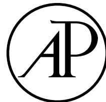
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Fungal Genetics and Biology 37 (2002) 197–204

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Identification of conidial-enriched transcripts in *Aspergillus nidulans* using suppression subtractive hybridization

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Received 5 February 2002; accepted 3 June 2002

Abstract

We have isolated and sequence-identified 12 genes whose transcripts are significantly enriched in *Aspergillus nidulans* conidia. To identify these genes, we used the method of suppressive subtraction hybridization (SSH). One of the 12 genes is similar to plant thaumatin-like proteins that have antifungal properties. Four genes encode metabolic enzymes crucial in the synthesis of glucose, carbohydrates, nucleic acid, and amino acid precursors. The rest are of unknown function. We have analyzed the pattern of expression of the 12 conidial-enriched transcripts in wild-type and mutant strains of *A. nidulans* blocked at different stages of conidial development. Our results indicate that the conidial-enriched transcripts can be divided into four classes based on their expression pattern in the wild-type and mutant strains. Study of the genes identified in this report may enhance our understanding of the process of conidial formation and germination.
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Keywords: *Aspergillus nidulans*; Conidial germination; Suppression subtractive hybridization

1. Introduction

The asexual spore or conidium plays a critical role in the life cycle of many filamentous fungi. The spore is the primary means for dispersion in the environment. While the process of conidial formation has been intensively studied at the molecular level in the filamentous fungus *Aspergillus nidulans* (review by Adams et al., 1998) the process of conidial germination remains surprisingly obscure. One approach to understanding this process is to utilize differential screening to identify and characterize genes that are uniquely transcribed in conidiating cultures and that are present in mature conidia.

Differential screening has been used to identify transcripts enriched in plant seeds (Nuccio and Thomas, 1999) in dormant spores from the slime mold *Dictyostelium discoideum* (Kelly et al., 1983) and in conidiating cultures of *Neurospora crassa* (Berlin and Yanofsky, 1985). Pioneering studies by Timberlake and co-workers showed that about 300 such genes exist in *A. nidulans*. Most of the clones were isolated but not sequence-identified (Timberlake, 1980; Zimmermann et al., 1980). Approximately 80% of these genes are organized in clusters (Orr and Timberlake, 1982). One of these, the *spoC1* cluster, has been studied in detail. It contains 14 conidial-specific genes of unknown function. Deletion of the entire region or of specific genes had no phenotypic effect (Aramayo et al., 1989; Stephens et al., 1999). Therefore, despite considerable effort, this approach has not yielded significant insights into the process of conidial germination.

Recently, using a genetic approach to analyze conidial germination, we showed that the process requires RAS signaling and protein synthesis (Osherov and May, 2000). We hypothesized that by isolating and cloning conidial-enriched transcripts and analyzing their translational control, we could identify the signaling elements that function during early conidial germination. To isolate conidial-enriched transcripts, we chose the method of suppression subtractive hybridization (SSH), because unlike earlier approaches, it eliminates intermediate

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PII: S1087-1845(02)00502-9

steps for physical separation of single-stranded and double-stranded cDNAs, requires only one subtractive hybridization round, and can achieve greater than 1000-fold enrichment for differentially expressed cDNAs (Diatchenko et al., 1996). SSH has been successfully applied in various systems, including the study of genes preferentially expressed in cancer cell lines (Hufton et al., 1999) in the study of endothelial cells during angiogenesis (Glienke et al., 2000), and in the comparison of virulent and avirulant strains of bacteria (Akopyants et al., 1998; Bogush et al., 1999).

Using SSH, we have identified 12 conidial-enriched transcripts (*cet*) whose genes we have designated *cetA–L*. Based on sequence homology, one of these genes is similar to plant thaumatin-like proteins that have antifungal properties; four genes encode metabolic enzymes involved in the synthesis of glucose, carbohydrates, nucleic acid, and amino acid precursors. The functions of the rest are unknown. These genes may provide intriguing insights into the early stages of conidial metabolism and germination.

