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Fungal Genetics and Biology xxx (2007) xxx–xxx
FUNGAL GENETICS AND BIOLOGY
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The *Aspergillus nidulans cetA* and *calA* genes are involved in conidial germination and cell wall morphogenesis

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Received 22 April 2007; accepted 7 July 2007

Abstract

The *Aspergillus nidulans* genes *cetA* (AN3079.2) and *calA* (AN7619.2) encode a novel class of fungal thaumatin-like proteins of unknown function. Deletion of *cetA* does not result in an observable phenotype [Greenstein, S., Shadkchan, Y., Jadoun, J., Sharon, C., Markovich, S., Osherov, N., 2006. Analysis of the *Aspergillus nidulans* thaumatin-like *cetA* gene and evidence for transcriptional repression of *pyr4* expression in the *cetA*-disrupted strain. Fungal Genet. Biol. 43, 42–53]. We prepared knockout *calA* and *calA*/*cetA* *A. nidulans* strains. The *calA* mutants were phenotypically identical to the wild-type. In contrast, the *cetA*/*calA* double mutant showed a synthetic lethal phenotype suggesting that the two genes affect a single function or pathway: most of its conidia were completely inhibited in germination. Many collapsed and underwent lysis. A few showed abnormal germination characterized by short swollen hyphae and abnormal hyphal branching. Nongerminated conidia contained a single condensed nucleus suggesting a block in early germination. This is the first functional analysis of the novel *cetA*/*calA* family of thaumatin-like genes and their role in *A. nidulans* conidial germination. We show that CETA and CALA are secreted proteins that together play an essential role in early conidial germination.

Keywords: *Aspergillus nidulans*; Conidial germination; Cell wall morphogenesis; Cell wall integrity; Thaumatin-like protein

1. Introduction

The asexual spore, or conidium, is vital in the life cycle of many fungi because it is their principal means of dispersion and serves as a 'safehouse' for the fungal genome under unfavorable environmental conditions. The process of conidial germination has been extensively studied in a number of fungi, including *Aspergillus nidulans*, *Aspergillus fumigatus*, *Neurospora crassa*, and several of the major plant-pathogenic fungi (Osherov and May, 2001). Germination is generally triggered by nutrient sensing and is characterized by conidial swelling, adhesion, nuclear reorganization and hyphal growth.

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

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doi:10.1016/j.fgb.2007.07.005

The conidium contains a pre-existing pool of mRNA molecules which are rapidly translated during early germination (Aramayo et al., 1989; Osherov and May, 2000). One of these, *cetA* (AN3079.2) is a member of a novel family of fungal genes of unknown function with homology to plant thaumatin-like (PR-5) defense proteins (Greenstein et al., 2006). *cetA* transcription is repressed by glucose and by the presence of protein kinase A (PKA). The CETA protein is highly expressed during the first 6 h of germination and is secreted into the medium. Disruption of the *cetA* gene however, results in no observable phenotype. *Aspergillus nidulans* contains an additional *cetA*-like gene, *calA* (AN7619.2), whose transcript is not expressed in dormant conidia but only during germination (Greenstein et al., 2006). We hypothesized that *cetA* and *calA* are involved in similar functions and that deletion of both genes may reveal their function.

In this report, we undertook a detailed study of the *calA* gene, analyzing its transcriptional regulation in wild-type and mutant strains of *A. nidulans*, as well as the expression pattern of the CALA protein. We prepared *A. nidulans* mutant strains in which *calA* alone or in combination with *cetA* was deleted. Our results indicate that while deletion of the *calA* gene alone results in no obvious phenotypic changes, deletion of both *calA* and *cetA* causes profound defects in germination. The implications of our findings 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 $MgCl_2$, 1.5% agar (w/v) for solid plates), supplemented with trace elements and vitamins (Bainbridge, 1971), was used for growth. Uracil (10 mM) and uridine (5 mM) (UU) were added to the *cetA*- and *cetA*/*calA*-deleted strains. Conidia were harvested in 0.2% (w/v) Tween 80, resuspended in double-distilled water (DDW) and counted in a hemacytometer. When specified, minimal medium (MM) was used (70 mM $NaNO_3$, 2% (w/v) glucose, 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 with 0.2% (w/v) BSA (bovine serum albumin).

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 previously described "hot SDS/phenol" method (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 approximately 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 described previously by May and Morris (1988). This additional grinding step was 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 described in Jadoun et al. (2004).

2.3. Deletion of the *A. nidulans calA* gene and generation of the double mutant *cetA*/*calA*

A 4516-bp DNA fragment flanking the *A. nidulans calA* gene was generated by PCR, using the Expand high-fidelity PCR system (Roche Diagnostic, Penzberg, Germany) and primers *Asc*I-*calA* 5′ and *Asc*I-*calA* 3′ (Table 2). These primers were designed to contain an AscI restriction site at their 5′ end (marked in italics in Table 2). The *calA* gene, including 779 bp upstream and 610 bp downstream of the open-reading frame (ORF) was then removed by digestion with XhoI and replaced with a hygromycin-selectable marker to produce the *calA*-K/O plasmid. The hygromycin cassette, containing 5′ and 3′ XhoI restriction sites was generated by PCR amplification using primers *Hyg 5′* and *Hyg 3′* (Table 2). For transformation, 10 μg of spin-purified AscI-digested *calA*-K/O plasmid was used. Transformation was performed as described by Osherov and May (2000), except for the following modifications: (i) Prior to transformation, conidial protoplasts were resus-

Table 1
Strains used in this study

Strain	Genotype	Source
R153	*wA3;pyroA4*	FGSC[a]
A23	*pabaA1; yA2;chaA1*	FGSC
ΔpkaA (TKIS18.11)	*pabaA1; yA2; ΔpkaA:: argB;_argB:: trpC; trpC801;veA1*	N.P. Keller
cetA-K/O1	*wA3; cetA-K/O:: pyr4;pyrG89;pyroA4*	Greenstein et al. (2006)
cetA-K/O12	*yA2;pabaA1; cetA-K/O:: pyr4;pyrG89*	This study
calA-K/O1	*wA3;pyroA4; calA-K/O::hyg*	This study
cetA/*calA*-K/O1	*wA3;pyroA4; calA-K/O::hgh; cetA-K/O:: pyr4;pyrG89*	This study
calA-myc1	*wA3; calA-myc:: pyr4;pyrG89;pyroA4*	This study
cetA-myc1	*wA3; cetA-myc:: pyr4;pyrG89;pyroA4*	Greenstein et al. (2006)

[a] FGSC, Fungal Genetics Stock Center.

