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ScienceDirect
Fungal Genetics and Biology 43 (2006) 775–788
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Coccidioides posadasii contains single chitin synthase genes corresponding to classes I to VII

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Received 21 September 2005; accepted 23 May 2006
Available online 20 July 2006

Abstract

Coccidioides posadasii is a dimorphic fungal pathogen of humans and other mammals. The switch between saprobic and parasitic growth involves synthesis of new cell walls of which chitin is a significant component. To determine whether particular subsets of chitin synthases (CHSes) are responsible for production of chitin at different stages of differentiation, we have isolated six *CHS* genes from this fungus. They correspond, together with another reported *CHS* gene, to single members of the seven defined classes of chitin synthases (classes I–VII). Using Real-Time RT-PCR we show their pattern of expression during morphogenesis. *CpCHS2*, *CpCHS3*, and *CpCHS6* are preferentially expressed during the saprobic phase, while *CpCHS1* and *CpCHS4* are more highly expressed during the parasitic phase. *CpCHS5* and *CpCHS7* expression is similar in both saprobic and parasitic phases. Because *C. posadasii* contains single members of the seven classes of CHSes found in fungi, it is a good model to investigate the putatively different roles of these genes in fungal growth and differentiation.

Index descriptors: Chitin synthase; CHS; Cell wall; *Coccidioides* spp; Valley fever; Real-time RT-PCR; Gene expression; Fungal pathogenesis

1. Introduction

Coccidioides species are the causal agent of coccidioidomycosis, commonly known as Valley Fever (Galgiani, 1999). The saprobic phase of this dimorphic fungus is found in desert soils of the southwestern United States (the San Joaquin Valley in California, Arizona and Texas), northern Mexico, and isolated areas of Central and South America. Based on phylogenetic analyses (Fisher et al., 2002), the genus *Coccidioides* was divided into two species, with isolates from California recognized as *Coccidioides immitis*, and all the non-California isolates re-classified as *Coccidioides posadasii*. In spite of this separation, there are no reports of differences in the disease caused by the two species, or in the regulation of their parasitic cycles. Coccidioidomycosis manifests as a community acquired pneumonia in the majority of affected people, but can disseminate, causing potentially life-threatening complications that require extensive antifungal therapy in some individuals (Galgiani et al., 2000).

The saprobic phase of *Coccidioides* is typical of filamentous ascomycetes with polar growth of septate hyphae. Sporulation occurs with aerial hyphae undergoing wall rigidification, followed by the thickening of the inner cell wall and autolysis of alternate compartments, with the remaining cells developing into arthroconidia (Cole et al., 1995). Eventually, the empty autolysed compartments fracture and cylindrical arthroconidia may be dispersed in the environment, where the cycle of saprobic growth and asexual sporulation can repeat itself. When an arthroconidium is inhaled, a unique parasitic phase is initiated in the lungs, ultimately producing a large multicellular structure called a

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doi:10.1016/j.fgb.2006.05.005

spherule that is capable of releasing infectious propagules known as endospores. The parasitic cycle can be divided into three stages (Cole et al., 1995; Cole and Hung, 2001). Briefly, spherule development initiates with the 3–5 μm barrel-shaped arthroconidium rounding up and expanding isotropically while the nuclei undergo multiple rounds of mitosis, ultimately forming a large multinucleate cell with a central vacuole. Spherule wall expansion requires biosynthesis of new cell wall components, as well as degradation of pre-existing ones. By 72 h after initiation, the spherule begins to undergo internal segmentation followed by development of uninucleate endospores. Invaginations from the spherule wall are synthesized and segment the protoplasm into compartments that surround a central vacuole. This process involves synthesis of large amounts of chitin (Cole et al., 1993, 1995). At about 96 h, these compartments differentiate into endospores with the synthesis of new cell walls within the segmentation walls. Between 120 and 132 h post-infection, the spherule may rupture and release 200–300 endospores that are capable of re-initiating the parasitic cycle and can also disseminate via blood or the lymphatic system to other parts of the body.

Because chitin is one of the main structural components of both the saprobic and parasitic cell wall of *Coccidioides* (Wheat et al., 1967; Hector and Pappagianis, 1982) and is not present in mammals, its biosynthesis is a logical target for antifungal therapies (Hector and Pappagianis, 1983; Hector et al., 1990). Chitin is a linear polymer of β-1,4-linked *N*-acetylglucosamine that is synthesized by a family of isoenzymes called chitin synthases (CHSes). CHSes have been divided into seven different classes according to sequence similarities (Munro and Gow, 2001; Roncero, 2002; Ruiz-Herrera et al., 2002; Nino-Vega et al., 2004). *Coccidioides* is particularly sensitive, both in vitro and in vivo, to Nikkomycin X and Nikkomycin Z (Hector et al., 1990), potential antifungal agents. Thus, a detailed understanding of how these agents affect chitin synthesis in the *Coccidioides* lifecycle is of interest for consideration of their use for treatment.