2. Materials and methods

2.1. *Aspergillus nidulans* strains and growth media

The strains used in this study were A4 (veA+, Glasgow wild type, available at the Fungal Genetics Stock Center) and the developmental mutant strains *brlA1* (AJC7.1), *abaA1* (G01), and *stuA1* (G0256). YAG, which consists of 0.5% yeast extract, 1% dextrose, 10 mM $MgCl_2$, trace elements and vitamins (Bainbridge, 1971) and 2% agar for solid medium, was used for routine propagation of these strains.

2.2. RNA preparation

RNA was prepared from freshly harvested A4 conidia and conidia that had been germinated in YAG liquid medium at 37 °C for the indicated time. Total RNA was prepared using the 'hot SDS/phenol' method described previously (May and Morris, 1988) with the following modifications. After lyophilization, fungal material was ground with a 1 ml blue tip in a 1.5 ml microcentrifuge 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 previously described. We found this additional grinding step essential to efficiently extract RNA from dormant conidia.

2.3. Subtractive hybridization and differential screening

Total RNA was prepared from conidia or 6 h germlings on YAG at 37 °C. cDNA was prepared from the total RNA using the SMART PCR cDNA synthesis kit by Clontech Laboratories (Palo Alto, CA). Conidial and germling cDNAs were then digested with *Rsa*I and purified by running through a chromaspin column. Adaptor oligonucleotides were ligated to the *Rsa*I-cut conidial cDNA ('tester' cDNA), and the tester cDNA was mixed and hybridized with an excess of germling cDNA ('driver' cDNA). A parallel reverse reaction to identify germling-enriched transcripts was also used as a control. Following subtraction, conidial-enriched and germling-enriched cDNA was preferentially amplified by polymerase chain reaction (PCR) using primers corresponding to the adaptor sequences. These amplified cDNAs were cloned into a bacterial plasmid using the Clontech pT-Advantage kit. In all cases, the precise instructions supplied by the manufacturer were followed without modifications.

To prepare the array, plasmid DNA prepared from 100 individual cDNA clones was digested with *Eco*RI, electrophoresed in duplicate tiers on a 1% agarose gel, and transferred to a nylon membrane.

2.4. 'Virtual' Northern hybridization

'Virtual' Northern blots were prepared by electrophoresing 1 μg/lane unsubtracted SMART PCR-amplified cDNA from total conidial or 6 h germling RNA on a 1% agarose gel and transferring it to a nylon membrane. The 'virtual' Northern blots were subsequently hybridized with [α-^{32}P]dCTP labeled radioactive probes prepared from the differentially expressed clones identified in the array.

2.5. Northern hybridization

Northern analysis was performed as previously described (May and Morris, 1987). Briefly, total RNA was prepared from ungerminated A4 conidia and conidia germinated in YAG medium for 6 h (germlings) 16 h (mycelium) and 48 h (conidiating mycelium) at 37 °C. For the developmental mutant strains *brlA1* (AJC7.1), *abaA1* (G01), and *stuA1* (G0256), total RNA was prepared following 48 h of growth of the mycelial inoculum in YAG medium at 37 °C. RNA samples were denatured and loaded at a concentration of 5 μg/lane on agarose gels containing 20 mM sodium borate buffer (pH 8.3), 0.2 mM ethylene diamine tetraacetic acid (EDTA), 1% agarose, and 3% formaldehyde. Gels were electrophoresed in 20 mM sodium borate buffer (pH 8.3) and transferred to nylon membranes using 20× SSPE as previously described (Maniatis et al., 1982). Normalization of gene expression (Table 2) was performed according to the following formula: {DR (*cet* gene expression, time point)/DR (*cet* gene expression in conidia)}/{DR (control rRNA time point)/DR (conidial rRNA)}, where DR is densitometric reading and rRNA

is ribosomal RNA. Briefly, for each gene, expression was normalized to the 0 h (ungerminated conidia) time point, and then further normalized for equal sample loading using rRNA as the control.