Please cite this article in press as: Belaish, R. et al., The *Aspergillus nidulans cetA* and *calA* genes are involved ..., Fungal Genet. Biol. (2007), doi:10.1016/j.fgb.2007.07.005

Table 2
Primers used in this study

Name	Sequence
calA 5′	5′-ATGCTCTTCAACAAGATCATCAG-3′
calA 3′	5′-TCATGAGTCGTAAGACCGCCTG-3′
Hyg 5′	5′-AT*CTCGAG*GTCGACAGAAGATGATATTGAAGG-3′
Hyg 3′	5′-AT*CTCGAGG*CTCTCCCTTATGCGACTCCTGCA-3′
*Asc*I-*calA* 5′	5′-TA*GGCGCGCC*AACTAGGTAGTAACAGGAATT-3′
*Asc*I-*calA* 3′	5′-TA*GGCGCGCCC*TGATTGCTGTTGACGAGGTA-3′
*calA Not*I 5′	5′-TAACGAG*GCGGCCGC*GAATGA-3′
*calA myc Not*I 3′	5′-TTGC*GCGGCCGC*TTACAAGTCCAGAAATGAGCTT TCTTCTTGGCC GCCCAAGTCCTCTTCAGAAATGA GCTTTTGGCCGCCGTTCGCTGTACGTGA-3′

pended in 0.5 ml of 50 mM $CaCl_2$, 0.6 M KCl, 10 mM Tris–HCl, pH 7.5, containing 1 mM dithiothreitol and incubated overnight at 4 °C. (ii) Following transformation with AscI-cut plasmid DNA and plating on MMUU supplemented with 1.1 M sorbitol, the plates were incubated for 12 h at room temperature and then 10 ml of MMUU-top agar medium supplemented with 1.1 M sorbitol and 0.5 mg/ml hygromycin B was added to each transformation plate. The plates were incubated overnight at room temperature and then for 2 more days at 37 °C.

Transformants that tested positive for *calA* deletion by Southern analysis were crossed with strain *cetA* K/O12 to generate the *cetA*/*calA* double-deletion strain.

2.4. Nucleic acid manipulation

For Southern blot analysis, 10 μg of fungal genomic DNA was digested with EcoRI and run on a 1% agarose gel. Transfer and hybridization conditions were 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 the probes were prepared by PCR amplification of genomic DNA using primer pairs *Hyg* 5′ and *Hyg* 3′ for Southern analysis and *calA* 5′ and *calA* 3′ for northern analysis (Table 2). Northern analysis was performed as previously described (May and Morris, 1987).

2.5. Construction of the pcalA-myc plasmid

The p*calA-myc* plasmid containing *calA* with two C-terminal c-*myc* tags was created as follows: a 1884-bp PCR fragment of *calA-myc* containing the *calA* promoter region and ORF was obtained by amplification of *A. nidulans* genomic DNA with the oligonucleotides *calA Not*I 5′ and *calA myc Not*I 3′ (Table 2). This fragment was gel-purified, subcloned into the pGEM T/A cloning vector (Promega Corp. Madison Wisconsin, USA), verified by sequencing and inserted into the previously described p*cetA-myc* plasmid (Greenstein et al., 2006). p*cetA-myc* was digested with NotI to remove the *cetA* promoter and ORF, which was replaced with the *calA-myc Not*I fragment. The resulting plasmid, p*calA-myc*, was subsequently transformed into *A. nidulans* strain GR5 to generate the *calA-myc1* strain expressing *myc*-tagged CALA protein (Table 1).

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, Darmstadt, Germany), washed once in distilled water and dried. Nongerminated, 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. Protein was extracted in urea sample-buffer as described previously (Osherov and May, 1998). Protein was transferred to a nitrocellulose membrane (BA 85; Whatman Schleicher and Schuell, Brentford, UK) in a Mini-PROTEAN 3 cell blotting apparatus according to the manufacturer's instructions (Bio-Rad, Hercules CA, USA). Blots were blocked by incubation with TBST (TBS containing 0.1% Tween 20) containing 5% (w/v) skimmed milk for 1 h at room temperature. CALA 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 each. The tagged protein was visualized by ECL (enhanced chemiluminescence) using a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, West Grove PA, USA) at 1:10,000 dilution.

2.7. Microscopy

Conidia of the mutant and R153 control (wild-type) *A. nidulans* strains were incubated at concentrations of 10^4 conidia/ml for the indicated time at 37 °C on glass cover slips in 24-well plates (Nunclon surface; Nunc, Roskilde, Denmark). For nuclear staining, cover slips were fixed in 10 mM KPO_4 pH 6.8, 4% (v/v) paraformaldehyde, 0.1% (w/v) Triton X-100 for 30 min at room temperature, stained with 100 ng/ml DAPI (4′,6-diamidino-2-phenylindole) for 15 min, washed twice in PBS and analyzed. For staining of dead conidia and hyphae, cells were stained with 1,3 dibutylbarbituric acid (DiBAC) (2 μg/ml in 100 mM MOPS buffer, pH 7.0) for 1 h at room temperature, washed twice and analyzed. Images were obtained by fluorescence microscopy on an Olympus BX40 microscope equipped for fluorescence with a UV filter for DAPI staining and a fluorescein iso-thiocyanate (FITC) filter for DiBAC staining, at a total magnification of 200× and 400×. Images were recorded with a digital Olympus DP70 camera.

For CETA-*myc* and CALA-*myc* immunostaining, conidia were grown on cover slips in MMUU for 8 h, incubated in fixation solution (3.7% formaldehyde, 50 mM Hepes pH 6.7, 5 mM $MgSO_4$, 25 mM EGTA pH 7.0) for 45 min and

washed in PBS–BSA (1% BSA) for 5 min. Mouse-derived primary antibody *c-myc* (9E10) (Santa Cruz Biotechnology Inc. Santa Cruz, CA) was diluted 1:100 in PBS–BSA and added to the cover slips for 1 h. Cover slips were washed twice with PBSP (PBS containing 0.1% w/v Nonidet P-40) for 5 min. Alexa Fluor 488 anti-mouse secondary antibodies were used according to the manufacturer's instructions (Signal-Amplification Kit for Mouse Antibodies, Invitrogen Molecular Probes, Carlsbad CA). Images were obtained by fluorescence microscopy on an Olympus BX40 microscope as already described.

For transmission electron microscopy (TEM), conidia (0 h) and germinated conidia (6 h) were fixed in 2.5% glutaraldehyde in PBS. They were then washed, postfixed in 1% OsO_4 in PBS and washed again. After dehydration in graded ethanol solutions, the cells were embedded in glycid ether 100 (Serva Electrophoresis Gmbh, Heidelberg, Germany). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a Jeol 1200 EX TEM. For scanning electron microscopy (SEM), conidia (0 h) and germinated conidia (6 h) were fixed with 2.5% glutaraldehyde in PBS, dehydrated in ethanol, CO_2—critical-point-dried, gold-coated and examined in a Jeol JSM 840A SEM.

