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The *Aspergillus fumigatus* StuA Protein Governs the Up-Regulation of a Discrete Transcriptional Program during the Acquisition of Developmental Competence[D]

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Members of the Asm1p, Phd1p, Sok2p, Efg1p, and StuAp (APSES) family of fungal proteins regulate morphogenesis and virulence in ascomycetes. We cloned the *Aspergillus fumigatus* APSES gene encoding StuAp and demonstrated that *stuA* transcription is markedly up-regulated after the acquisition of developmental competence. *A. fumigatus* Δ*stuA* mutants were impaired in their ability to undergo asexual reproduction. Conidiophore morphology was markedly abnormal, and only small numbers of dysmorphic conidia were produced, which exhibited precocious germination. Whole genome transcriptional analysis during the onset of developmental competence was performed and identified a subset of developmentally regulated genes that were *stuA* dependent, including a cluster of putative secondary metabolite biosynthesis genes, genes encoding proteins implicated in the regulation of morphogenesis, and genes encoding allergens and other antigenic proteins. Additionally, hyphae of the Δ*stuA* mutant displayed reduced expression of the catalase gene *CAT1* and were hypersusceptible to hydrogen peroxide.

INTRODUCTION

As increasing numbers of patients undergo immunosuppressive medical procedures such as solid organ and bone marrow transplantation, the incidence of invasive fungal infections has increased (Marr *et al*., 2002). Despite the availability of new therapeutic agents, mortality from infections due to the most common mold, *Aspergillus fumigatus*, remains at least 50% (Herbrecht *et al*., 2002). A more comprehensive understanding of the biology of *A. fumigatus* and its interactions with the host is a critical step in the development of new strategies aimed at the prevention and treatment of infections caused by this opportunistic pathogen.

Aspergillus species have a complex life cycle with distinct developmental stages. Airborne uninucleate conidia are deposited on organic matter where they become metabolically active, swell and germinate, producing filamentous septate hyphae, which grow by apical extension (Adams *et al*., 1998). After a defined period of growth (~8–15 h in submerged culture in vitro), these hyphae are able to respond to a variety of stimuli and initiate asexual reproduction or conidiation (Adams *et al*., 1998). Hyphae after this transition are termed developmentally competent and can be maintained in this phase of development indefinitely if grown in submerged culture. When exposed to a suitable stimulus, such as a static-air interphase, developmentally competent hyphae enter into the final stage of development. This stage is characterized by the production of conidiophores, which are multicellular asexual reproductive structures consisting of highly specialized, morphologically distinct cell types that produce uninucleate conidia via apolar budding (Miller *et al*., 1992; Adams *et al*., 1998). These conidia are then dispersed by air currents for the cycle to continue. Humans are infected incidentally by the inhalation of small numbers of spores. In the absence of a robust immune response, these conidia germinate to form hyphae that invade and destroy pulmonary tissue (Latge, 1999). Conidiation does not occur during invasive aspergillosis, and hyphae are the only invasive fungal forms observed by histopathology after the initiation of infection (Fraser, 1993). Sexual reproduction has not been observed in the pathogenic *A. fumigatus*, but it does occur in some other *Aspergillus* species, including the well studied model organism *A. nidulans*.

In fungi, the genes involved in the regulation of fungal development often directly regulate the expression of key virulence factors (Lengeler *et al*., 2000). In ascomycetes, members of the APSES group of transcription factors have been identified as key regulators of fungal development that often also control virulence traits. This group of proteins was named for the original members (Asm1p, Phd1p, Sok2p, Efg1p, and StuAp) and comprises a family of transcription factors that share a common DNA-binding motif (Dutton *et al*., 1997; Stoldt *et al*., 1997; Sonneborn *et al*., 1999a,b; Doedt *et al*., 2004). This motif can be structurally modeled as a basic helix-loop-helix (bHLH) protein analogous to eukaryotic

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Table 1. PCR primers used in this study

Primer name	Target gene	Sequence 5′–3′
F1	*stuA*	TCGAGAGCGAACAGTTGGTA
F2	*stuA*	TCCTGTGTGAAATTGTTATCCGCT
F3	*stuA*	CCAACACCTCGGTTGGTAGT
F4	*stuA*	GTCGTGACTGGGAAAACCCTGGCG
M13F	*hph*	GGTGTTCTGACCTCGCTGAT
M13R	*hph*	CTCTGTCCTCGCTTTCTTGC
HY	*hph*	CGCCAGGGTTTTCCCAGTCACGAC
YG	*hph*	AGCGGATAACAATTTCACACAGGA
S1 sense	*stuA*	GGATGCCTCCGCTCGAAGTA
S2 antisense	*stuA*	CGTTGCAAGACCTGCCTGAA
StuA RT sense	*stuA*	ACGATTGATCATTGCCTTG
StuA RT antisense	*stuA*	AACGAATGCAAAGTCGGAAG
TEF1RT sense	*TEF1*	GAGGACGAAGGGAGTCTCTG
TEF1RT antisense	*TEF1*	ACCGTTGATCATGTGGTTGT
CC9 sense	Clock controlled gene 9	CCATGTGTGTCGAGTCCTTC
CC9 antisense	Clock controlled gene 9	GAACGTACAGCAACAGTCTGG
IME2 sense	*IME2*	ACGTCTTGGACTCGTATCCC
IME2 antisense	*IME2*	GGCGAGATGGGACATTACTT
CAT1 sense	*CAT1*	CAGAATTCCGACTTGAACGA
CAT1 antisense	*CAT1*	GATTTGGACAGGGACGTTTC
IgE sense	IgE binding protein	CGCTGAGACCGAACAAGTTA
IgE antisense	IgE binding protein	GCTGAGTGTCCAGGTACGAA
RosA sense	Repressor of sexual development	CGAAGGCACTCAAACTGTTC
RosA antisense	Repressor of sexual development	GAGTGAGCGGTGGTGAACT
		ATAGGGACACCAGAGAAGGC
		ATCACACTTCTTCCTTCGCA

Myc and Max proteins (Dutton *et al*., 1997). Each of these proteins has been shown to play an important role in controlling fungal morphogenesis and development.

The link between the regulation of fungal development and virulence by an APSES protein has been best studied in *Candida albicans* (Lo *et al*., 1997; Stoldt *et al*., 1997). *C. albicans* Efg1p not only plays a key role in mating and the yeast-to-hypha transition, but it is required for normal adherence to and invasion of host cells as well as virulence in murine models of disseminated and oral candidiasis (Lo *et al*., 1997; Stoldt *et al*., 1997; Sonneborn *et al*., 1999a,b; Doedt *et al*., 2004; Park *et al*., 2005).

In the minimally pathogenic *Aspergillus nidulans*, both sexual and asexual reproduction are controlled by the APSES protein StuAp (Wu and Miller, 1997). Mutants deficient in StuAp produce shortened conidiophores that lack phialides and produce low numbers of conidia by direct budding from the vesicle head (Miller *et al*., 1992). Although the exact function of StuAp in asexual reproduction is not known, it has been suggested that StuAp functions predominately as a transcriptional repressor, governing the spatial temporal expression of the conidiation genes *brlA* and *abaA* (Dutton *et al*., 1997). However, transcription of *stuA* is not restricted to conidiation, but rather it is temporally associated with the onset of developmental competence and is further up-regulated at the initiation of conidiation (Miller *et al*., 1991). In addition, StuAp expression is diffusely distributed in competent hyphae and not restricted to specific cell types (Miller *et al*., 1992). These findings suggest the hypothesis that in addition to functioning as a transcriptional repressor during conidiation, StuAp may govern other cellular processes in competent hyphae. This possibility is of particular interest given that hyphae are the invasive form of *A. fumigatus* during human infection, and conidiation is not observed during invasive pulmonary aspergillosis (Fraser, 1993).

In this study, we cloned, disrupted, and characterized the function of the *stuA* gene of the pathogen *A. fumigatus*. To understand the role of StuAp in preconidiation competent hyphae, whole genome transcriptional analysis was used to identify the *stuA*-dependent components of the regulatory program associated with the onset of developmental competence.

MATERIALS AND METHODS

Strains and Growth Conditions

A. fumigatus strain Af293, (a generous gift from P. Magee, University of Minnesota, St. Paul, MN) was used as the basis for molecular manipulations. Except where indicated, strains were propagated on YEPD agar (1% yeast extract, 2% peptone, 2% glucose, solidified with 1.5% agar). For germination time courses, liquid YEPD medium was inoculated with 1×10^6 conidia of the strain of interest per milliliter and grown in shaking culture at 37°C, while exposed to light. To determine the time of acquisition of developmental competence, we used a modification of the method of Axelrod *et al*. (1973). Briefly, conidia of strain Af293 were germinated in liquid YEPD as described above, and serial aliquots were subcultured to solid YEPD agar and incubated at 37°C. The duration of time required for the production of visible conidiophores for each sample was recorded. Precompetent hyphae require a period of maturation before responding to induction (subculture to solid medium), which manifests as an increase in the delay between induction and conidiophore production. In contrast, competent hyphae can immediately respond to the induction stimulus and therefore produce conidiophores with a fixed delay after induction regardless of the cultures age. Therefore, the acquisition of developmental competence was defined as the time of growth in submerged culture after which the delay between induction and production of conidiophores became constant.

