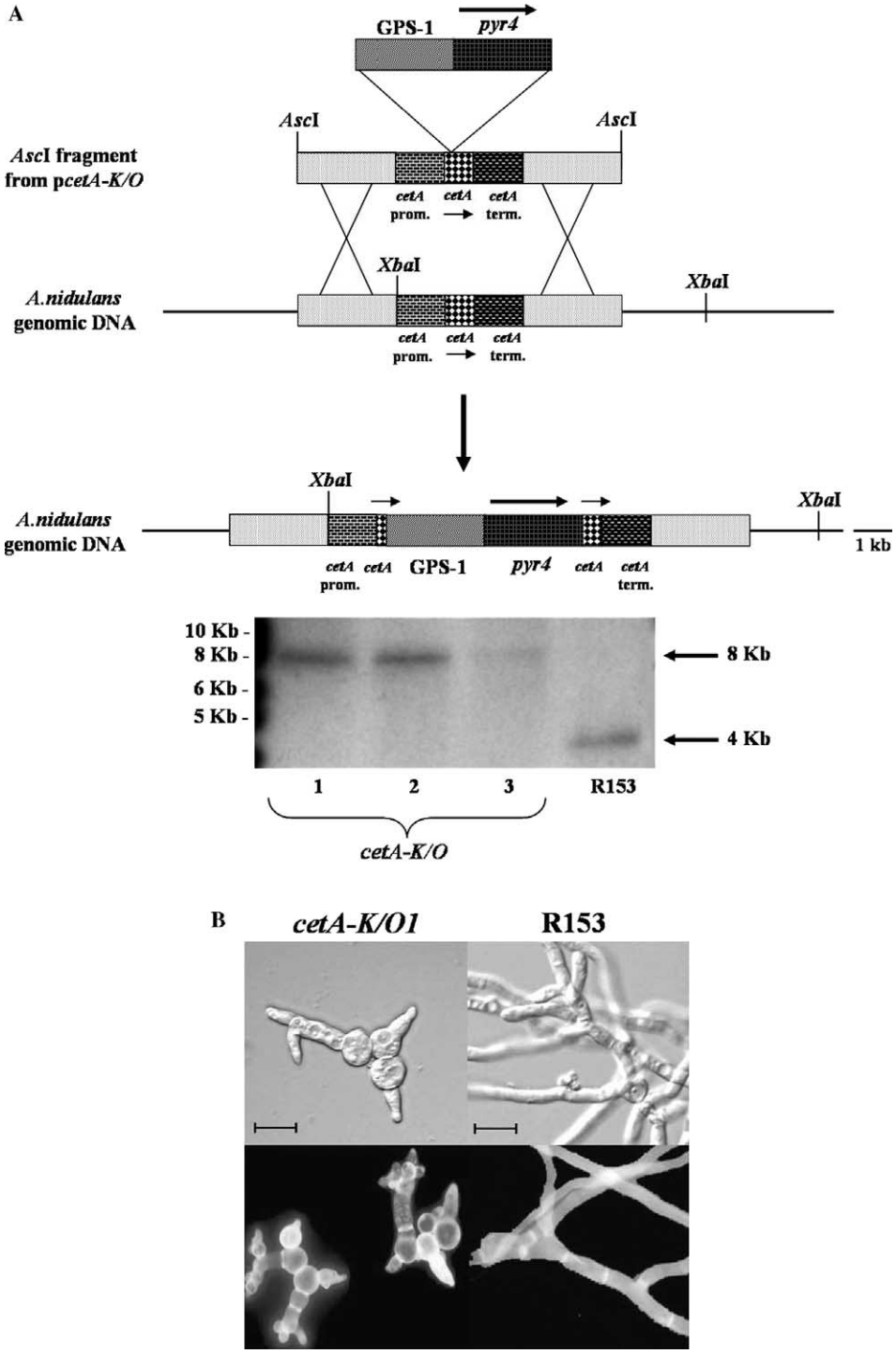

Fig. 3. Disruption of the *cetA* gene in *A. nidulans*. (A) Schematic representation of the *cetA* wild-type locus and the plasmid pK/O *cetA Asc*I-cut insert used for disruption and the Southern blot analysis of the *A. nidulans* control (R153 strain) and *cetA* disrupted strains (1–3). Genomic DNA (10 μg/well) was digested with *Xba*I, resulting in a ~4 kb fragment for the R153 control strain and a ~8 kb fragment for the *cetA* disrupted strains. (B) The *cetA* disrupted strain exhibits abnormal hyphal morphology and calcofluor cell-wall staining. *CetA-K/O1* and R153 control strains were grown for 12 h on glass coverslips in YAG medium and DIC/Nomarski microscopy (top panel) or fluorescence microscopy for calcofluor staining (lower panel) were performed. Bar, 20 μm.

have been isolated from many plant species, and are believed to be involved in plant defense because their synthesis is induced or strongly enhanced upon challenge of the plants by pathogens or stress. They are highly conserved, low molecular-weight (15–30 kDa) acidic or basic secreted proteins resistant to heat or acid treatments. Eight conserved disulfide bridges contribute to the stability of the PR5 proteins. Indeed, four cysteine residues in the CETA C-terminal region of the protein (Cys136, 141, 148, and 164) are conserved among fungal and plant PR-5 proteins.