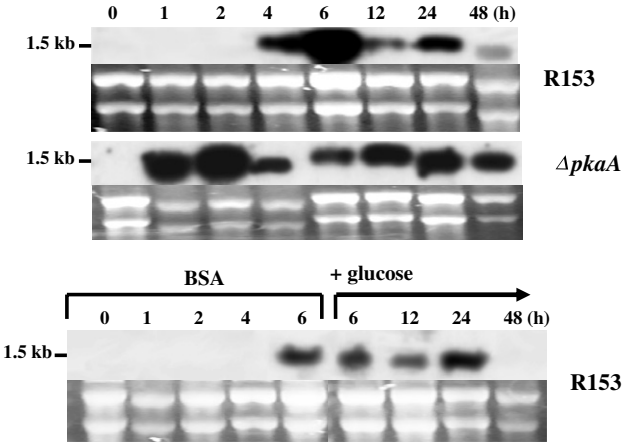

Fig. 1. Expression of *calA* mRNA in *A. nidulans*. Northern blot analysis of *A. nidulans calA* expression in the wild-type (R153) strain (top panel), and *ΔpkaA* mutant strain (middle panel), in MM defined medium containing glucose or following glucose addition to BSA (lower panel). Each lane contains 5 μg of total RNA from ungerminated conidia (0 h), swollen germinating conidia (1, 2, 4 h), germlings (6 h), early and late mycelium (12, 24 h), and conidiating mycelium (48 h). Each blot was hybridized with the *calA* ^{32}P-labeled DNA probe. Ethidium-bromide staining of rRNA in the agarose gel was performed to control the loading (lower panels).

3. Results

3.1. Northern analysis of A. nidulans calA mRNA expression

We have previously demonstrated that *cetA* expression is inhibited by PKA activity and is subject to glucose repression. We therefore examined the expression of *calA* in an *A. nidulans* mutant lacking the catalytic subunit of PKA (strain *ΔpkaA*) and in the wild-type strain (R153) following glucose shift. We found that as for *cetA*, deletion of *pkaA* results in constitutive expression of *calA* throughout the vegetative life cycle of the fungus, suggesting that PKA activity inhibits or attenuates *calA* expression (Fig. 1, top panel). To test the effect of glucose addition on *calA* transcription, *A. nidulans* conidia were allowed to germinate for 6 h in the presence of albumin as the sole energy source, after which glucose was added to the medium and *calA* mRNA levels monitored over time (Fig. 1, lower panel). Addition of 2% glucose after 6 h did not result in down-regulation of *calA* expression, indicating that unlike *cetA*, *calA* is not subject to glucose repression.

3.2. Western analysis of CALA expression

Our previous experiments showed that CETA is synthesized during early germination and is a secreted protein. To investigate the temporal expression pattern and localization of the CALA protein, an integrative plasmid, p*calA-myc*, containing the *calA* gene tagged with two C-terminal *c-myc* epitopes under the control of its endogenous promoter (Fig. 2a), was constructed as described in Section 2. After transformation into GR5, *pyrG*+ transformants were examined by Southern blot. One of the transformants, *calA-myc1* in which the plasmid had integrated in a single copy at the *calA* locus was selected for further study. We examined the expression pattern of the tagged CALA protein by Western blot. Total protein was extracted from dormant and germinating *calA-myc1* conidia at different time points and probed with *c-myc*-specific monoclonal antibodies. CALA-*myc* protein was expressed as a ~23-kDa band which was not detectable in nontransformed control R153 cells (Fig. 2b). The *calA-myc1* strain germinated and developed normally on MM, suggesting that expression of the *myc*-tagged CALA protein has no deleterious effects on morphogenesis (not shown). CALA protein was not detectable in dormant *A. nidulans* conidia, but was expressed at increasing levels during the first 12 h of germination (Fig. 2b, top panel). Analysis of culture medium for the presence of extracellular CALA protein indicates that it is indeed a secreted protein and that it accumulates mainly during the first 12 h of germination (Fig. 2b, lower panel).

Immunofluorescence analysis was performed on *calA-myc1* and *cetA-myc1* conidia germinated for 8 h on coverslips and analyzed by immunofluorescence using *c-myc*-specific monoclonal antibodies. Both CETA and CALA proteins were labeled in the cell walls of the germinating conidia but not in the germtubes (Fig. 2c). Surprisingly, both proteins were also labeled in a diffuse halo surrounding the cells, suggesting that they may incorporate into a secreted extracellular matrix.

3.3. Deletion of calA and generation of a calA/cetA double mutant

To investigate the effect of loss of function of the *calA* gene in *A. nidulans*, a deletion plasmid (p*calA*-K/O) was