2.6. Sequence analysis

Plasmids containing differentially expressed cDNA were sequenced using the T7 primer. The sequences derived were used to search the DNA databases using the blast program at the NCBI WEB site, the TIGR *A. fumigatus* fungal database. (http://www.tigr.org/tdb/fungal/), and the Oklahoma University *A. nidulans* cDNA sequencing project (http://www.genome.ou.edu/fungal.html).

3. Results

3.1. Array analysis of cet transcripts

Following subtractive hybridization and cloning of the subtracted conidial-enriched and control germling-enriched cDNAs into the Clontech AdvanTAge plasmid vector, plasmid DNA was prepared from 100 independent bacterial colonies, digested with *Eco*RI, and run in duplicate on a 1% agarose gel. After transfer to a nylon membrane, the plasmid DNA was probed with conidial-enriched or germling-enriched cDNA for one of the duplicate blots. A representative portion of the array containing 15 clones is shown (Fig. 1). The results show that both the conidial- and germling-enriched clones hybridized exclusively to the conidial- and germling-enriched cDNA probes, respectively, indicating that the subtraction step had eliminated clones expressed in both stages of growth at the same time. Those colonies selectively hybridizing to the conidial-enriched probe (approximately 60% of the total) were selected for sequencing.

3.2. Identification of conidial-enriched transcripts

We sequenced the cDNA inserts of a total of 33 independent plasmids hybridizing with the conidial-enriched cDNA (Table 1). A comparison of these sequences indicated they fell into 15 contigs. Three of these proved to be false positive (described below) and are not reported in Table 1. At this stage, we began to repeatedly identify identical overlapping sequences (e.g., six *cetA* and four *cetB*, *C*, and three *cetD* clones) and therefore did not sequence additional clones. It is therefore likely that additional rare *cet* genes remain to be found in our arrays.

The sequences were used to identify possible gene function through homology with known genes in the data banks. One of the sequences is homologous to thaumatin-like plant-defense proteins (*cetA*), five are homologous to genes involved in metabolism (*cetD*, *E* (*acuD*), *G*, *H*, *I*), two are homologous to genes of unknown function (*cetB* and *cetJ*), and the rest have no significant homology to sequences in the databases (Fig. 2).

3.3. Virtual Northern analysis of conidial-enriched transcripts

Differential expression of the 15 putative *cet* clones was initially confirmed using 'virtual' Northern blots made with PCR-amplified unsubtracted cDNA instead