Analysis of the transcriptional and translational control of the *cetA* gene indicated a high level of complexity. Tran-

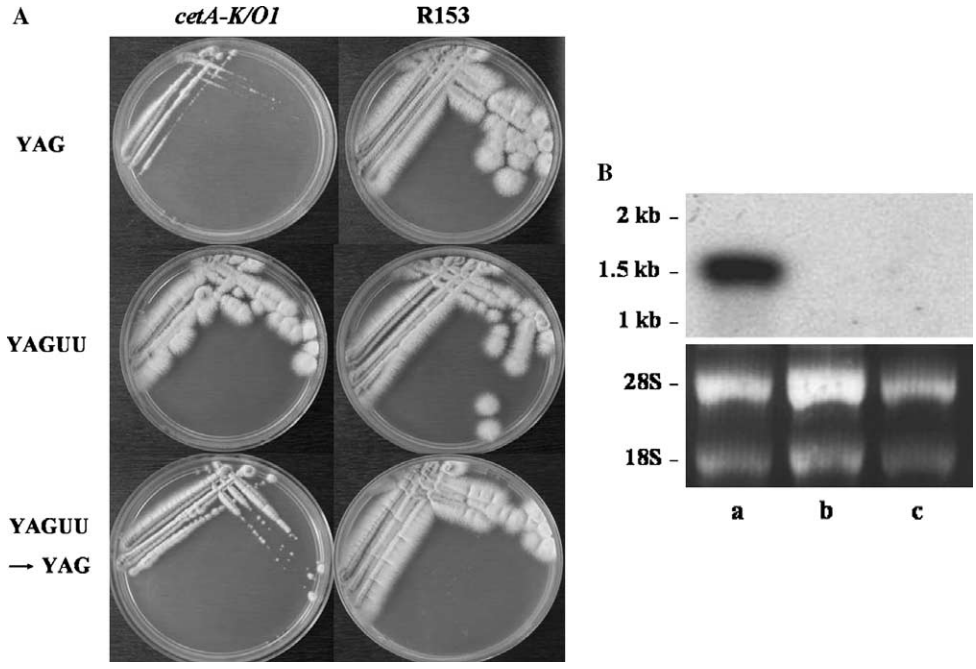

Fig. 4. The *cetA* disrupted strain is delayed in growth when grown in the absence of uracil/uridine (UU) but this phenotype is remediable in the presence of uracil/uridine (UU). (A) *CetA-K/O1* and R153 control strains were grown for 48 h on YAG agar plates in the absence (top panel) or presence (middle panel) of UU. Conidia taken from these plates were restreaked on YAG plates without UU, resulting in the reappearance of the mutant phenotype (lower panel). (B) Northern blot analysis of *cetA* mRNA levels in freshly harvested conidia collected from: lane (a) control R153 wild-type. (b) *CetA-K/O1* grown on YAG, and (c) *CetA-K/O1* grown on YAGUU.

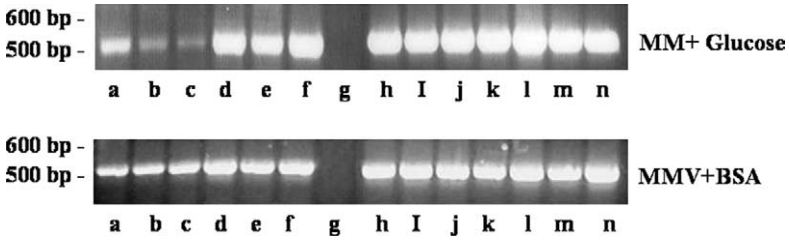

Fig. 5. The *pyr4* selectable marker disrupting the *cetA* gene undergoes glucose-dependent transcriptional repression. (A) RT-PCR analysis of *pyr4* mRNA expression in *CetA-K/O1-3* strains (lanes a–c) and three random *pyr4* integrants outside of the *cetA* locus (lanes d–f), in the presence of MM containing glucose (top panel) or albumin (lower panel) as sole carbon sources. Loading was controlled by running parallel PCR using *gpdA* primers (lanes h–n). Lane (g) contains R153 wild-type reverse-transcribed RNA amplified with the *pyr4* primers as a negative control.