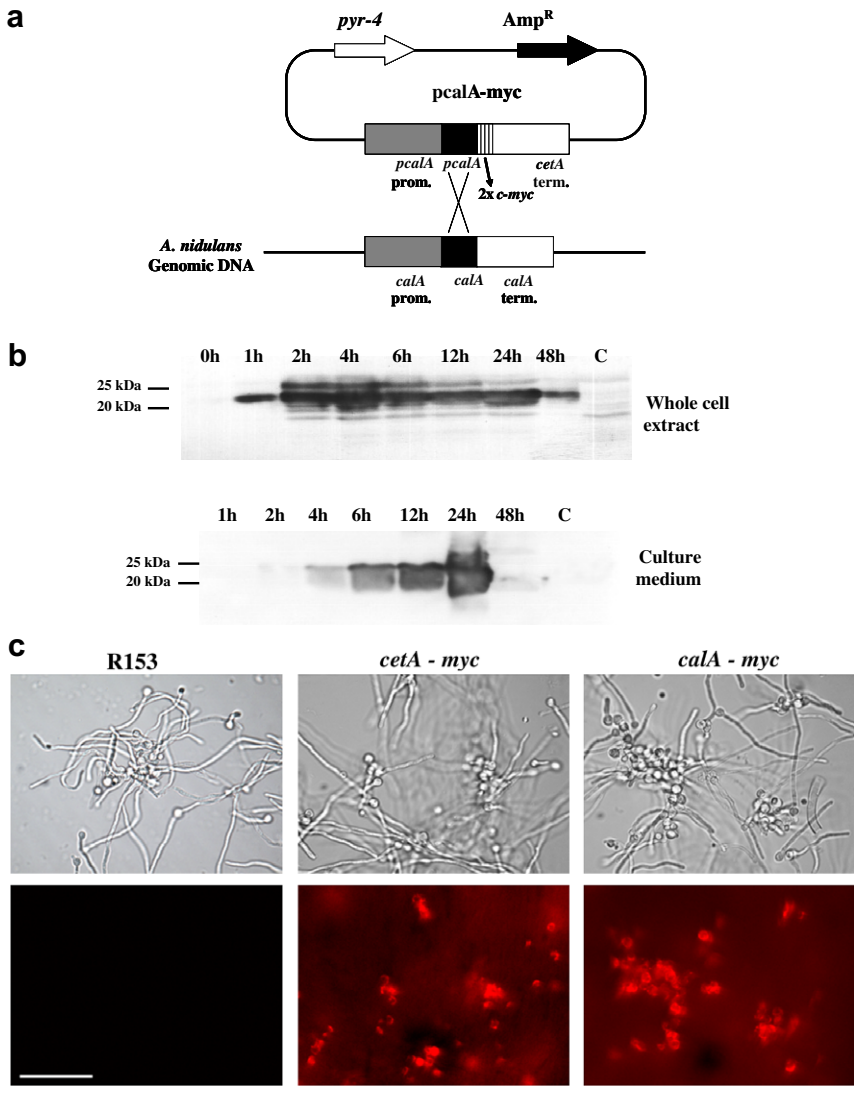

Fig. 2. CALA protein is expressed and secreted during early germination and is found primarily in the cell wall and extracellular matrix. (a) Schematic representation of the p*calA-myc* plasmid used to generate the CALA-*myc*-expressing *A. nidulans* strain. p*calA-myc* contains the *calA* promoter and coding sequences tagged with two C-terminal *c-myc* epitopes and the *cetA* terminator region. (b) Western blot analysis of CALA protein expression in the *calA-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, 4 h), germlings (6 h), early and late mycelium (12, 24 h), and conidiating mycelium (48 h). Lane C contains control wild-type R153 whole cell extract. Hybridization was performed with the *c-myc* specific monoclonal antibody mAb 9E10, as described in Section 2. (c) Immunofluorescence analysis of CETA and CALA protein localization in germinated (8 h) conidia expressing CETA-*myc* or CALA-*myc*. No immunofluorescence is detectable in untransformed control wild-type (R153) cells. Top panel: immunolabeled cells; lower panel: corresponding DIC images.

constructed as described in Section 2 (Fig. 3a). After transformation of p*calA*-K/O into R153, 25 *hyg*^{r} transformants were purified and screened by PCR for insertion into the *calA* locus. Three putative mutants were identified and further characterized by Southern blot analysis (Fig. 3b). Based on this analysis, all three transformants, (*calA*-K/O1-3) showed deletion of the *calA* gene. When the *calA*-K/O strains were outcrossed to a tester strain (A23, Table 1), *calA*-deletion as deduced by PCR analysis and hygromycin resistance co-segregated at the expected 1:1 ratio ($n = 50$). The *cetA*/*calA* double mutant strain was generated by crossing *calA*-K/O1 with *cetA*-K/O12 (Table 1). Six *cetA*/*calA* double knockout strains resulting from this cross were initially characterized and all exhibited the same mutant phenotype. One of them, *cetA*/*calA*-K/O1, was used in this study.

3.4. Deletion of both cetA and calA results in the inhibition of germination

The growth phenotypes of the *calA*-K/O1, *cetA*-K/O1, *and calA*/*cetA*-K/O1 strains were compared to that of the wild-type R153 strain by streaking (Fig. 4a, top panel) on YAGUU agar plates. No differences were seen in the growth rates or colony morphologies of the four strains. However, microscopic analysis of the streaked agar plates

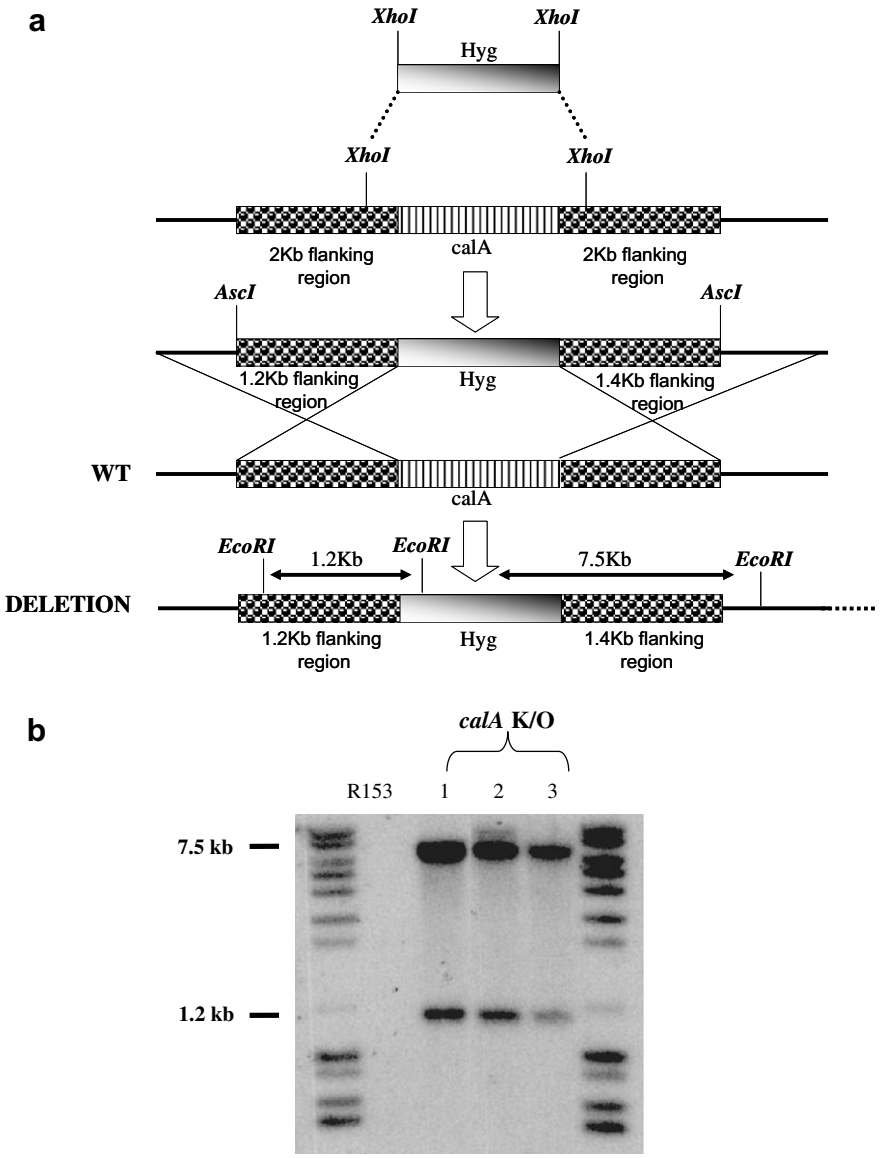

Fig. 3. Deletion of the *calA* gene in *A. nidulans*. (a) Schematic representation of the *calA* wild-type locus and the plasmid p*calA*-K/O AscI-cut insert used for deletion and (b) Southern blot analysis of the *A. nidulans* control (R153 strain) and *calA*-deleted strains (1–3). Genomic DNA (10 μg/well) was digested with EcoRI, and probed with *hyg* ^{32}P-labeled DNA probe, resulting in a 7.5 kb fragment for the *calA*-deleted strain and no hybridization for the R153 control strain.

after 8 h of incubation revealed that most of the *calA*/*cetA*-K/O1 double-mutant conidia ($80 \pm 2\%$; $n = 200$) had failed to germinate (Fig. 4b). Of the 20% germinated conidia, most (19%) grew abnormally, producing short swollen hyphae. A small fraction (1%; $n = 1000$) developed normally, producing colonies that were indistinguishable in size or morphology from the wild-type. Importantly, conidia collected from these colonies exhibited the double-mutant phenotype, indicating that they were not revertants. A similar pattern was seen in submerged liquid culture; $91 \pm 2\%$ ($n = 400$) of the double-mutant conidia were completely inhibited in germination (Fig. 4c). This defect could not be remediated by the addition of osmotic stabilizers or of wild-type culture filtrate.