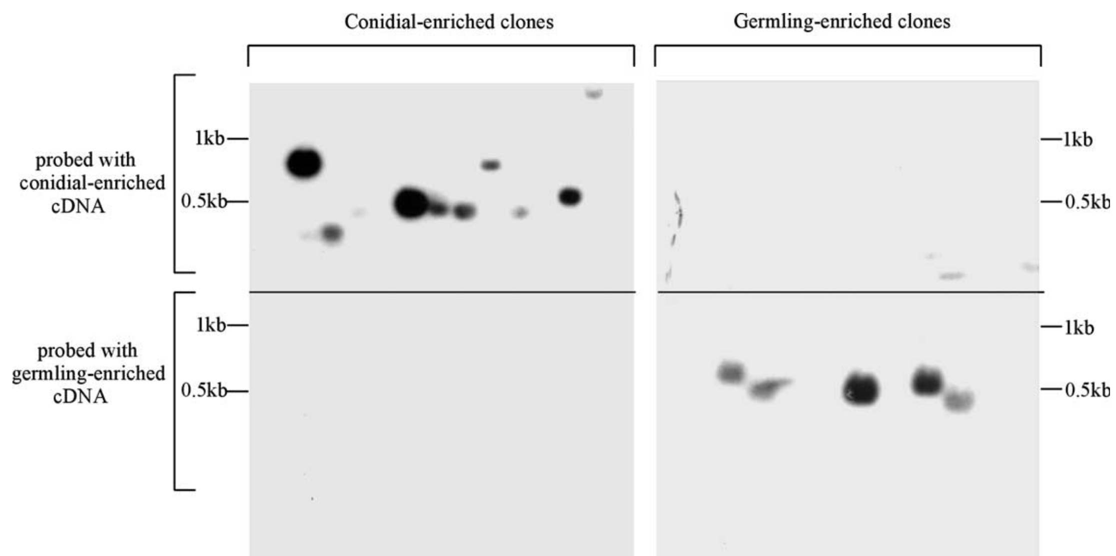

Fig. 1. Arrays of subtracted conidial-enriched (left panel) and germling-enriched (right panel) transcripts probed with subtracted conidial-enriched (top) or germling-enriched cDNA respectively. Agarose gel electrophoresis and Southern transfer were performed as described in Section 2. Arrays were exposed to film for 6 h at −80 °C.

Table 1

Gene	Frequency	Length (bp)	Putative homologue	*E*-value	Function of homologue	Oklahoma database Accession No.
cetA	6	1015	Thaumatin-like protein (*A. fumigatus*)	10^{-60}	Plant antifungal protein	Contig 1747
cetB	4	1047	Unknown gene, *A. fumigatus* contig 3618 in TIGR database	10^{-28}	Unknown	Contig 1784
cetC	4	929	Unknown	—	Unknown	Contig 1616
cetD	3	535	Glucose-repressible gene *grg1* (*Neurospora crassa*)	10^{-16}	Glucose-repressed gene	Contig 1859
acuD (cetE)	2	866	Isocitrate lyase, *acuD* (*A. nidulans*)	—	Glycoxylate cycle	X62696 (Genbank)
cetF	2	530	Unknown	—	Unknown	c49o5a1.r1
cetG	1	797	Transketolase (*Thermogota maritima*)	10^{-12}	Pentose phosphate pathway	NA
cetH	1	1059	UDP-galactose epimerase-related protein (*A. fumigatus*)	10^{-113}	Glucose metabolism	Contig 691
cetI	1	720	PEP carboxykinase (*S. cerevisiae*)	10^{-115}	Gluconeogenesis	i8g09a1.f1
cetJ	1	574	Hypothetical protein SPAC869.06c (*S. pombe*)	10^{-28}	Unknown	c2a01a1.f1
cetK	1	450	Unknown	—	Unknown	o6a04a1.r1
cetL	1	711	Unknown	—	Unknown	f5d10a1.r1