Table 3
Radial growth of the cetA-K/O and WT strains on agar plates containing alternative carbon sources[a]

Medium	*cetA-K/O*	WT	% of WT
YAG	0.07 ± 0.03 [b]	1.2 ± 0.12	5.8
MM + Glucose	0.16 ± 0.03	1.37 ± 0.07	11.7
MM + BSA	0.72 ± 0.08	0.79 ± 0.02	91.1

[a] Strains were streaked on plates and grown for 24 h at 37 °C.
[b] Averaged colony diameter (in centimeters) of 20 individual colonies.

scription and translation are temporally uncoupled; *cetA* mRNA is a stored conidial transcript which is only translated during early germination. In this aspect, *cetA* differs from the previously characterized conidial-specific genes SpoC1-C1C and *CatA*, encoding a non-essential gene of unknown function and a conidial-specific catalase, respectively, in which both transcript and protein are expressed during conidiogenesis and rapidly disappear during germination (Navarro et al., 1996; Stephens et al., 1999). Surprisingly, unlike *cetA* the *A. fumigatus AfCalA* and *AfCalB* homologues are not stored as transcripts in dormant spores. This suggests that species-specific differences in the nature of stored transcripts between related *Aspergillus* species may exist.

We also found that *cetA* transcription is subject to glucose-mediated carbon catabolite repression (CCR). In *A. nidulans* CCR is regulated by the DNA-binding transcriptional repressor *creA* (Felenbok et al., 2001), and several putative CREA binding sites are present in the *cetA* promoter region. We also show that *cetA* is not subject to CCR in a mutant lacking *pkaA*, suggesting a connection between *cetA* transcription and the PKA pathway. A substantial fraction of conidial enriched genes may be transcriptionally regulated by the availability of glucose. Four of the twelve

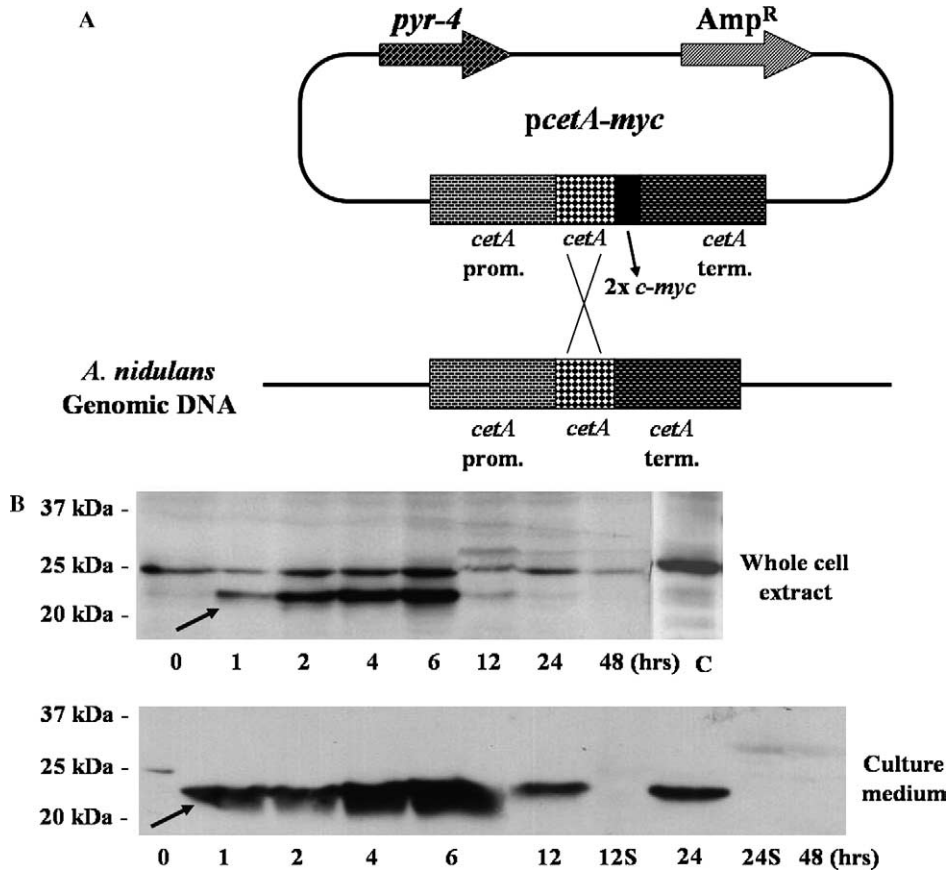

Fig. 6. CETA protein is expressed and secreted during early germination. (A) Schematic representation of the p*cetA-myc* plasmid used to generate the CETA-myc-expressing *A. nidulans* strain. p*cetA-myc* contains the *cetA* promoter and coding sequences tagged with two C-terminal c-myc epitopes and the *cetA* terminator region. (B) Western blot analysis of CETA protein expression in the *cetA-myc1* strain in whole cell extracts (upper panel) and culture medium (lower panel). Each lane contains 10 μg of protein extracted from ungerminated conidia (0 h), swollen germinating conidia (1, 2, and 4 h), germlings (6 h), early and late mycelium (12 and 24 h),and conidiating mycelium (48 h). Lane C contains control R153 whole cell extract. Hybridization was performed with the c-myc specific monoclonal antibody mAb 9E10, as described in Materials and methods. The arrow denotes the location of CETA protein.