In contrast, we were unable to identify any phenotypic changes in the *calA*-K/O1 and *cetA*-K/O1 single mutants compared to the wild-type strain, both under the above-described conditions or when grown on either solid or liquid MM at elevated (42 °C) and reduced (25 °C) temperatures, on several different sole carbon sources (glycerol, acetate, fructose, raffinose, bovine serum), nitrogen sources (ammonium sulfate, nitrate), and either high or low salt concentrations. Therefore, further phenotypic analysis was performed on the *cetA*/*calA*-K/O1 double mutant.

3.5. Abnormal nuclear staining and cell death in the cetA/calA-K/O1 double mutant

To further investigate the microscopic changes occurring in the *cetA*/*calA*-K/O1 double mutant, conidia were allowed to germinate for 8 h in liquid MMUU and stained with either DAPI (nuclear staining) or DiBAC (staining of

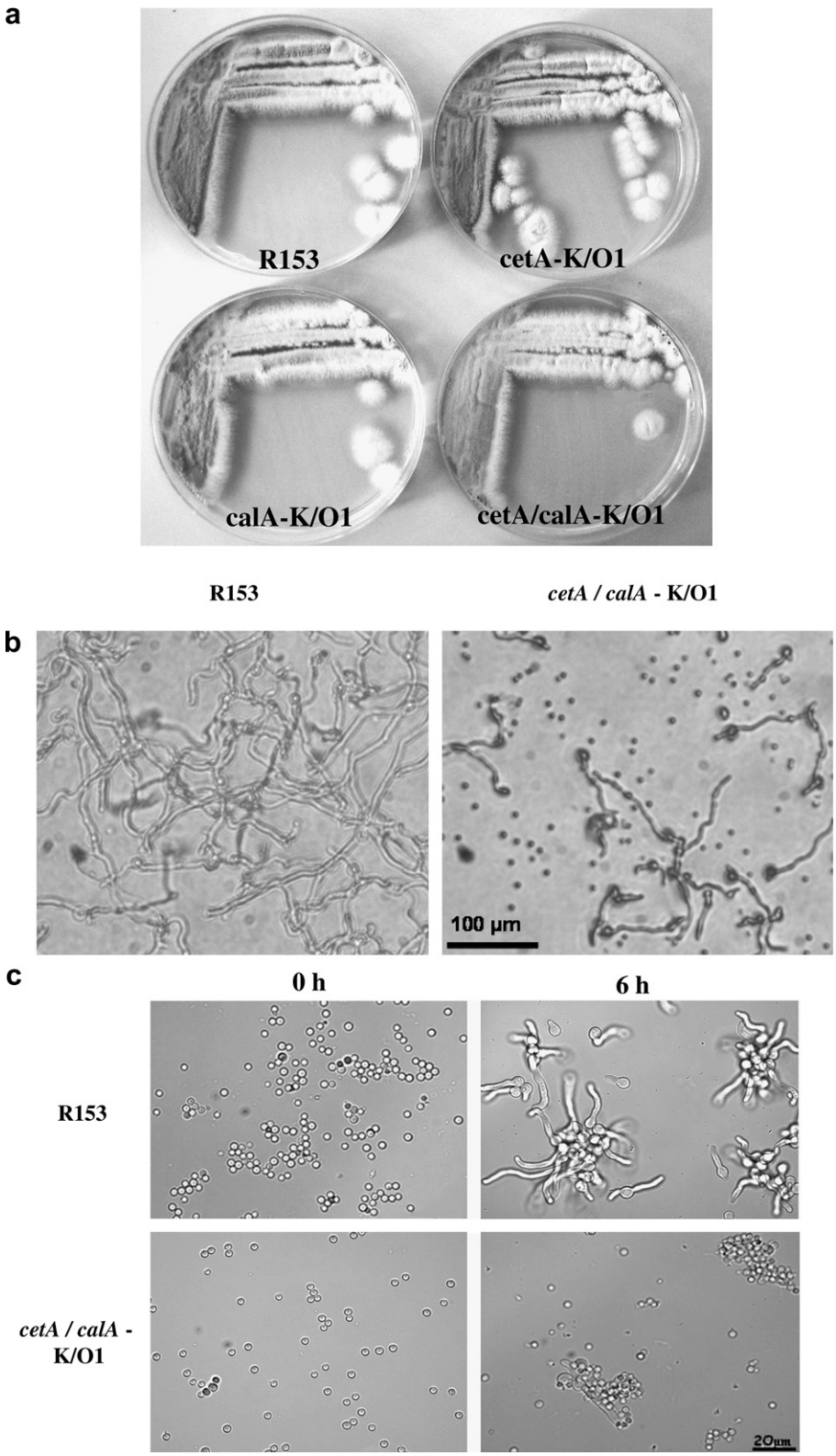

Fig. 4. The *cetA*/*calA*-K/O1 double mutant is blocked in germination. (a) R153 control strain, *cetA*-K/O1, *calA*-K/O1, and *cetA*/*calA*-K/O1 mutant strains were grown for 48 h at 37 °C on MMUU agar plates and appeared to develop normally. However, microscopic analysis of the *cetA*/*calA*-K/O1 double mutant growing for 8 h on MMUU agar (b) or liquid media (c) revealed that the majority of conidia failed to germinate.

dead cells). DAPI staining revealed the presence of a single condensed nucleus in most of the mutant conidia that failed to germinate (Fig. 5). This inability to undergo nuclear decondensation and mitosis, suggests a defect in the initiation of early germination. In some conidia, following germination, the nucleus appeared to be extruded from