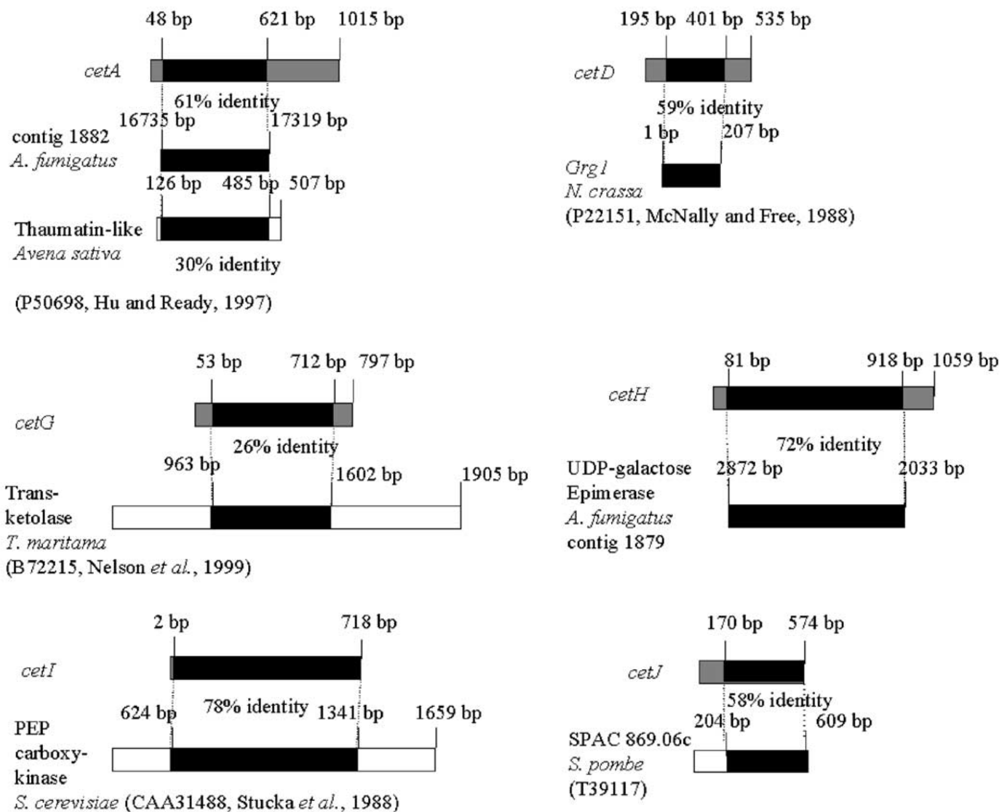

Fig. 2. Schematic representation of comparisons between *cetA*, *D*, *G–J* to known genes in the database. Sequence data for *cetA*, *D*, *G–J* were used to run Blast database searches to identify possible *cet* gene homologues. Regions of significant identity and their range are shown below the homologous gene. GenBank accession numbers and references are given in parenthesis. *A. fumigatus* contigs can be accessed at the TIGR *A. fumigatus* database.

of total or poly(A) RNA, which is used in standard Northern blots.

We prepared conidial and 6-h germling cDNA using the PCR cDNA synthesis kit (Clontech). Fifteen blots were prepared; for each one, equal amounts of conidial and germling cDNA were electrophoresed on a 1% agarose gel and transferred to a nylon membrane. Each blot was hybridized to one of the 15 cloned cDNA inserts (clones 1–15). The results indicate that all clones hybridize selectively to the conidial cDNA, validating the success of the subtractive hybridization (data not shown).

3.4. Northern analysis of conidial-enriched transcripts

To unambiguously verify that clones 1–15 are enriched in dormant conidia, Northern analysis was performed. This analysis has advantages over 'virtual' Northern analysis in that it eliminates spurious 'false positive' virtual Northern readings and reveals the size of the mRNA transcript. Total RNA was prepared from ungerminated conidia and conidia germinated in rich medium for 6 h (germlings), 16 h (mycelium), and 48 h (conidiating mycelium). Fifteen blots were prepared; for each one, equal amounts of total RNA from the various stages of growth were electrophoresed on a 1% agarose gel and transferred to a nylon membrane. Each blot was hybridized to one of the 15 cloned cDNA inserts. The results indicate that 12 of the *cet* cDNAs (*cetA–L*) are highly enriched in dormant conidia (Fig. 3). In most of these, small quantities of transcript were also expressed at the conidiating mycelial stage (48 h), probably because they contain large amounts of ungerminated conidia. Surprisingly, unlike the results found for the 'virtual' Northern blots, clones 3, 12, and 14 were primarily expressed at the mycelial (16 h) growth stage. These clones were regarded as 'false positives' and were not further studied. A possible reason for the appearance of false positives at the virtual Northern stage is that during the cDNA amplification step that precedes it, some cDNA fragments are, by chance, amplified preferentially in the tester cDNA even though they are equally represented in the driver cDNA.