Molecular Genetic Manipulations

Total *A. fumigatus* RNA and DNA were isolated as described previously (Monroy and Sheppard, 2005). The genomic sequence of *stuA* was identified by BLAST search of the unfinished *A. fumigatus* genome sequence assembly (http://tigrblast.tigr.org/er-blast/index.cgi?project = afu1). The predicted open reading frame of *stuA* was identified via the Institute for Genomic Research annotation pipeline using the Eukaryotic Genome Control software package (Nierman *et al*., 2005). The *stuA* gene was cloned by high-fidelity PCR using primers S1 and S2 (Table 1), and the resulting amplicon was cloned into pGEM-T-Easy (Promega, Madison, WI) for sequencing. This product was used as a probe for subsequent Northern and Southern blotting experiments.

Transformation of *A. fumigatus* by electroporation is simple and rapid, but it is hampered by a low rate of homologous integration, making targeted

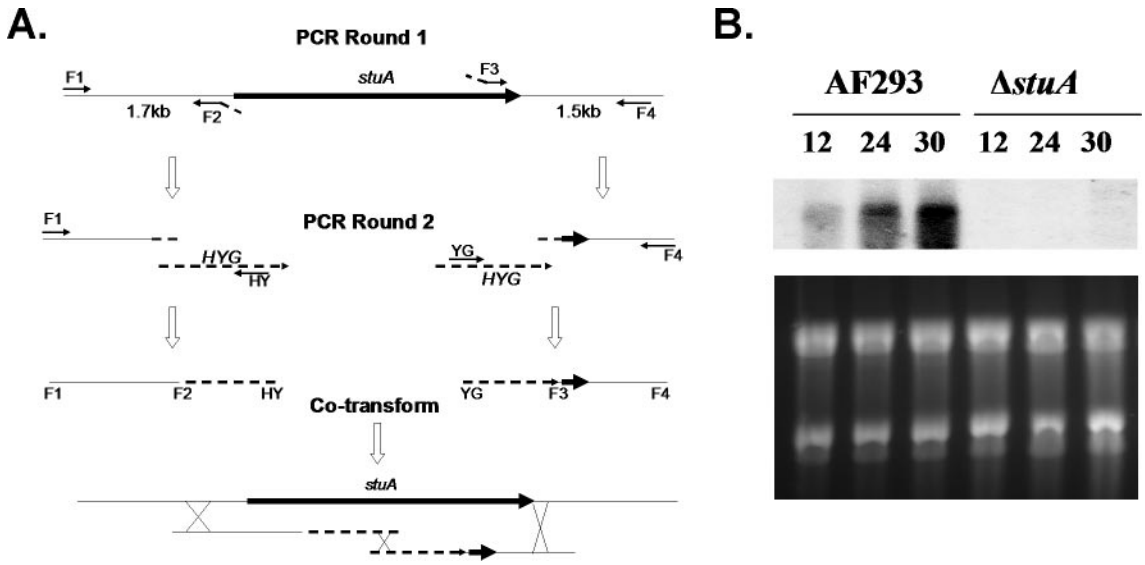

Figure 1. Disruption of *stuA*. (A) Split marker strategy for the deletion of *stuA*. Two deletion fragments were generated by successive rounds of PCR. First flanking regions were amplified containing M13 sequences at the *stuA* flanking ends. Next, a fusion PCR was performed using each fragment and the HYG resistance cassette, both containing homologous M13 sequences, as template to generate the final deletion fragments. Finally, Af293 was cotransformed with the two deletion fragments. A triple crossover event resulted in replacement of *stuA* by the HYG resistance cassette. F1, F2, F3, F4, HY, and YG, primers used for amplification. HYG, hygromycin resistance cassette amplified from pAN7–1. (B) Northern blot of Af293 and the *stuA* null mutant strain. Total RNA from hyphae grown in YPD at 37°C was probed with the entire open reading frame of *stuA*, confirming an absence of *stuA* mRNA in the *stuA* null mutant strain.

integration a challenge (Sheppard *et al*., 2004). We therefore used a split marker electroporation strategy to generate a *stuA*-deficient strain of *A. fumigatus* (Figure 1A) (Catlett *et al*., 2003). Briefly, *A. fumigatus* strain Af293 was cotransformed with two DNA constructs, each containing an incomplete fragment of the dominant selection marker HYG (hygromycin phosphotransferase) fused to 1 kb of *stuA* flanking sequence. These marker fragments shared an approximately 450-bp overlap within the HYG cassette, which served as a potential recombination site during transformation. During transformation, homologous integration of each fragment into the genome flanking *stuA* allows recombination of the HYG fragments and generation of the intact resistance gene at the site of recombination. Each fragment was generated by two rounds of PCR. First, each flanking region was amplified from Af293 genomic DNA using primer pairs F1-F2 and F3-F4. These produced 1-kb regions of *stuA* flanking sequence, fused to a 24-bp conserved M13 sequence at their respective internal ends. In parallel, the HYG resistance cassette was amplified from plasmid pAN7-1 by M13F and M13R primers. To generate the final transformation fragments, fusion PCR was performed to combine each flanking fragment with the M13 HYG resistance cassette and amplifying using the F1-HY, and the YG-F4 primer set. This produced two *stuA* flanking regions, each linked by M13 sequences to incomplete fragments of the HYG resistance cassette. Strain Af293 was then transformed by electroporation using 5 μg of each fragment, and the transformants were retrieved by hygromycin selection. Deletion of *stuA* was confirmed by PCR (our unpublished data) and Northern blotting to ensure a complete absence of *stuA* mRNA (Figure 1B). To generate a *stuA*-complemented strain, an intact *stuA* insert that overlapped the deletion as well as 1.5 kb of each intact flank was generated using high-fidelity PCR with primers F1 and F4. The resulting amplicon was cloned into pGEM-T and sequence verified. The *stuA* deletion mutant was then transformed with 10 μg of this insert by spheroplasting and regenerated without selection. The resulting transformants were screened for clones that displayed normal conidiation. Transformants that exhibited normal conidiation were tested by PCR to ensure correct integration of the amplicon at the *stuA* native locus and subsequent loss of the HYG cassette then by Northern blot to ensure normal restoration of *stuA* transcription (our unpublished data).

Transcriptional Profiling

To identify genes that were differentially regulated during developmental competence, we determined the transcriptional profile of *A. fumigatus* during germination and hyphal growth in liquid YEPD at 37°C, while exposed to light. RNA was extracted from wild-type Af293, the *stuA* null mutant, and the *stuA* complemented strain after 8, 24, and 30 h of growth. After 8 h, the organisms had formed short, precompetent hyphae. At the later time point, the organisms had formed long hyphae that were developmentally competent.

The *A. fumigatus* Af293 DNA amplicon microarray containing 9516 genes (Nierman *et al*., 2005) was used in this study. Labeling reactions with RNA, and hybridization were conducted as described in The Institute for Genomic Research (TIGR, Rockville, MD) standard operating procedures found at http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml. In the first set of experiments, the sample from 8 h served as reference in all hybridizations with samples from later time points within each strain to identify genes exhibiting altered transcription in competent hyphae. In addition, in separate experiments, direct hybridizations were performed using RNA pools from wild-type Af293 and the Δ*stuA* mutant strain after 12, 24, and 30 h of growth. All the hybridizations were repeated in dye-swap sets. Hybridized slides were scanned using the Axon GenePix 4000B microarray scanner and the TIFF images generated were analyzed using TIGR Spotfinder (http://www.tigr.org/software/; TIGR, Rockville, MD) to obtain relative transcript levels. Data from TIGR Spotfinder were stored in MAD, a relational database designed to effectively capture and store microarray data. Data were normalized using a local regression technique LOcally WEighted Scatterplot Smoothing (LOWESS) for hybridizations using a software tool MIDAS (http://www.tigr.org/software; TIGR). The resulting data were averaged from triplicate genes on each array and from duplicate flip-dye arrays for each experiment, taking a total of six intensity data points for each gene. Differentially expressed genes at the 95% confidence level were determined using intensity-dependent Z-scores (with $Z = 1.96$) as implemented in MIDAS for all experiments. To identify genes that were differentially expressed during the acquisition of developmental competence, we identified all genes exhibiting differential expression in both the wild-type and *stuA*-complemented strain at 24 or 30 h. Next, the resulting data were organized and visualized using k-means algorithm to find the genes that are differentially expressed between the wild type and the Δ*stuA* mutant. The results of this analysis were then compared with the list of genes exhibiting differential expression in the direct hybridizations of RNA from the wild type and the Δ*stuA* mutant at 24 and 30 h of growth. Genes that were found to have significantly different expression in both sets of experiments were then organized based on similar expression vectors using Euclidean distance and hierarchical clustering with average linkage clustering method with TIGR MEV (http://www.tigr.org/software; TIGR).

Real-Time RT-PCR

To test that the genes identified by the microarray studies were both developmentally regulated and *stuA* dependent, we performed real-time RT-PCR analysis using five genes. The primers used for each gene are shown in Table 1. To obtain RNA, YEPD broth was inoculated with 1×10^6 conidia of either strain Af293 or the Δ*stuA* mutant and incubated at 37°C. Samples were removed at 8 and 24 h, and total RNA was extracted as in the microarray experiments. Samples were then treated with TurboDNase (Ambion, Austin,

TX) before first strand synthesis with RetroScript (Ambion). Real-time PCR was then performed using an ABI 7000 thermocycler (Applied Biosystems, Foster City, CA), and amplification products were detected with SYBR Green (QIAGEN, Valencia, CA). Gene expression was then normalized to *A. fumigatus TEF1* expression, and relative expression was estimated using the formula $2^{\Delta Ct}$, where $\Delta Ct = [Ct_{target\ gene} - Ct_{TEF1}]$. To verify the absence of genomic DNA contamination, negative controls were used for each gene set in which reverse transcriptase was omitted from the mix.