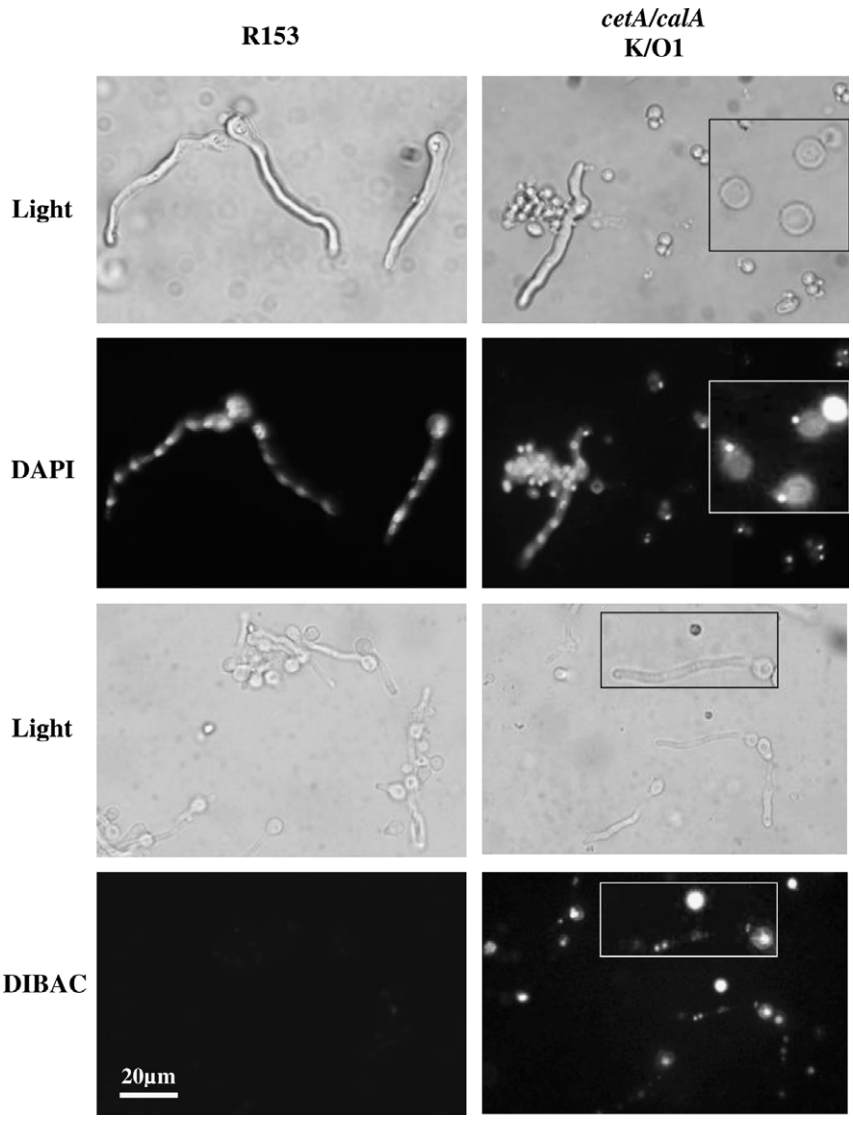

Fig. 5. The *cetA*/*calA*-K/O1 double mutant is blocked in nuclear decondensation that takes place during early germination and undergoes cell death. R153 control and *cetA*/*calA*-K/O1 mutant conidia were allowed to germinate for 6 h at 37 °C in liquid MMUU on glass coverslips. Cells were subsequently stained with either DAPI (top two panels) or DiBAC (lower two panels) and visualized by fluorescence microscopy.

the cells, suggesting that the cell wall had ruptured (DAPI panels, enlarged inset). A few conidia underwent abnormal germination producing swollen short hyphae and abnormal hyphal branching (Fig. 5). DiBAC morbidity staining showed that the mutant conidia were viable when they were harvested (data not shown) but that most died after 8 h of germination (Fig. 5).

3.6. cetA/calA conidia exhibit cell wall defects and lyse during germination

The *cetA*/*calA* double-mutant phenotype was analyzed at greater resolution. Nongerminated conidia and 6 h germlings of the wild-type and *cetA*/*calA*-K/O1 double mutant were collected and analyzed by TEM and SEM (Fig. 6a and b, respectively). In contrast to the smooth-surfaced wild-type conidia, the mutant conidia exhibited a crinkled cell surface and a poorly layered, electron-dense cell wall (Fig. 6). Following 6 h of germination, most of the mutant conidia exhibited a partial or complete loss of cytosol (note conidial 'ghosts' in Fig. 6c), suggesting that they had undergone a process of collapse and disintegration (Fig. 6a–c). This lytic process probably resulted from a weakness in the cell wall or its inability to develop and mature during germination.

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 other unknown pathways), and the transcription and translation of specific genes (Osherov and May, 2001). Previously, we used sup-