3.5. Northern analysis of conidial-enriched transcripts in *A. nidulans* conidiation mutants

To better define the temporal expression of the *cet* genes, we utilized *A. nidulans* strains that are defective at different stages of the conidial development program (Adams et al., 1998). The *brlA1* and *abaA1* mutants are blocked in conidiophore vesicle swelling and phialide formation, respectively (Boylan et al., 1987). The *stuA1* mutant lacks phialides and metulae but produce some viable conidia directly on the conidiophore swelling (Miller et al., 1991). Total RNA was prepared for 0 h (wild-type ungerminated conidia), 16 h (mycelium), and 48 h (conidiating mycelium) and from *brlA1*, *abaA1*, and *stuA1* mutant strains grown for 48 h in parallel. Twelve identical blots were prepared and hybridized to one of the cloned *cetA–L* cDNA inserts. The results indicate that the *cet* transcripts can be divided into four classes based on their expression pattern in the developmental mutants (Table 2). Class A, represented by *cetC* alone, shows almost no detectable expression in the three developmental mutant strains, and is only expressed in wild-type conidia and conidiating cultures. Class B, represented by *cetA*, *B*, *E* (*acuD*), and *L* were expressed at very low levels in the *brlA1* and *abaA1* mutant strains as compared to the wild type control at 48 h, but at surprisingly high levels in the *stuA1* mutant strain. Class C, represented by *cetF–K* were expressed at high levels in the *stuA1* mutant strain, at levels comparable to the wild type control at 48 h in the *brlA1* strain and at very low

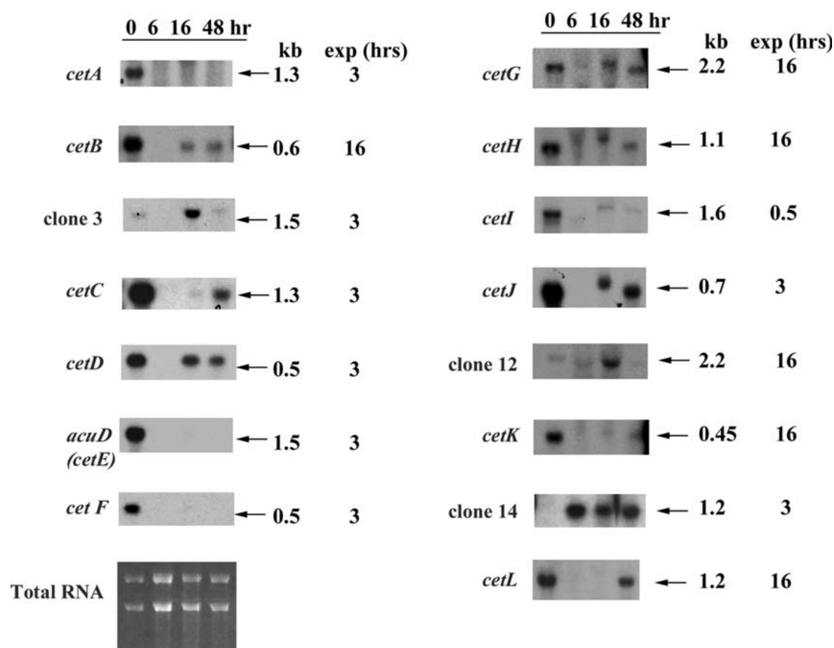

Fig. 3. Confirmation of putative differentially expressed cDNAs by Northern blot. Each lane contains 5 μg of total RNA from ungerminated conidia (0 h), germlings (6 h), mycelium (16 h), and conidiating mycelium (48 h). Each blot was hybridized to the indicated radioactively labeled cDNA clones 1–15, and autoradiography was performed. A picture of the ethidium-stained agarose gel confirms that lanes were loaded equally (lower left). The relative size in kilobases of the transcripts and the exposure time to film are given. Conidial-enriched transcripts were subsequently renamed *cetA–L* as illustrated in the figure.

Table 2
Differential expression of *cetA–L* in wild-type conidia (0 h), during mycelial growth (16 h) and conidiation (48 h), compared with their expression after 48 h of growth in the conidiation mutant strains *brlA1*, *abaA1*, and *stuA1*

	Class A	Class B				Class C						Class D
	cetC	*cetA*	*cetB*	*acuD* (*cetE*)	*cetL*	*cetF*	*cetG*	*cetH*	*cetI*	*cetJ*	*cetK*	*cetD*
0 h	1[a]	1	1	1	1	1	1	1	1	1	1	1
16 h	0.1	0.05	0.05	0.05	0.12	0.1	0.1	0.05	0.15	0.05	0.05	0.5
48 h	0.42	0.25	0.21	0.125	0.79	0.23	0.83	0.21	0.21	0.75	0.3	1.2
brlA1	0.01	0.1	0.07	0.03	0.13	0.2	0.8	0.2	0.67	0.9	0.35	1
abaA1	0.01	0.05	0.05	0.01	0.06	0.02	0.05	0.04	0.17	0.06	0.1	0.8
stuA1	0.02	1.25	1.5	1.5	1.57	1	0.45	1.25	1.43	1.5	1.05	1.25