In this report, we describe the identification and isolation of six *CHS* genes of *C. posadasii* strain Silveira. An additional *CHS* gene has been identified in *C. posadasii* (GenBank Accession No. AAP74955). *C. posadasii* contains a single member of each of the seven phylogenetically distinct class of fungal chitin synthases. We also report the expression patterns of these seven chitin synthases during saprobic growth and at different stages of spherule development using Real-time RT-PCR.

2. Materials and methods

2.1. Strain and culture conditions

Coccidioides posadasii strain Silveira was maintained on 2× GYE medium (2% glucose, 1% yeast extract). For isolation of genomic DNA and RNA from mycelia, 1×10^8 arthroconidia were inoculated into 100 ml of 2× GYE and shaken at 180 rpm at 37 °C for 48 h. For RNA isolations from developing spherules, 6×10^8 arthroconidia were inoculated in 1-liter cultures of Converse medium (Converse and Besemer, 1959) with 50 mg/liter of enzymatic hydrolysate of casein (ICN Biomedicals, Aurora, OH), incubated at 39 °C with 8% CO_2 and grown with shaking at 150 rpm prior to RNA isolation at 48, 72, and 96 h.

2.2. Nucleic acid isolation

High molecular weight genomic (HMW) DNA was isolated from liquid mycelial cultures using a modified protocol of Sweigard et al. (1990). Briefly, mycelia were grown for 48 h from arthroconidia, filtered and resuspended in 1 M sorbitol with zymolyase (10 U/ml, Sigma, St. Louis, MO) and chitinase (0.9 U/ml, Sigma). This mixture was incubated at 37 °C for 2–4 h with gentle shaking to generate protoplasts. The protoplasts were gently lysed and DNA was recovered as described (Sweigard et al., 1990).

RNA was isolated using a modified acid–phenol method (Collart and Oliviero, 1997). Briefly, cells were pelleted from liquid cultures by centrifugation at 4000*g* for 40 min at 4 °C, washed with cold PBS and the fungal material was resuspended in ice-cold TNE buffer (200 mM Tris–HCl, pH 7.6, 0.5 M NaCl, and 10 mM EDTA pH 8.0). After being transferred to 1.5-ml O-ring screw-cap tubes, an equal volume of acid phenol was added and tubes were filled with sterile 0.45 μm glass beads. Cells were disrupted by shaking the tubes three times for 2 min at 3000 rpm, separated by incubation on ice for 1 min, using a Vortex with a MoBio 2-ml adapter (MoBio, Solana Beach, CA). Homogenates were extracted twice with equal volumes of phenol and once with chloroform. Following extractions, supernatants were precipitated with ethanol and the resultant pellets were resuspended in DEPC-treated H_2O. RNA was precipitated in 2 M LiCl and resuspended in DEPC-treated H_2O. RNA quality was assessed on an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Poly(A)$^+$ RNA was isolated from 300 μg of total RNA using the PolyATtract® mRNA Isolation System III (Promega, Madison, WI) following the manufacturer's recommendations. Poly(A)$^+$ RNA concentrations were estimated using the RiboGreen® RNA quantitation reagent (Molecular Probes, Inc, Eugene, OR) and an FLX 800 fluorometer (Bio-Tek Instruments, Inc, Winooski, VT).

2.3. Genomic and cDNA libraries

A cosmid library of Silveira genomic DNA was constructed using HMW DNA partially digested with *Mbo*I, ligated to the pMOCosX vector digested with *Xho*I and partially filled-in, as described elsewhere (Orbach, 1994).

Poly(A)$^+$ RNA from mycelia was used to synthesize first strand cDNAs with oligo-dT_{12-18} and SuperScript Reverse Transcriptase II (Invitrogen, San Diego, CA) following the manufacturer's protocol. Following treatment with RNAse H (Invitrogen), cDNAs were synthesized with oligonucleo-

tide primers specific for each *CHS* gene using High-Fidelity Platinum® *Taq* DNA Polymerase (Invitrogen) according to the manufacturer's specifications. Amplified fragments were cloned directly into pGEM® T-Easy (Promega) or pCR2.1 (Invitrogen).