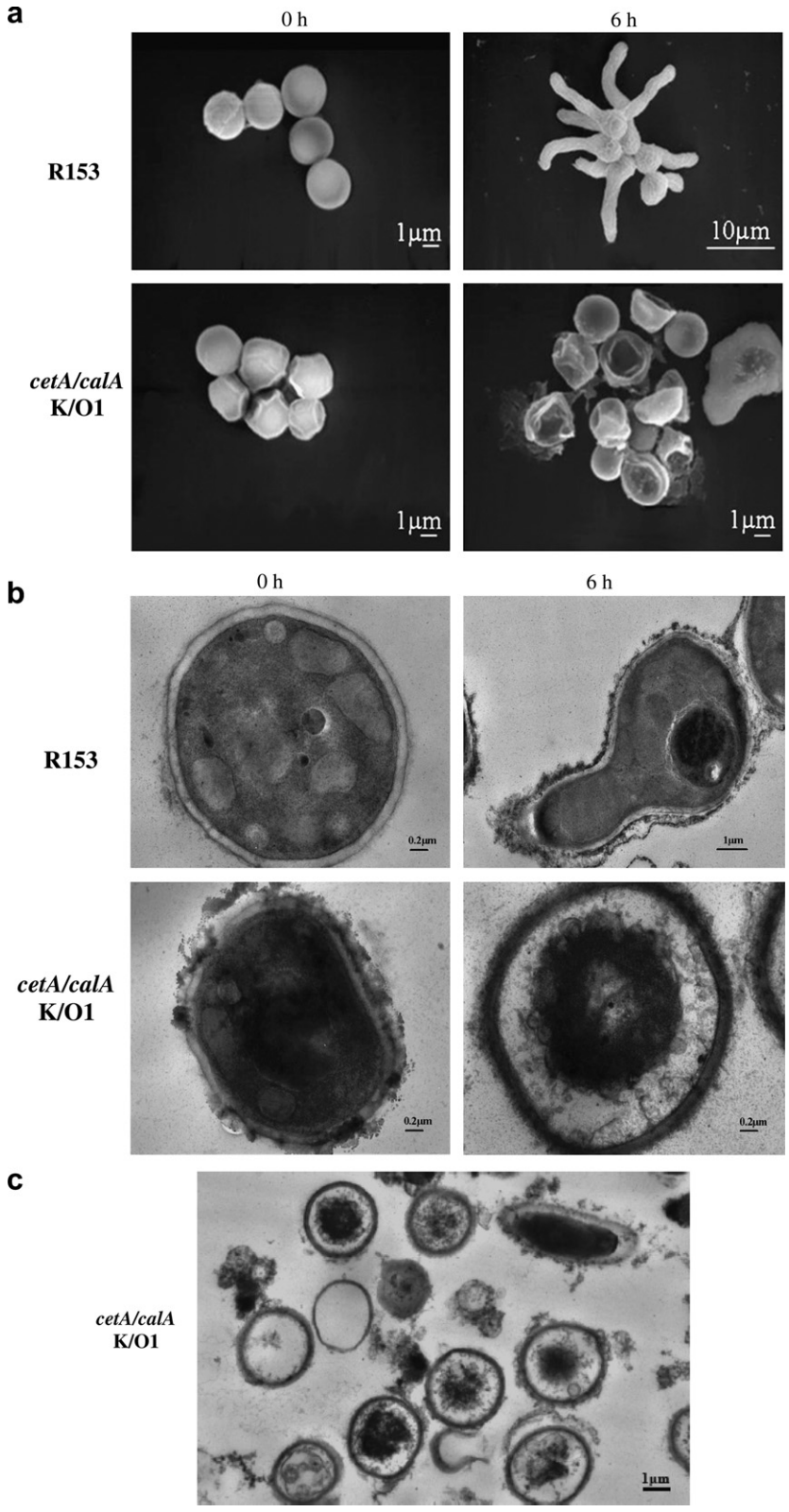

Fig. 6. The *cetA*/*calA*-K/O1 double mutant has a defective cell wall and undergoes autolysis during germination. Dormant conidia (0 h) and conidia that were allowed to germinate in liquid MMUU for 6 h at 37 °C (6 h) of R153 control and *cetA*/*calA*-K/O1 mutant strains were prepared as described in Section 2 and visualized by (a) scanning electron microscopy (SEM) and (b and c) transmission electron microscopy (TEM).

pressive-subtraction hybridization (SSH) to identify conidium-enriched transcripts (*cet* genes) (Osherov et al., 2002). Of these, the *cetA* gene was particularly intriguing, in that it encodes a protein with similarity to plant thaumatin-like proteins which have antifungal activity. CETA-like proteins are only found in the ascocarp-producing ascomycetes (Pez-

izomycotina) such as *Coccidioides immitis*, *Magnaporthe grisea*, and *N. crassa* and are not found in nonascocarp-producing ascomycete yeast such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* or in basidiomycetes such as *Cryptococcus neoformans* and *Ustilago maydis*. Three *cetA*-like homologs are found in *A. fumigatus* (*Afu3g09690*, *Afu8g01710*, and *Afu3g00510*) and two of these are expressed during early germination (Greenstein et al., 2006). An additional *cetA*-like gene, *calA* (AN7619.2), is also found in *A. nidulans* and is likewise expressed during early germination. However, unlike *cetA*, *calA* mRNA is not found in the dormant conidium, and is transcribed only after germination has begun (Greenstein et al., 2006). We have previously shown that conidial *cetA* mRNA is translated during early germination and that the protein is secreted. However, disruption of *cetA* does not result in an observable phenotype, suggesting that it may have a redundant function (Greenstein et al., 2006). In this report, we describe the functional analysis of the *calA* gene and the generation of *calA* and *cetA*/*calA*-deleted mutant strains.

We demonstrate that like CETA, the CALA protein is not found in dormant conidia, but is expressed and secreted during germination and early growth. Using immunofluorescence microscopy, we show that during germination both proteins are found in the cell wall and in the matrix surrounding the cells. Very little information is available regarding the extracellular matrix in *Aspergillus* species. *A. fumigatus* in culture produces an amorphous polysaccharide-rich extracellular matrix which can be visualized by cryo-SEM and confocal microscopy (Beauvais et al., 2007). However, the proteins incorporated in this matrix in *A. nidulans* have yet to be identified. CETA and CALA may serve as a starting point to further investigate this matrix.

We show that the *cetA* and *calA* genes are synthetically lethal: while deletion of either one results in no obvious phenotypic changes, deletion of both genes is lethal, resulting in inhibition of germination at an early stage, preceding nuclear decondensation and mitosis.

Mutations that block germination at its earliest visible steps are rare. We previously conducted a screen for such mutants and identified mutations in five genes, *sgdA-E*, that lead to the early arrest of germination. Four of the five genes encode proteins directly involved in protein synthesis and folding (Osherov and May, 2000). In contrast, *cetA* and *calA* define a novel category of genes involved in early germination with a possible role in cell wall remodeling.

The conidial cell wall in *A. nidulans* undergoes profound changes in polysaccharide and protein content during germination, changing from a dense melanized protective shield into a dynamic interacting sieve (Bernard and Latge, 2001). We speculate that in the absence of CALA and CETA this process is damaged, causing a block in cell wall maturation, the subsequent collapse of the cell wall and conidial lysis. A phenotype characterized by conidial lysis has been previously described in *A. nidulans* strains mutated in *chsD* which encodes a chitin synthase (Borgia et al., 1996) and *orlA* encoding a trehalose-6-phosphate phosphatase (Specht et al., 1996). However, the lytic phenotype of the *cetA*/*calA* double mutant differs significantly from these mutant strains in that it does not undergo conidial swelling and is not osmotically remediable. It also differs from the conidial lysis seen in strains mutated in the transcriptional regulator *wetA*, which occurs during late conidiogenesis and before germination (Marshall and Timberlake, 1991).

One of this study's findings was surprising. A small proportion (~1%) of the *cetA*/*calA* mutant conidia underwent seemingly normal germination and growth on agar plates, despite the severity of the phenotype seen in most of the conidia. Importantly, conidia collected from these colonies exhibited the double-mutant phenotype, indicating that they were not revertants. One possible explanation for this phenomenon, in addition to activation of general compensatory mechanisms, is that other, more distantly related *cetA*/*calA*-like genes are upregulated in these conidia. Indeed, the genome of *A. nidulans* contains two additional genes (AN8043.3 and AN1863.3) encoding small proteins (<200 amino acids in length) with low homology (25–30% identity) to CETA and CALA.

In summary, our results show that CETA and CALA are expressed during germination and vegetative growth. While the *cetA* transcript is stored in the dormant conidium and is rapidly translated during germination, *calA* is both transcribed and translated during germination. While *cetA* transcription is repressed by glucose (Greenstein et al., 2006), *calA* transcription is not. Both CETA and CALA proteins accumulate in the cell wall during secretion. They are subsequently secreted into the extracellular matrix and surrounding medium. What could the biological functions of CETA and CALA be? 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 (Trudel et al., 1998; Grenier et al., 1999; Osmond et al., 2001; Menu-Bouaouiche et al., 2003). In the fungi, thaumatin-like proteins from several basidiomycetes exhibited (1,3)-β-glucanase activity (Grenier and Asselin, 2000; Sakamoto et al., 2006). Corresponding to the phenotype we observed, the CETA and CALA proteins may have a similar function in *A. nidulans* during germination, perhaps acting as cell wall-softening agents by binding or hydrolyzing conidial (1,3)-β-glucans. Ongoing experiments in our laboratory are being conducted to identify the biochemical activities of CETA and CALA.