[a] The numbers indicate fold-increase or decrease over conidial (0 h) expression. The experiment was independently performed twice with similar results. Gene expression was normalized according to the formula described in Section 2.

levels in the *abaA1* mutant strain. Class D, represented by *cetD* alone, was expressed at high levels in all the developmental mutant strains as well as during vegetative growth. This is consistent with the results of the Northern analysis in Fig. 3, showing that this gene is down-regulated during early germination (6 h) and up-regulated during vegetative growth (16 h).

4. Discussion

Conidia contain a pre-existing pool of mRNAs, primed for translation. Upon receiving a signal activated by glucose, pre-stored mRNAs undergo rapid translation (Horikoshi et al., 1965; Mirkes, 1974). We previously showed that conidia containing a mutant constitutively active for of the gene *rasA* germinate in the absence of an external carbon source (Osherov and May, 2000). This result suggests that *rasA* may function as part of a sensory transduction mechanism that initiates conidial germination. We also demonstrated that conidial germination in *A. nidulans* requires translation as an early first step. Heat sensitive mutant strains defective in genes involved in protein synthesis produce conidia that are completely blocked in germination at the restrictive temperature. Unlike normal conidia, they do not swell, adhere, or undergo nuclear decondensation. To identify the signal transduction mechanism that links *rasA* and glucose sensors to the essential and early onset process of translation, we isolated unique conidial transcripts and analyzed their transcriptional and translational regulation. Here, we report our results of using SSH to find conidial-enriched transcripts. We have identified 12 *cet* genes and analyzed their expression pattern during *A. nidulans* development.

4.1. Identification of cetE (acuD), G–I that show similarity to metabolic enzymes

cetE is identical to *acuD*, the gene encoding isocitrate lyase, a metabolic enzyme specifically involved in the glyoxylate pathway (Gainey et al., 1992). This plant- and fungal-specific pathway is primarily used to convert acetyl residues derived from the β-oxidation of fatty acids into carbohydrates and glucose via succinic acid. In *A. nidulans*, isocitrate lyase is induced by growth on C2 compounds and long-chain fatty acids and repressed by glucose. Isocitrate lyase is highly induced in *Saccharomyces cerevisiae* and *Candida albicans* during nutrient deprivation and following ingestion by macrophages. *C. albicans* isocitrate lyase deleted mutant strains show reduced virulence in a mouse model for candidemia (Lorenz and Fink, 2001). We speculate that isocitrate lyase and the glyoxylate pathway are activated during conidial development because of increasing nutrient deprivation in the conidia. During conidial germination, the pathway probably plays an important role in supplying the germling with glucose from pre-stored fatty acids. Presumably, inhibitors of the glyoxylate pathway should block nutrient availability and prevent germination of conidia. Since this pathway is not found in animals, it is a prime target for antifungal therapy. Three of the four metabolic *cet* genes (*cetE* (*acuD*), *H*, *I*) are probably involved in the generation of glucose and carbohydrates from various carbon sources, thereby providing the energy resources needed for conidial germination. The fourth (*cetG*) probably functions in the pathway that provides the precursor molecules necessary for the synthesis of nucleotides and amino acids.

4.2. cetA shows similarity to thaumatin-like plant defense proteins

Thaumatin-like proteins are small molecular weight (17–25 kDa) proteins produced by plants in response to fungal infection (Hu and Reddy, 1997). They inhibit fungal growth, but the mechanisms by which they do this are unknown. We speculate that in fungi they may play a role in inhibiting germination until conditions are optimal. Interestingly, a database search in the *A. fumigatus* TIGR database and the *A. nidulans* Oklahoma University EST database reveals that at least two additional closely related thaumatin-like genes exist in these organisms. No homologs were found in the

Schizosaccharomyces pombe (Sanger Institute), *S. cerevisiae*, or *Candida albicans* (Stanford Institute) databases.