Hydrogen Peroxide Susceptibility Assay

To determine the effects of *stuA* deletion on susceptibility to oxidative stress, we tested the susceptibility of conidia and hyphae to hydrogen peroxide. Conidia were harvested as described above, and either used directly (for conidia sensitivity) or allowed to germinate in Sabouraud dextrose broth at 37°C for 8 h. Both conidia and germlings were suspended to a concentration of 10^5 cells/ml, and 10 μl of suspension was added to microtiter wells containing hydrogen peroxide (0–100 mM) and incubated for 30 or 60 min. At each time point, an aliquot was removed and replica-plated to YEPD agar for recovery. Plates were incubated overnight at 37°C and examined for growth.

Virulence Studies

The virulence of the Δ*stuA* mutant was compared with that of the wild-type Af293 strain and *stuA*-complemented strain in an intranasal model of invasive murine aspergillosis. Male BALB/c mice (National Cancer Institute, Bethesda. MD), weighing 18–22 g, were immunosuppressed with 250 mg/kg cyclophosphamide (Western Medical Supply, Oklahoma City, OK) and cortisone acetate (Sigma-Aldrich, St. Louis, MO) 2 d before infection. For each strain, 10 mice were then anesthetized with isoflurane and infected by intranasal instillation of 10^6 conidia of each strain in 25 μl of phosphate-buffered saline (PBS) + 0.1% Tween. A second dose of immunosuppression was given 3 d of infection consisting of 200 mg/kg cyclophosphamide and 250 mg/kg cortisone acetate. Mice were monitored for signs of illness and time to euthanasia was recorded. Differences in survival between experimental groups were compared using the log-rank test. All procedures involving mice were approved by the Institutional Animal Use and Care Committee, according to the National Institutes of Health guidelines for animal housing and care.

RESULTS

Identifying the Onset of Developmental Competence in A. fumigatus

Because *stuA* expression has been linked to the acquisition of developmental competence (Miller *et al*., 1991), it was critical to determine the time required for the onset of this developmental stage in *A. fumigatus* strain Af293. When submerged hyphae were subcultured to solid agar, the delay between induction by exposure to the air–surface interphase, and production of visible conidiophores decreased with increasing hyphal maturation. However, after 9–10 h of hyphal growth, a fixed delay of 4.5 h between exposure to the air–solid interphase and conidiophore production was observed. Thus, for the conditions used in this study, developmental competence occurred after 9–10 h of submerged growth.

Cloning and Transcriptional Characterization of A. fumigatus stuA

BLAST searches of the ongoing *A. fumigatus* genome sequencing project identified a single putative open reading frame with high homology to the *A. nidulans stuA* gene. The predicted protein sequence of this protein contains a bHLH domain that is highly similar to that of the other APSES proteins (Figure 2, A and B). Surprisingly, this domain is slightly more closely related to the *Penicillium marneffei stuA* (Borneman *et al*., 2002) rather than that of *A. nidulans* (Miller *et al*., 1992), although the overall homology of the predicted proteins is higher between the *A. nidulans* and *A. fumigatus* proteins (Figure 2A). This sequence has been subsequently deposited with GenBank by the genome sequence project under accession no. EAL93087.

Transcriptional analysis by Northern blotting and real-time RT-PCR during germination and hyphal maturation in submerged culture demonstrated a marked increase in *stuA* transcription after 12 h (Figure 3), corresponding to the onset of developmental competence. This expression of *stuA* mRNA was maximal by 24 h of growth and this gene was highly expressed in competent hyphae at all subsequent time points that we have examined. This pattern of transcription is similar to that seen with *stuA* in *A. nidulans* (Miller *et al*., 1992), suggesting that the proteins encoded by these two genes likely share some similarities in function.

Morphological Effects of stuA Deletion

To determine the function of StuAp in *A. fumigatus*, we constructed a Δ*stuA* mutant by gene deletion. The resulting Δ*stuA* mutant was then complemented by transformation with an intact copy of *stuA*, to ensure that the observed phenotypes were specifically a result of the deletion of *stuA*.

Deletion of *stuA* produced a dramatic morphological phenotype (Figure 4). Wild-type *A. fumigatus* colonies produced a vivid green color due to the production of abundant pigmented spores borne on condiophores (Figure 4A). In contrast, strains deficient in *stuA* produced whitish colonies, which became slightly yellow on prolonged incubation. The explanation for this difference was readily apparent upon microscopic examination. The Δ*stuA* mutants displayed markedly abnormal conidiophores (Figure 4, D–F). First, the broad, aseptate conidiophore stalk upon which the vesicle is borne was completely absent from the Δ*stuA* mutant (Figure 4D). The vesicles were produced directly from septate hyphae, and although relatively normal in shape, they were often lacking phialides entirely (Figure 4D). When phialides were present, they were swollen and dysmorphic (Figure 4D). Furthermore, secondary conidiophores were observed emerging from some vesicles, often producing conidia themselves (Figure 4F). The number of conidia was greatly reduced compared with wild-type strain Af293. Conidia were not only produced by phialides as in wild-type *A. fumigatus* but also were seen budding directly from the vesicle head (Figure 4D), and even from what seemed to be normal hyphae without any detectable vesicle production (Figure 4E). These Δ*stuA* mutant conidia were approximately twice the size of normal conidia when examined microscopically, and they varied in both shape and size (Figure 4, D–F). Complementation of the Δ*stuA* mutant with a wild-type allele of *stuA* restored normal conidiation (Figure 4C), although the conidia produced by this strain were smaller than the original parent Af293.

The Δ*stuA* mutant also had accelerated germination. Wild-type Af293 conidia and the *stuA*-complemented strain produced visible germ tubes after 6–8 h of incubation at 37°C. In contrast, germ tubes of the Δ*stuA* were observed as early as 3–4 h of incubation (Figure 5, A and B). The germ tubes and hyphae of the Δ*stuA* mutant were indistinguishable from that of wild-type Af293 or the complemented strain. Radial growth on solid media was also similar among the three strains.

Given the impaired conidiation of the Δ*stuA* mutant, and the correlation of *stuA* transcription with developmental competence, we tested the effects of *stuA* deletion on the time course of developmental competence. Conidiophore production by the Δ*stuA* mutant was markedly delayed compared with wild-type Af293 and the *stuA*-complemented strain. This delay was attributable to a much slower production of conidiophores after induction (8–9 h for the Δ*stuA* strain compared with 4–5 h for the wild-type and *stuA*-complemented strain). The time to acquisition of developmental competence by the Δ*stuA* strain was not significantly different from the wild-type or *stuA*-complemented strain. Collectively, these data suggest a model whereby

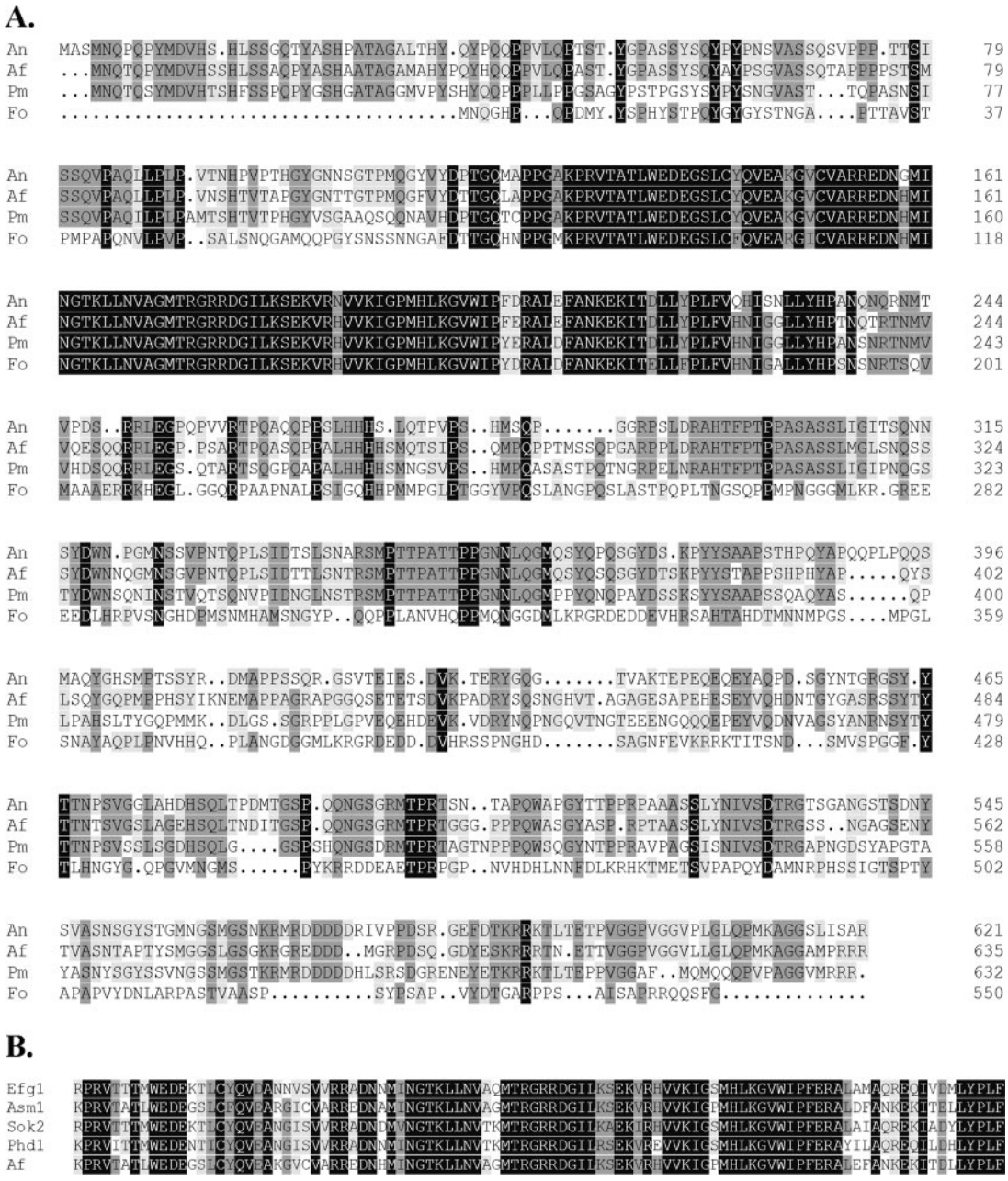

Figure 2. Sequence alignments of the members of the APSES protein. (A) Alignment of the predicted protein sequence of the StuA proteins identified from filamentous fungi. An, *A. nidulans*; Af, *A. fumigatus*; Pm, *Penicilllium marneffei*; and Fo, *Fusarium oxysporum*. (B) Alignment of the basic-helix-loop-helix domains of *A. fumigatus* StuAp with other APSES family members. Efg1 from *C. albicans*, Asm1 from *Neurospora crassa*; and Phd1 and Sok2 from *S. cerevisiae*.