Acknowledgment

This work was funded by the Israel Academy of Sciences, Grant 280/06, to N.O.

References

Aramayo, R., Adams, T.H., Timberlake, W.E., 1989. A large cluster of highly expressed genes is dispensable for growth and development in *Aspergillus nidulans*. Genetics 122, 65–71.

Bainbridge, B.W., 1971. Macromolecular composition and nuclear division during spore germination in *Aspergillus nidulans*. J. Gen. Microbiol. 66, 319–325.

Beauvais, A., Schmidt, C., Guadagnini, S., Roux, P., Perret, E., Henry, C., Paris, S., Mallet, A., Prevost, M.C., Latge, J.P., 2007. An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. Cell. Microbiol. 9, 1588–1600.

Bernard, M., Latge, J.P., 2001. *Aspergillus fumigatus* cell wall: composition and biosynthesis. Med. Mycol. 39 (Suppl. 1), 9–17, Review.

Borgia, P.T., Miao, Y., Dodge, C.L., 1996. The *orlA* gene from *Aspergillus nidulans* encodes a trehalose-6-phosphate phosphatase necessary for normal growth and chitin synthesis at elevated temperatures. Mol. Microbiol. 20, 1287–1296.

Chang, M.H., Chae, K.S., Han, D.M., Jahng, K.Y., 2004. The GanB Galpha-protein negatively regulates asexual sporulation and plays a positive role in conidial germination in *Aspergillus nidulans*. Genetics 167, 1305–1315.

Fillinger, S., Chaveroche, M.K., Shimizu, K., Keller, N., d'Enfert, C., 2002. cAMP and ras signalling independently control spore germination in the filamentous fungus *Aspergillus nidulans*. Mol. Microbiol. 44 (4), 1001–1016.

Fortwendel, J.R., Panepinto, J.C., Seitz, A.E., Askew, D.S., Rhodes, J.C., 2004. *Aspergillus fumigatus rasA* and *rasB* regulate the timing and morphology of asexual development. Fungal Genet. Biol. 41, 129–139.

Greenstein, S., Shadkchan, Y., Jadoun, J., Sharon, C., Markovich, S., Osherov, N., 2006. Analysis of the *Aspergillus nidulans* thaumatin-like *cetA* gene and evidence for transcriptional repression of *pyr4* expression in the *cetA*-disrupted strain. Fungal Genet. Biol. 43, 42–53.

Grenier, J., Potvin, C., Trudel, J., Asselin, A., 1999. Some thaumatin-like proteins hydrolyse polymeric beta-1,3-glucans. Plant J. 19, 473–480.

Grenier, A., Asselin, A., 2000. Some fungi express β-1,3-glucanases similar to thaumatin-like proteins. Mycologia 92, 841–848.

Han, K.H., Seo, J.A., Yu, J.H., 2004. Regulators of G-protein signalling in *Aspergillus nidulans*: RgsA downregulates stress response and stimulates asexual sporulation through attenuation of *GanB* (G-alpha) signaling. Mol. Microbiol. 53, 529–540.

Jadoun, J., Shadkchan, Y., Osherov, N., 2004. Disruption of the *Aspergillus fumigatus argB* gene using a novel in vitro transposon-based mutagenesis approach. Curr. Genet. 45, 235–241.

Lafon, A., Seo, J.A., Han, K.H., Yu, J.H., d'Enfert, C., 2005. The heterotrimeric G-protein GanB(alpha)-SfaD(beta)-GpgA(gamma) is a carbon source sensor involved in early cAMP-dependent germination in *Aspergillus nidulans*. Genetics 171, 71–80.

Marshall, M.A., Timberlake, W.E., 1991. *Aspergillus nidulans wetA* activates spore-specific gene expression. Mol. Cell. Biol. 11, 55–62.

May, G.S., Morris, N.R., 1987. The unique histone H2A gene of *Aspergillus nidulans* contains three introns. Gene 58, 59–66.

May, G.S., Morris, N.R., 1988. Developmental regulation of a conidiation-specific β-tubulin in *Aspergillus nidulans*. Dev. Biol. 128, 406–414.

Menu-Bouaouiche, L., Vriet, C., Peumans, W.J., Barre, A., Van Damme, E.J., Rouge, P.A., 2003. Molecular basis for the endo-beta 1,3-glucanase activity of the thaumatin-like proteins from edible fruits. Biochimie 85, 123–131.

Osherov, N., May, G.S., 1998. Rapid and efficient protein extraction from *Aspergillus nidulans*. Fungal Genet. Newsl. 45, 38–41.

Osherov, N., May, G.S., 2000. Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis. Genetics 155 (2), 647–656.

Osherov, N., May, G.S., 2001. The molecular mechanisms of conidial germination. FEMS Microbiol. Lett. 199 (2), 153–160.

Osherov, N., Mathew, J., Romans, A., May, G.S., 2002. Identification of conidial-enriched transcripts in *Aspergillus nidulans* using suppression subtractive hybridization. Fungal Genet. Biol. 37, 197–204.

Osmond, R.I., Hrmova, M., Fontaine, F., Imberty, A., Fincher, G.B., 2001. Binding interactions between barley thaumatin-like proteins and (1,3)-beta-D-glucans. Kinetics, specificity, structural analysis, and biological implications. Eur. J. Biochem. 268, 4190–4199.

Sakamoto, Y., Watanabe, H., Nagai, M., Nakade, K., Takahashi, M., Sato, T., 2006. *Lentinula edodes tlg1* encodes a thaumatin-like protein that is involved in lentinan degradation and fruiting body senescence. Plant Physiol. 141, 793–801.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: a Laboratory Manual, third ed. CSH Laboratory Press, Cold Spring Harbor, NY.

Specht, C.A., Liu, Y., Robbins, P.W., Bulawa, C.E., Iartchouk, N., Winter, K.R., Riggle, P.J., Rhodes, J.C., Dodge, C.L., Culp, D.W., Borgia, P.T., 1996. The *chsD* and *chsE* genes of *Aspergillus nidulans* and their roles in chitin synthesis. Fungal Genet. Biol. 20, 153–167.

Trudel, J., Grenier, J., Potvin, C., Asselin, A., 1998. Several thaumatin-like proteins bind to beta-1,3-glucans. Plant Physiol. 118, 1431–1438.

Please cite this article in press as: Belaish, R. et al., The *Aspergillus nidulans cetA* and *calA* genes are involved ..., Fungal Genet. Biol. (2007), doi:10.1016/j.fgb.2007.07.005