4.3. *cetD shows a striking similarity to the glucose-repressible genes grg-1 and PaGrg1 of N. crassa and Podospora anserina*

grg-1 is a light-regulated catabolite-repressed gene predicted to encode a short (71 amino acids) protein of unknown function (McNally and Free, 1988). It has been suggested that *grg-1* encodes a general stress protein (Kimpel and Osiewacz, 1999). *PaGrg-1* is a catabolite-repressed gene whose mRNA levels increase gradually during growth and aging of *P. anserina* (Kimpel and Osiewacz, 1999). Interestingly, the *grg-1* promoter region shows a high degree of similarity to the promoter region of the *eas* (easily wettable) gene in *N. crassa* and *rodA* of *A. nidulans* that encodes the rodlet protein that constitutes a hydrophobic sheath covering the conidium (Lauter et al., 1992). c*etD* is unusual among the *cet* genes isolated in that it is expressed throughout late vegetative growth in both the wild-type and conidiation mutants, indicating that it is expressed in both mature hyphae and conidia. It is probably activated during the late growth phase as a result of glucose depletion and loss of carbon catabolite repression.

4.4. *Classification of the cet genes into four groups based on their expression pattern in conidiation mutants*

To better define the temporal expression of the *cet* genes, we measured their expression in the *A. nidulans* conidiation mutants *brlA1, abaA1* and *stuA1*. *brlA* and *abaA* encode transcription factors that form a linear pathway activating expression of many sporulation-specific genes (reviewed in Adams et al., 1998). *stuA* encodes a transcriptional repressor that probably regulates both developmental and cell-cycle-specific genes during conidiophore development (Dutton et al., 1997). Our results indicate that the *cet* genes can be divided into four classes (A–D) based on their expression pattern in the wild-type and mutant strains. *cetC*, the only class A gene, is probably accumulated primarily during conidial development as it is poorly expressed in all three developmental mutant strains. *cetA*, *B*, *E* (*acuD*), and *L*, class B genes, are also probably expressed primarily in conidia, as they are found at significantly reduced levels in the aconidial strains *brlA1* and *abaA1* as compared with the wild-type strain grown for 48 h. They are however, expressed at surprisingly high levels (2–12 fold compared with the wild-type strain grown for 48 h) in the *stuA1* mutant strain. This is consistent with previous findings demonstrating that STUA is a transcriptional repressor with potential binding sites in the promoters of the developmental regulators *brlA* and *abaA*, the cell cycle regulators, *nimE* and *O* and *awh11*, a conidial-expressed gene with homology to small heat-shock proteins (Dutton et al., 1997). Class C, represented by *cetF–K* were expressed at high levels in the *stuA1* mutant strain and at levels comparable to the wild-type strain grown for 48 h in the *brlA1* mutant strain. Surprisingly, they were expressed at low levels in the *abaA* 1 mutant strain, even though it is blocked at a later developmental stage than *brlA1* and is transcriptionally regulated by BRLA. Similar results have been previously demonstrated for the conidiation-accumulating transcripts pCAN11, 65, and 77 (Boylan et al., 1987). A possible explanation for this result is that BRLA and ABAA function as transcriptional repressor and activator, respectively, towards class C *cet* genes, leading to their activation and inactivation, respectively, in the corresponding null strains. Class D, represented by *cetD* alone, was expressed at high levels in all the developmental mutant strains as well as during vegetative growth. This gene is transcribed in vegetative hyphae before the onset of conidial development and is therefore not strictly conidiation specific.

Acknowledgments

The developmental mutant strains *brlA1* (AJC7.1), *abaA1* (G01), and *stuA1* (G0256) were kindly provided by Dr. Bruce Miller. This investigation was funded by the National Institute of Health Grant GM53027 to G.S.M. and the Cancer Center Support Grant CA-16672 for the core DNA sequencing facility.

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