StuAp does not govern developmental competence but rather functions as an effector of this process.

Transcriptomes during the Acquisition of Developmental Competence

Transcriptional profiling is ideally suited to the study of development in *Aspergillus* sp. Developmental competence occurs in a highly coordinated manner within growing cultures and is unaffected by nutritional deficiencies (Axelrod *et al*., 1973; Pastushok and Axelrod, 1976). Furthermore, the majority of known developmental genes in *Aspergillus* spp. exhibit transcriptional regulation (Adams *et al*., 1992). Therefore, to identify genes whose expression was linked to developmental competence, we performed whole-genome transcriptional profiling of the wild-type and *stuA*-complemented strains. Comparing the expression of genes between precompetent (8 h) and postcompetent hyphae (both at 24 and 30 h) identified 720 differentially expressed genes in the wild-type strain Af293, and 597 genes in the *stuA*-complemented strain. The subset of 445 genes that were found to be differentially expressed in both strains was used in subsequent analysis.

Next, to identify which of these developmentally regulated genes were dependent on StuAp, we performed tran-

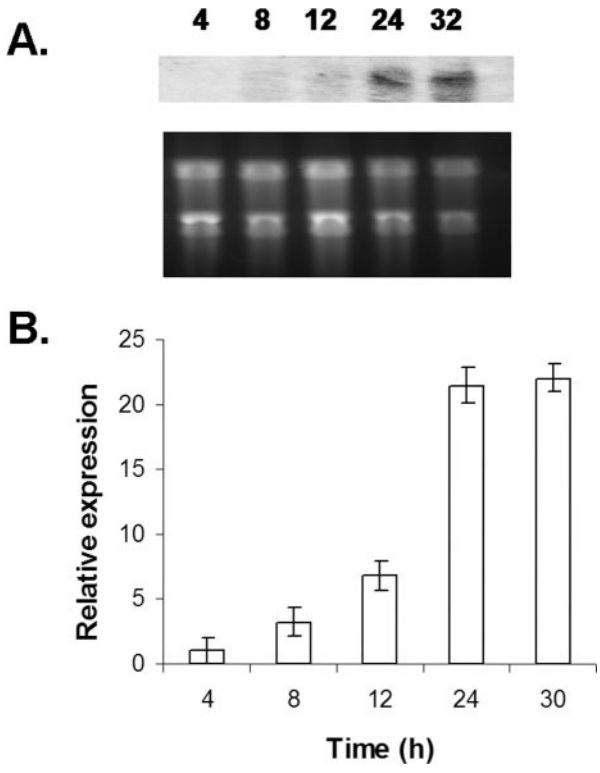

Figure 3. Time course of *stuA* expression. (A) Northern blot of *stuA* expression during germination and hyphal development. Total RNA was extracted from organisms growing at 37°C in YEPD broth. Top, blot probed with the entire *stuA* open reading frame. Bottom, total RNA stained with ethidium bromide. (B) Real-time RT-PCR of *stuA* expression in an independent experiment. Total RNA was extracted from identical time points, and *stuA* expression normalized to *A. fumigatus TEF1* is displayed on the *y*-axis. Error bars indicate SD.

scriptional profiling of the Δ*stuA* mutant using the same time points as described above. Of the 445 competence-associated genes identified in the wild-type hybridizations, 104 genes differed in expression level between the Δ*stuA* mutant and both the wild-type and complemented strains at 24 and 30 h (when *stuA* expression is maximal). To confirm these results, we next performed direct hybridizations of RNA isolated from wild-type and Δ*stuA* hyphae after 24 and 30 h of incubation. Of the 104 candidate *stuA*-dependent genes identified in the previous experiments, 94 of these again showed a statistically significant difference in expression between the wild-type and the Δ*stuA* mutant at both 24 and 30 h. Hierarchical cluster analyses of all developmental genes, and of the *stuA*-dependent developmental genes, are presented in Figure 6, A and 6B.

The *stuA*-dependent developmental genes identified by the microarray studies could be broadly classified into several groups according to their predicted function (Table 2). As expected, given the role of *stuA* in conidiation, this included genes encoding putative proteins either known or expected to be involved in the regulation of morphogenesis and development and genes encoding proteins involved in cell metabolism. Interestingly, several genes encoding enzymes involved in secondary metabolite biosynthesis and export were identified as well as the genes encoding proteins that are antigenic during invasive and allergic disease.

Six of the *stuA*-dependent genes identified by the microarray analysis were found to be clustered in proximity on chromosome 8. Examination of this region revealed a putative biosynthetic gene cluster containing at least 36 hypothetical genes. These genes are predicted to encode a variety of proteins required for the biosynthesis of both sterigmatocystin/aflatoxin and ergot alkaloid metabolites (Figure 6C). This cluster is interrupted by the presence of a retrotransposon-like element that is found in at least three other locations within the genome (Afu3g09410-30, Afu4g02620-40, and Afu4g14860-70, identified by BLAST search of the *A. fumigatus* genome at http://tigrblast.tigr.org/er-blast/index.cgi?project = afu1). With the exception of the putative retrotransposon element, the majority of the genes in this cluster exhibit of *stuA*-dependent up-regulation during the onset of developmental competence (Figure 6C).

Real-Time Reverse Transcription (RT)-PCR Confirmation of Transcriptomes

To test the accuracy of the microarray experiments, we used real time RT-PCR to analyze the expression of five putative members of the *stuA*-regulated developmental program. These results (Figure 7) confirmed that all five of these genes were up-regulated during the acquisition of developmental competence by wild-type *A. fumigatus*. Furthermore, this induction was significantly reduced or absent for all five of these genes in the Δ*stuA* mutant strain.

Susceptibility of ΔstuA Mutants to Oxidative Stress

The results of the microarray analysis and real-time PCR studies provided strong evidence that competent hyphae of Δ*stuA* strains are deficient in catalase production. To determine the functional significance of these results, we compared the susceptibility of the Δ*stuA* strain to the wild-type and complemented strain. Hyphae of the Δ*stuA* mutant were markedly more susceptible to oxidative stress from hydrogen peroxide than either the wild-type or *stuA*-complemented strain (Figure 8). Even the lowest concentration of hydrogen peroxide, for the shortest exposure, completely inhibited subsequent growth of the organism. These results mirror the observations from the transcriptional profiling experiments, suggesting that for StuAp governs the response to oxidative stresses in competent hyphae by increasing expression of hyphal catalase.

Virulence of the ΔstuA Mutant Strain

The Δ*stuA* mutant strain displayed altered regulation of multiple genes that may play a role in virulence. We therefore compared the virulence of the Δ*stuA* mutant strain to the wild-type Af293 and the *stuA* complemented strain during experimental murine pulmonary aspergillosis. Mice infected with the *stuA* mutant exhibited a trend toward increased survival compared with both wild-type and the *stuA*-complemented strain (Figure 9); however, this difference was not statistically significant ($p = 0.1$ and 0.4, respectively, by the log-rank test).