conidial enriched transcripts we identified previously, including *cetA*, *grg1*, *acuD* (isocitrate lyase), and *acuF* (PEP carboxykinase) are repressed by glucose (Osherov et al., 2002).

We demonstrate that CETA protein is not found in dormant conidia, suggesting it is not involved in conidiogenesis. CETA is rapidly synthesized during early germination and subsequently secreted into the culture medium. This suggests that the CETA protein performs an extracellular function. However, no obvious phenotype was associated with *cetA* disruption, suggesting that *cetA* may have redundant function with the closely related homolog *AnCalA*. To test this possibility, we are now creating a *cetA*/*calA*-disrupted mutant. Recent observations show that several of the plant thaumatin-like proteins bind and hydrolyze (1,3)-β-glucans of the type commonly found in fungal cell-walls (Grenier et al., 1999; Menu-Bouaouiche et al., 2003; Osmond et al., 2001; Trudel et al., 1998). It is tempting to speculate that the CETA and CALA proteins may have a similar function in *A. nidulans* during germination, perhaps acting as cell-wall softening agents by hydrolyzing conidial (1,3)-β-glucans.

An interesting and unexpected finding revealed during disruption of the *cetA* gene is that the *pyr4* selectable marker used in the disruption undergoes glucose-dependent transcriptional repression. This results in greatly reduced transcription of *pyr4* and a phenotype characterized by delayed germination and impaired hyphal growth. These severe morphological consequences may be the result of a reduction in the level of the *pyr4* gene product, orotidine 5′ phosphate (OMP) decarboxylase, a key enzyme in the biosynthesis of uridine diphosphate (UDP). UDP is required in the biosynthesis of chitin and glucan, the major polysaccharide components of the fungal cell wall. We further demonstrate that the mutant phenotype is wholly a result of *pyr4* repression as it is fully remediated in the presence of uracil/uridine. Indeed, the *cetA-K*/*O* strains, when grown in the presence of uracil/uridine, show no obvious growth defects under a wide variety of conditions (high or low temperature, pH, osmolarity, addition of cell-wall destabilizing agents, and antifungal compounds), (Our unpublished data), as compared to the wild-type strain, indicating that the *cetA* gene is not essential.

Transcriptional repression of selectable markers has not been previously documented in *Aspergillus* species. In contrast, differences in the expression of the URA3 marker in *Candida albicans* depending on its genetic locus

("position effects") have been well documented (Lay et al., 1988; Staab and Sundstrom, 2003). Such "position effects" in *C. albicans* mutants prepared with the URA3 marker can affect both adherence and virulence in infected mice leading to erroneous conclusions regarding the function of the deleted target gene. We demonstrate that a similar problem may be encountered in *Aspergillus* species.

What might be the mechanism through which *pyr4* repression occurs? It is unlikely that *pyr4* transcription is repressed by silencing, which normally occurs in telomeric regions and mating-type (*MAT*) loci (Pirrotta and Gross, 2005), as the *cetA* locus is situated in the middle of chromosome 6 and the *MAT*-1 and *MAT*-2 mating loci are not found in its vicinity. The most likely explanation is that the *pyr4* cassette undergoes glucose-dependent transcriptional repression by the *cetA* promoter. The *pyr4* cassette is located 1.8 kb downstream of the *cetA* promoter in the 5′–3′ direction, necessitating repression at a distance. Activation at a distance has been previously demonstrated for the *podG* tRNA synthetase-encoding gene by the *niaD* promoter (Osherov et al., 2000). *podG* expression was activated at a distance of 10 kb from the *niaD* promoter and in the antisense orientation. This result however, was obtained in an AMA-1 based autonomously replicating vector and not in a chromosomal locus. It is possible that additional selectable markers used to transform *Aspergillus* strains may be similarly repressed by strong promoters in the vicinity of insertion. We therefore suggest that as a precautionary measure, following isolation and mapping, mutant *Aspergillus* strains be grown both with and without selection to confirm that the phenotypes remain the same. Additionally, all mutant strains should be outcrossed to a reference strain to confirm that the mutant phenotype segregates at a 1:1 ratio.

Acknowledgments

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