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The *Aspergillus nidulans cetA* and *calA* genes are involved in conidial germination and cell wall morphogenesis

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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.
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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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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.

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