DISCUSSION

The initiation of *A. fumigatus stuA* transcription coincides with the onset of developmental competence, and after 24 h remains relatively constant in competent hyphae during all time points tested. Disruption of *stuA* produced a dramatic oligo-conidial phenotype with reduced or absent conidiophores, few to no phialides, and dysmorphic conidia. These results parallel the abnormalities seen in *stuA*-deficient *A. nidulans* (Miller *et al.*, 1991), although the production of conidia in the absence of vesicle heads, and the production of compound vesicles (conidiophores arising from vesicle

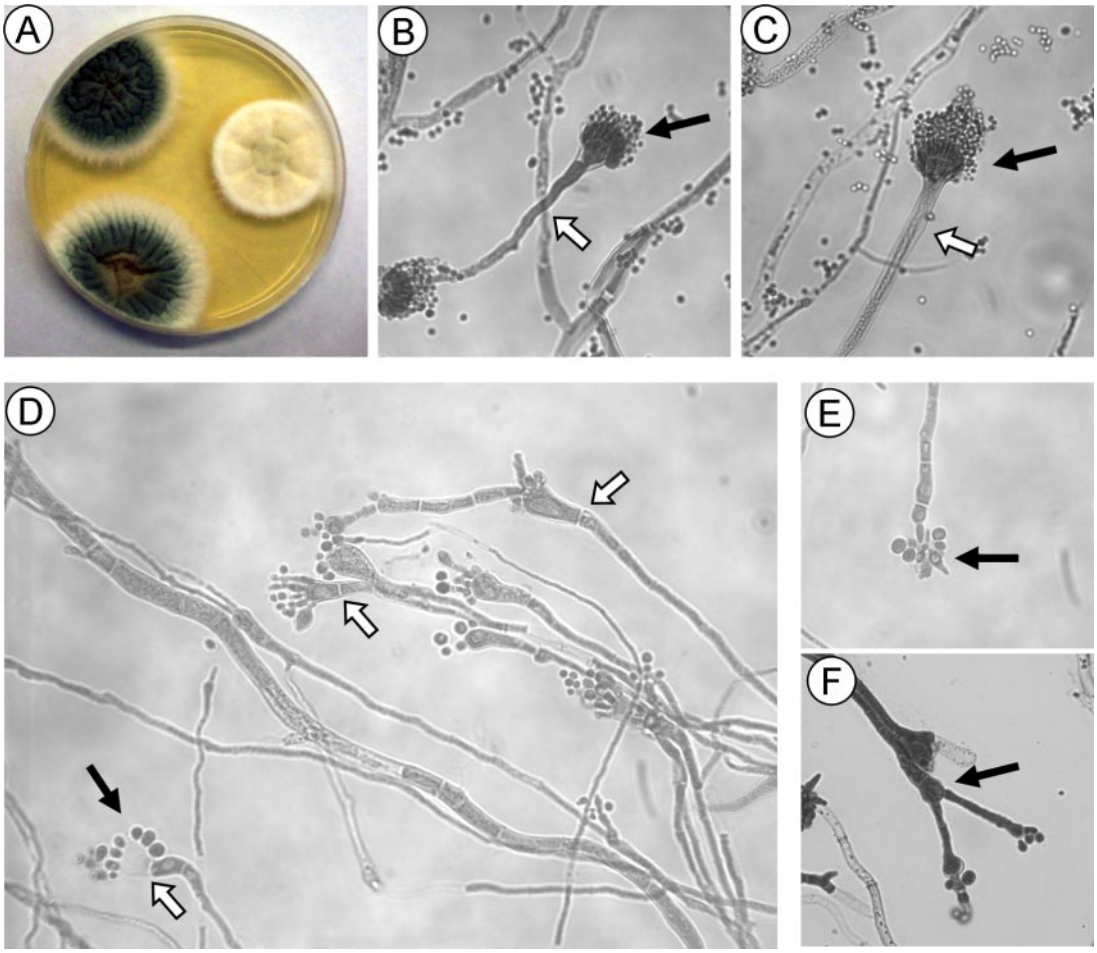

Figure 4. Morphology of Δ*stuA* mutant. (A) Colonial morphology of *A. fumigatus* strains Af293 (top left), Δ*stuA* mutant (middle right), and *stuA*-complemented strain (bottom left) grown for 4 d at 37°C on YEPD agar. The Δ*stuA* mutant strain displays markedly impaired conidiation in vitro. (B–F) 400× magnification view of conidiophores. (B) Wild-type strain Af293 produces long aseptate conidiophore (open arrow) and chaining conidia produced by phialides (solid arrow). (C) The *stuA*-complemented strain produces normal conidiophores indistinguishable from strain Af293. (D–F) Conidophores of the Δ*stuA* mutant strain. (D) The Δ*stuA* mutant produces abnormally short to absent conidiophores (open arrows) and conidia that often emerge directly from the vesicle (solid arrow). (E) Production of condia by the Δ*stuA* mutant in the absence of a visible vesicle. (F) Compound conidiophores with conidiophores emerging from the vesicle head (solid arrow).

heads) has not been described in these mutants. In addition, the rapid germination of *A. fumigatus* Δ*stuA* conidia has not been reported in *A. nidulans* Δ*stuA* strain, although it is unclear whether this phenomenon has been specifically examined.

A. nidulans has long served as a model system for studying the regulation of conidial development. The majority of this work, however, has focused on the events surrounding conidiation, and not the molecular events underlying the acquisition of developmental competence. In contrast, because *A. fumigatus* does not undergo conidiation during invasive disease, the preconidiation stage of developmental competence is of great interest from the perspective of pathogenicity. Indeed, recent studies from our group have found that hyphae from mice infected with *A. fumigatus* express *stuA* in vivo (Doedt *et al*., 2005), suggesting that competent hyphae are present during infection

We therefore performed whole-genome transcriptional analysis to identify the genes displaying differential regulation during the acquisition of developmental competence and to identify the subset that exhibit *stuA* dependence. Although microarrays are a powerful tool for the identification of transcriptional programs, validation of these results is critical. We used multiple complementary strategies to ensure that the results of these experiments were robust, including dye-swaps, in-slide triplication of hybridizations target spots, the use of the complemented and wild-type strain for biological replicates, and an experimental design that used both time-course studies as well as direct hybridizations between strains. Furthermore, several lines of evidence support the validity of these data. In addition, direct verification of expression by real-time PCR using RNA from an independent experiment was performed. These results confirmed that the expression of all five candidate genes was both developmentally regulated and *stuA* dependent. Finally, the hypersusceptibility of the Δ*stuA* mutant strain to hydrogen peroxide provides a phenotypic confirmation of *stuA*-dependent hyphal catalase expression. Collectively, these results suggest that the gene list generated by the microarray experiments is an accurate one. Conversely, it is important to note that this list of *stuA*-dependent genes is not exhaustive. In particular, by focusing our analyses on the

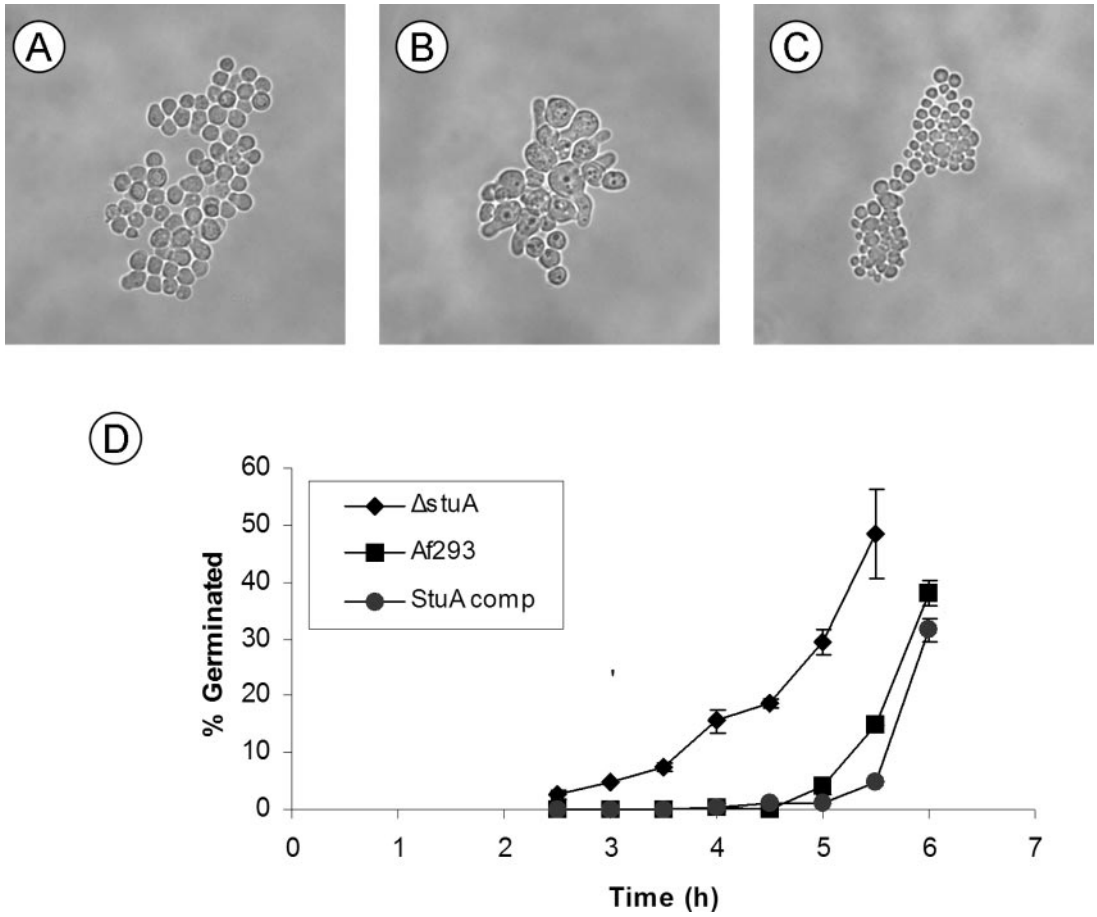

Figure 5. Precocious germination of Δ*stuA* mutants. Photomicrograph (400× magnification) of conidia grown for 4 h in YEPD broth at 37°C. (A) Wild-type Af293. (B) Δ*stuA* mutant strain showing germination and early hyphal formation. (C) *stuA*-complemented strain. (D) Time course of germination of conidia in YEPD broth at 37°C. Samples removed at each time interval were examined microscopically, and the number of conidia with visible germ tubes were counted. A minimum of 100 organisms were counted in triplicate for each time point. Error bars indicate SD of three separate experiments.

later time points of 24 and 30 h (when *stuA* expression is maximal), genes with transient alterations in expression coincident with the onset of *stuA* expression, or other *stuA*-dependent genes that are not developmentally regulated, would not have been identified.

Because much of the *A. fumigatus* genome remains either unannotated, or annotated only by automated algorithms, a detailed analysis of genes by functional category from the results of microarray studies is not yet possible. Indeed, almost half (48/94) of the *stuA*-dependent genes identified in these experiments are of unknown function. However even given these limitations, interesting patterns of gene expression can be discerned. As expected, several *stuA*-dependent genes were homologous to genes known to be involved in the developmental regulation of other fungi, including a homologue of the *Saccharomyces cerevisiae IME2*, which positively regulates meiosis during sporulation (Smith and Mitchell, 1989). Opsin 1 is homologous to the Nop-1 protein of *Neurospora crassa*, which encodes a photoreactive protein involved in light sensing, and whose expression is highest during conidiation (Bieszke *et al*., 1999). A homologue of the *Blastomyces dermatitidis bys1* gene was also identified. This gene encodes a protein of unknown function whose transcription is tightly linked to morphogenesis and is expressed only during the pathogenic yeast phase of this organism (Burg and Smith, 1994; Bono *et al*., 2001). Expression of *rosA* was *stuA* dependent and associated with developmental competence in *A. fumigatus*. Interestingly, in *A. nidulans*, high level expression of *rosA* was found only after the initiation of conidiation and not in submerged competent hyphae (Vienken *et al*., 2005). In addition, deletion of *rosA* was associated with increased *stuA* expression, suggesting either a direct or feedback regulatory link between these two proteins (Vienken *et al*., 2005). A novel finding was the decreased expression of a *phiA* homologue in the *stuA* mutant during development. The cell wall protein encoded by the *phiA* gene of *A. nidulans* plays a critical role in the development of normal phialides, with *phiA*-deficient mutants displaying abnormal phialides and marked reductions in conidiation (Melin *et al*., 2003). Whether the abnormal *phiA* expression in *stuA* null mutants is responsible for, or results from, the marked abnormalities in conidiphore growth is not known. Finally, clock-controlled gene 9 encodes a trehalose synthase required for normal conidiation in response to light cycling in *N. crassa* (Shinohara *et al*., 2002)

Several other *stuA*-dependent developmental genes are predicted to encode proteins that are involved in the immune response to *A. fumigatus* during allergic or invasive disease. The elaboration of IgE-binding proteins by *A. fu-*

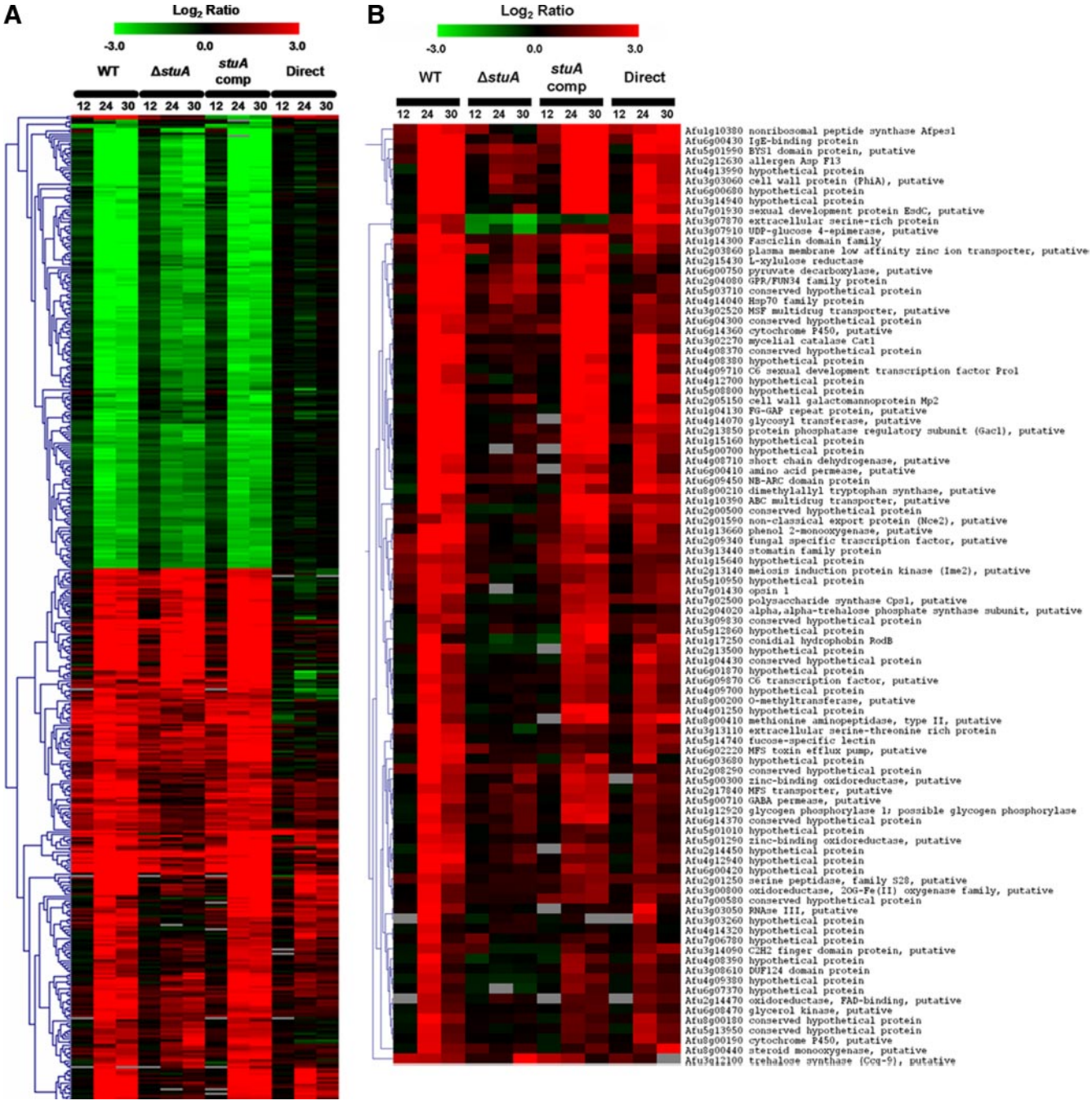

Figure 6. Transcriptional analysis during the acquisition of developmental competence. (A) Hierarchical clustering of genes displaying significantly different expression ($Z > 1.96$) after the onset of developmental competence in both wild-type Af293 and the *stuA*-complemented strain. (B) Hierarchical clustering of genes showing *stuA*-dependent expression during the onset of developmental competence. (C) Comparative expression of genes comprising the *stuA*-dependent putative biosynthetic cluster, arranged by genomic location. For all figures, the mean-fold changes ($\log_2$) in gene expression are represented by colored squares. Red blocks indicate up-regulation of the gene of interest in the test pool compared with the control pool and green blocks indicate down-regulation. The first nine columns present the relative transcript level at each time point compared with the expression at 8 h for the same strain. The last two columns indicate the relative transcript level comparing the Δ*stuA* mutant and wild-type Af293 directly at each time point. Cluster groupings on the left (A and B) indicate similar gene expression trends within groups of significant genes. WT, wild-type strain Af293; Δ*stuA*, Δ*stuA* mutant strain; *stuA* comp, *stuA*-complemented strain; Direct, direct comparison of wild-type and Δ*stuA* RNA; EA, putative ergot alkaloid synthetic subcluster; Tn, putative retrotransposon; and ST, putative sterigmatocystin/aflatoxin synthetic subcluster.

migatus (including rAsp f13, an alkaline serine protease; and IgE-binding protein Afu6g00430) is a key stimulus for the development of allergic bronchopulmonary aspergillosis (Kurup *et al*., 2002; Stevens *et al*., 2003), suggesting that StuAp may play an important role in the pathogenesis of this condition. *MP2* encodes a surface protein in *A. fumigatus* that elicits a specific antibody response during invasive disease, although its role in protective or allergic immunity has

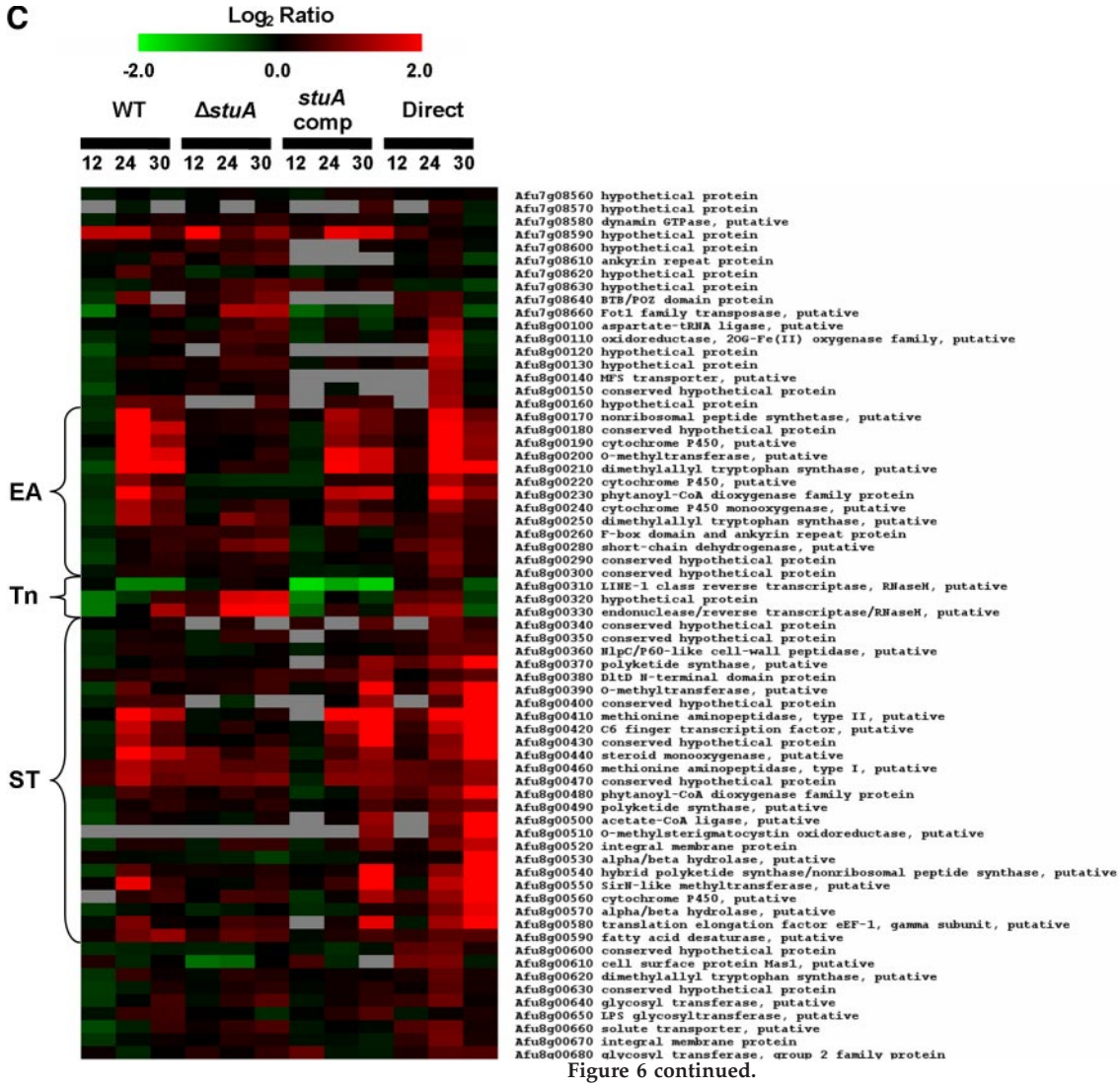

Figure 6 continued.

not yet been defined (Chong *et al*., 2004). Finally, *rodB* encodes a hydrophobin that does not mediate rodlet formation or oxidative resistance in conidia of *A. fumigatus* (Paris *et al*., 2003a). The function of this protein in developmentally competent hyphae however is unknown. In other filamentous fungi, expression of hyphal hydrophobins is required for the production of aerial hyphae or normal conidiophores (Fuchs *et al*., 2004). These hydrophobins can also contribute to virulence by mediating adherence to host tissues (Talbot *et al*., 1996). Collectively, these data suggest that *stuA* may regulate virulence-associated phenotypes during both invasive and allergic disease.

Perhaps the most interesting finding of the transcriptional analysis studies is the identification of a *stuA*-dependent putative secondary metabolite biosynthesis cluster. Unlike most other genes in *Aspergillus* spp., genes encoding proteins that mediate the synthesis of these mycotoxins are often found physically linked in clusters such as the aflatoxin/sterigmatocystin biosynthesis clusters of *Aspergillus parasiticus*, *Aspergillus flavus*, and *Aspergillus*. *nidulans* (Keller and Adams, 1995; Brown *et al*., 1996; Yu *et al*., 2004) and the gliotoxin biosynthesis cluster of *A. fumigatus* (Gardiner and Howlett, 2005). In *A. fumigatus*, two biosynthetic clusters involved in the synthesis of ergot alkaloids such as fumigaclavine have been identified (Coyle and Panaccione, 2005; Unsold and Li, 2005) as has a polyketide synthesis cluster responsible for the production of conidial pigment (Tsai *et al*., 1999). A recent genomic analysis of *A. fumigatus* found at least 26 of these putative clusters throughout the genome of strain Af293 (Nierman *et al*., 2005).

The composition of the cluster identified in this study is unique in that it contains both genes encoding synthetic enzymes required for both sterigmatocystin/aflatoxin synthesis as well as ergot alkaloid synthesis with each set of genes clustered at one end of the cluster. The ergot alkaloid synthetic arm of the cluster is composed of two dimethylallyl tryptophan synthetases, three cytochrome p450 enzymes, an *o*-methyltransferase, a nonribosomal peptide synthetase, and several open reading frames encoding proteins of unknown function (Figure 6C). A similar group of genes is found in both other ergot alkaloid synthesis clusters identified from *A. fumigatus* as well as the archetypal ergot alkaloid synthesis cluster first described in *Claviceps purpurea* (Tudzynski *et al*., 1999; Haarmann *et al*., 2005). Interestingly, although a homologue of the nonribosomal peptide synthetase in the *stuA*-dependent *A. fumigatus* cluster is found in *C. pupurea*, this gene is not found in either of the other *A. fumigatus* ergot biosynthesis clusters previously described,

Table 2. Subset of StuAp-dependent developmentally regulated genes identified by microarray studies arranged by putative function

Functional group	Locus	Common name/description
Development and morphogenesis	Afu3g03060	PhiA protein
	Afu7g01930	ESDC
	Afu7g01430	Opsin 1
	Afu2g13140	Meiosis induction protein kinase ime2/sme1
	Afu4g09710	Repressor of sexual development/Pro1
Antigenic proteins	Afu2g12630	rAsp f 13
	Afu2g05150	Cell wall galactomannoprotein MP2
	Afu6g00430	IgE-binding protein
	Afu1g17250	Conidial hydrophobin RodB
Metabolism	Afu2g15430	L-Xylulose reductase
	Afu2g04020	α,α-Trehalose phosphate synthase subunit
	Afu2g13850	Protein phosphatase regulatory subunit (Gac1)
	Afu3g12100	Trehalose synthase (Ccg-9)
	Afu4g08710	Short chain dehydrogenase
	Afu2g04080	GPR/FUN34 family protein
	Afu6g08470	Glycerol kinase
	Afu1g12920	Glycogen phosphorylase 1
	Afu3g07910	UDP-glucose 4-epimerase
	Afu6g00750	Pyruvate decarboxylase
Secondary metabolite biosynthesis	Afu6g14360	Cytochrome P450
	Afu1g13660	Phenol 2-monooxygenase
	Afu4g14070	Glycosyl transferase
	Afu1g10380	Nonribosomal peptide synthase Afpes1
	Afu3g00800	Oxidoreductase, 2OG-Fe(II) oxygenase family, putative
	Afu5g00300	Zinc-binding oxidoreductase, putative
	Afu3g02270	Mycelial catalase Cat1
	Afu5g01290	Zinc-binding oxidoreductase, putative
	Afu2g14470	Oxidoreductase, FAD-binding, putative
	Afu8g00190	Cytochrome P450
	Afu8g00210	Dimethylallyl tryptophan synthase
	Afu8g00200	*O*-Methyltransferase
	Afu8g00440	Steroid monooxygenase
	Afu8g00410	Methionine aminopeptidase, type II
Transport	Afu1g10390	ATP-binding cassette (ABC) multidrug transporter
	Afu2g01590	Nonclassical export protein (Nce2)
	Afu2g03860	Plasma membrane low affinity zinc ion transporter
	Afu2g17840	Major facilitator superfamily (MFS) transporter
	Afu3g02520	MSF multidrug transporter
	Afu5g00710	GABA permease
	Afu6g02220	MFS toxin efflux pump
	Afu6g00410	Amino acid permease

suggesting that different mycotoxins are produced by individual clusters.

Similarly, the ~25 putative proteins encoded by the genes in the sterigmatocystin/aflatoxin subcluster are very similar to members of the conserved ~25 gene sterigmatocystin/aflatoxin synthetic cluster of *A. flavus*, *A. nidulans*, and *A. parasiticus* (Keller and Adams, 1995; Yu *et al*., 1995, 2004; Brown *et al*., 1996). All four clusters include monooxygenases, reductases, a polyketide synthase, and a $Zn(II)_2$, Cys_6 domain protein homologous to *aflR*, the pathway-specific transcription factor required for sterigmatocystin/aflatoxin biosynthesis (Figure 6C) (Yu *et al*., 1996; Fernandes *et al*., 1998). Interestingly, the presence of an associated ergot alkaloid synthetic cluster in association with this sterigmatocystin cluster has not been reported in any of these *Aspergillus* species and seems to be unique to *A. fumigatus*. The specific synthetic products elaborated by both these subclusters remain unknown, and are under study in our laboratory.

The identification of a *stuA*-dependent biosynthetic cluster provides a new link between development and secondary metabolite production. The mechanisms underlying this regulation are not yet elucidated; however, as noted above, the *A. fumigatus* sterigmatocystin/aflatoxin biosynthesis subcluster contains an *aflR* homologue, which exhibits *stuA*-dependent expression (Figure 6C). In other *Aspergillus* spp., *aflR* is required for sterigmatocystin/aflatoxin biosynthetic gene activation (Ayer and Eisenman, 1993; Yu *et al*., 1996; Fernandes *et al*., 1998). Thus, *stuA*-mediated control of the sterigmatocystin/aflatoxin biosynthetic subcluster is likely mediated at least in part by *aflR*. Further studies will be necessary to confirm how *stuA* integrates with *aflR* and other regulatory pathways governing development and secondary metabolite production including G protein-dependent signaling (Yu and Keller, 2005), the global regulator *laeA* (Bok and Keller, 2004) and the *FluG-brlA* pathway (Yu and Keller, 2005).

In addition to the identification of individual genes involved in development, the results of the *A. fumigatus* microarray studies provide insight into the overall role of *stuA* in the developmental program governing competence. Genes dependent on *stuA* made up >20% (94/445) of the total number of genes displaying differential regulation during development. Thus, although other regulatory pathways likely contribute significantly to the acquisition of develop-

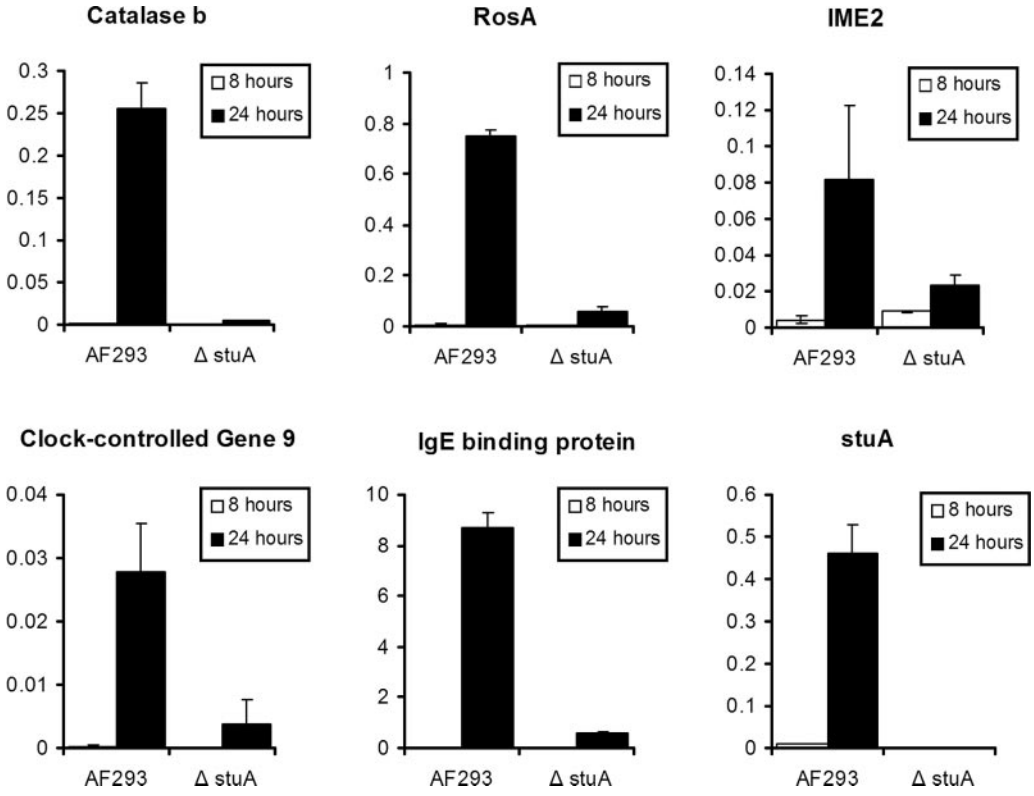

Figure 7. Real-time RT-PCR of *stuA* dependent developmental genes. The expression of five candidate *stuA*-dependent developmental genes was analyzed using RNA extracted from hyphae grown for 8 h (precompetent) and 24 h (postcompetent) in YEPD broth at 37°C. Relative expression (normalized to *A. fumigatus TEF1* expression) is shown on the *y*-axis. Error bars indicate the SD for each result. All five genes exhibited *stuA*-dependent up-regulation in developmentally competent hyphae. *stuA* expression in both strains is shown for comparison in the final graph.

mental competence, StuAp plays a central role in the regulation of this biological process.

The transcriptional analysis performed in this study also suggests that *stuA* may function as a transcriptional activator during the acquisition of developmental competence. Indeed, although almost an identical number of genes were up- and down-regulated during the acquisition of developmental competence (Figure 6A), it is striking that all of the *stuA*-dependent genes identified by these analyses were up-regulated at these time points (Figure 6B) and none were down-regulated. Indeed, approximately one-half of the genes showing increased expression during developmental

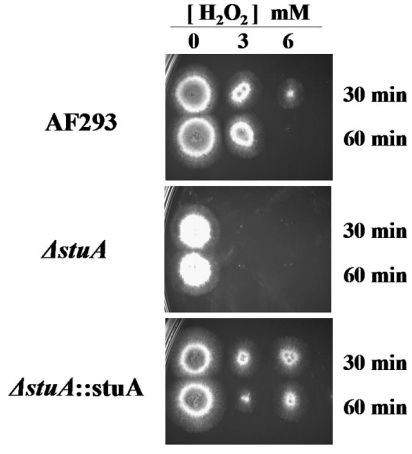

Figure 8. Hyphae from Δ*stuA* mutants are hypersusceptible to hydrogen peroxide. Hyphae of wild-type Af293, Δ*stuA* mutant, and *stuA*-complemented strains were exposed to varying concentrations of hydrogen peroxide for 30 or 60 min. Aliquots were then removed and plated to YEPD agar and incubated overnight at 37°C.

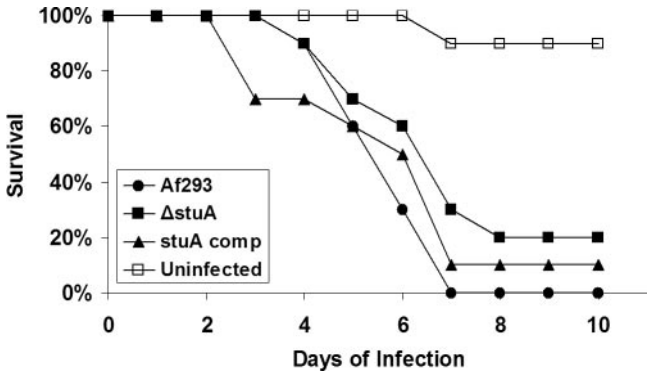

Figure 9. Virulence of Δ*stuA* mutants during murine pulmonary infection. Immunosuppressed mice were infected intranasal with 1×10^6 conidia of each strain and monitored daily. Δ*stuA*, Δ*stuA* null mutant; Af293, wild-type parent strain; stuA comp, *stuA*-complemented strain; and uninfected, sham infection using PBS + 0.1% Tween alone.

competence exhibit *stuA* dependence. These data contrast with studies of conidiation in *A. nidulans* in which StuAp has been shown to function as a transcriptional repressor (Dutton *et al*., 1997). One explanation is that StuAp may function as a transcriptional activator at the level of expression seen in competent hyphae and act as a transcriptional repressor at the higher levels seen during conidiation. Similar threshold effects for StuAp function have been shown in *A. nidulans*, with sexual reproduction requiring higher StuAp expression than asexual reproduction (Wu and Miller, 1997). Furthermore, a similar model of concentration-dependent transcriptional activation and repression has been proposed for other bHLH proteins, including Efg1p in *C. albicans* (Stoldt *et al*., 1997) and Myc in mammalian cells (Ayer and Eisenman, 1993; Ayer *et al*., 1993; Li *et al*., 1994). Alternately, StuAp may repress an intermediate regulatory element that is itself normally responsible for repression of the rest of the *stuA*-dependent program. By this model, StuAp would still function as a transcriptional repressor and yet produce upregulation of many downstream elements.

We found that hyphae of Δ*stuA* mutants were hypersusceptible to hydrogen peroxide and exhibited markedly reduced levels of *catB* (*CAT1*) mRNA. Previously, Paris and colleagues found that disruption of *catB* (*CAT1*) was not sufficient to render hyphae susceptible to hydrogen peroxide and that disruption of the second mycelial catalase (*CAT2*) was required (Paris *et al*., 2003b). Although these results may reflect methodological differences in determining hydrogen peroxide susceptibility, it is likely that the observed increased susceptibility of the Δ*stuA* mutant to hydrogen peroxide reflects the contribution of other *stuA*-dependent proteins. Indeed, examination of the microarray data reveals another putative catalase-peroxidase (locus Afu8g01670) that exhibits significant *stuA*-dependent expression but that was only significant at 12 h of growth and fell below the z-score cutoff by 24 and 30 h (z-scores of 4.67 at 12 h, 1.82 at 24 h, and 1.12 at 30 h in strain Af293), which resulted in exclusion from our initial analysis of *stu*A-dependent genes.

Finally, when tested in vivo, the Δ*stuA* mutant exhibited only a nonsignificant trend toward reduced virulence compared with the wild-type parent or the *stuA*-complemented strain. This result is somewhat surprising in light of the number of putative virulence factors found to have reduced expression in this strain. One possible explanation for these findings is that the accelerated germination of the Δ*stuA* mutant strain may have contributed to increasing the virulence of this strain despite the loss of several other virulence factors. Indeed, in other fungi, such as *C. albicans*, the rate of growth and germination is an important independent predictor of virulence (Rieg *et al*., 1999). Deletion of genes critical for individual *stuA*-dependent processes, such as those controlling the expression of secondary metabolites, may be helpful to resolve this issue.

In summary, we have demonstrated that not only does the APSES protein StuAp play an important role in governing conidiation of *A. fumigatus* but also that it is required for the normal expression of a large subset of the genes that are differentially expressed between competent and precompetent hyphae. StuAp is not required for normal virulence in a mouse model of experimental aspergillosis. In contrast to its role as a transcriptional repressor during conidiation, StuAp functions, either directly or indirectly, as a transcriptional activator in developmentally competent hyphae. In addition, we have found evidence for *stuA*-mediated expression of a unique mycotoxin biosynthetic cluster predicted to mediate production of ergot alkaloid and sterigmatocystin/aflatoxin.

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