2.4. Primers and PCR conditions

Oligonucleotide primers were designed based on published sequences of conserved regions of different fungal CHSes (Table 1). Degenerate primers OAM158, 159, and 160 are based on the oligonucleotide sequences published by Bowen et al. (1992) for class I, II, and III *CHS* genes. Primers OAM161 and OAM162 were designed based on the regions QVFEY and WKFDDF, conserved between *Saccharomyces cerevisiae* Chs3p and *Candida albicans* Chs3p that belong to class IV CHSes (Valdivieso et al., 1991; Sudoh et al., 1993). Primers OAM171 and 172 are based on the *C. immitis* class II *CHS* partial sequence available in GenBank (Accession No. U60123, deposited by D. Jiang and P. Szaniszlo), and OAM407 and 409 are based on the oligonucleotide sequences used by Liu et al. (2001) and Park et al. (1999) to amplify class V *CHS* genes. PCR amplifications of conserved regions were performed using *Taq* polymerase (Promega) following the manufacturer's recommended protocol as follows: 50–100 ng of Silveira HMW genomic DNA was incubated in a final volume of 25 μl with 10× *Taq* buffer (minus $MgCl_2$), 1 mM $MgCl_2$, 200 μM dNTPs, 400 nM or 2 μM each oligonucleotide primer (specific or degenerate primer sequences, respectively), and 1 U *Taq* polymerase. The reactions were performed in an ABI 2400 PCR machine (Applied Biosystems, Foster City, CA) or a DNA Engine™ thermal cycler (MJ Research, Inc, Waltham, MA) under the following conditions: one cycle of 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final cycle of 72 °C for 10 min. One microliter of each amplified product was used for cloning into the pGEM® T-easy vector (Promega). The degenerate set of primers OAM158/OAM159 and OAM160 amplified fragments corresponding to *CHS* genes from classes I and III, but not from class II. With the specific set of primers OAM171 and OAM172 we amplified a fragment that corresponds to a class II *CHS*. Primers OAM161 and OAM162 amplified only one band that corresponds to class IV *CHS* genes. Primers OAM407 and OAM409 produced two bands that correspond to *CHS* genes from classes V and VII (Table 1).

2.5. Genomic library screening and sub-cloning

Radiolabeled PCR fragments representing each class of *CHS* gene were used to screen a λ-GEM12 library of Silveira genomic DNA (Peng et al., 1999) and a cosmid library. Inserts from isolated clones were subcloned into pBlueScript SK+ (Stratagene, La Jolla, CA) or pGEM 7Z f(+) (Promega).

Following is a summary corresponding to each *CHS* gene: classes I and III: primers OAM159 and OAM160 amplified two bands, which were cloned and sequenced. Most of the sequenced clones corresponded to an amplified fragment of 603 bp, which has similarities with class I *CHS* genes, and only one corresponded to a fragment of 690 bp, which is homologous to class III *CHS* genes. One of the class I clones, pAM979, was used to screen the λ-GEM12 Silveira genomic library. Several λ clones were obtained, and one of these, λ1.1, was chosen. Two overlapping clones, a 4 kb *Bam*HI fragment and a 3.6 kb *Sac*I/*Eco*RI fragment were subcloned (clones pAM1041 and pAM1090, respectively) and sequenced to obtain the full class I *CHS* gene of *C. posadasii*, which was named *CpCHS1* (Fig. 1). The λ genomic library was screened with the class III clone, pAM978, and two partially overlapping λ clones were selected for subcloning. Analysis of the combined sequence of a 3.8 kb *Hin*dIII fragment from λ3 and a 4 kb *Eco*RV fragment from λ6.1 (clones pAM1042 and pAM1053, respectively) revealed the complete class III *CHS* gene of *C. posadasii*, which was named *CpCHS3* (Fig. 1). Class II: the specific primers OAM171 and OAM172 amplified a fragment of 567 bp, which was cloned, and generated clone pAM990. The sequence of pAM990 matched the GenBank sequence used to design primers OAM171 and OAM172, and is homologous to class II *CHS* genes. Screening the λ library with this class II *CHS* gene fragment identified several positive clones,

Table 1
Primers used to PCR-amplify conserved regions from *C. posadasii CHS* genes

Protein region	Oligonuleotide sequence	Primer	Primer combination	Clone	CHS gene	Class
TMYNED	*ctgaagctt* ACN ATG TAY AAY GAR GAT	OAM158	OAM158/OAM160	AM975	CpCHS1	I
TMYNED	*ctgaagctt* ACN ATG TAY AAY GAR GAC	OAM159	OAM159/OAM160	AM978	*CpCHS3*	III
QNFE(Y/C)K	*gttctcgag* YTT RYA YTC RAA RTT YTG	OAM160				
EINFTR	*ctgaagctt* GAA ATC AAC TTC ACT CGC	OAM171	OAM171/OAM172	AM990	*CpCHS2*	II
NPLVAS	*ctgaagctt* AGA TGC CAC AAG AGG ATT	OAM172				
QVFEY	*ctgaagctt* CAR GTN TTY GAR TA	OAM161	OAM161/OAM162	AM976	*CpCHS4*	IV
WKFDDF	*gttctcgag* AAR TCR TCR AAY TTC CA	OAM162				
QVYEYY	*tggggatcc* CAT GTY TAY GAR TAY TA	OAM407	OAM407/OAM409	AM1175	CpCHS5	V
Q(S/R)(S/R)(S/R)WIN	*atagaattc* TTS ATC CAI CKI CKI CKY TG	OAM409		AM1174	CpCHS7	VII

Shown are the targeted conserved amino acid regions from different CHS enzymes, the degenerate oligonucleotides derived from them, the generated PCR clones and the corresponding genes with their class.

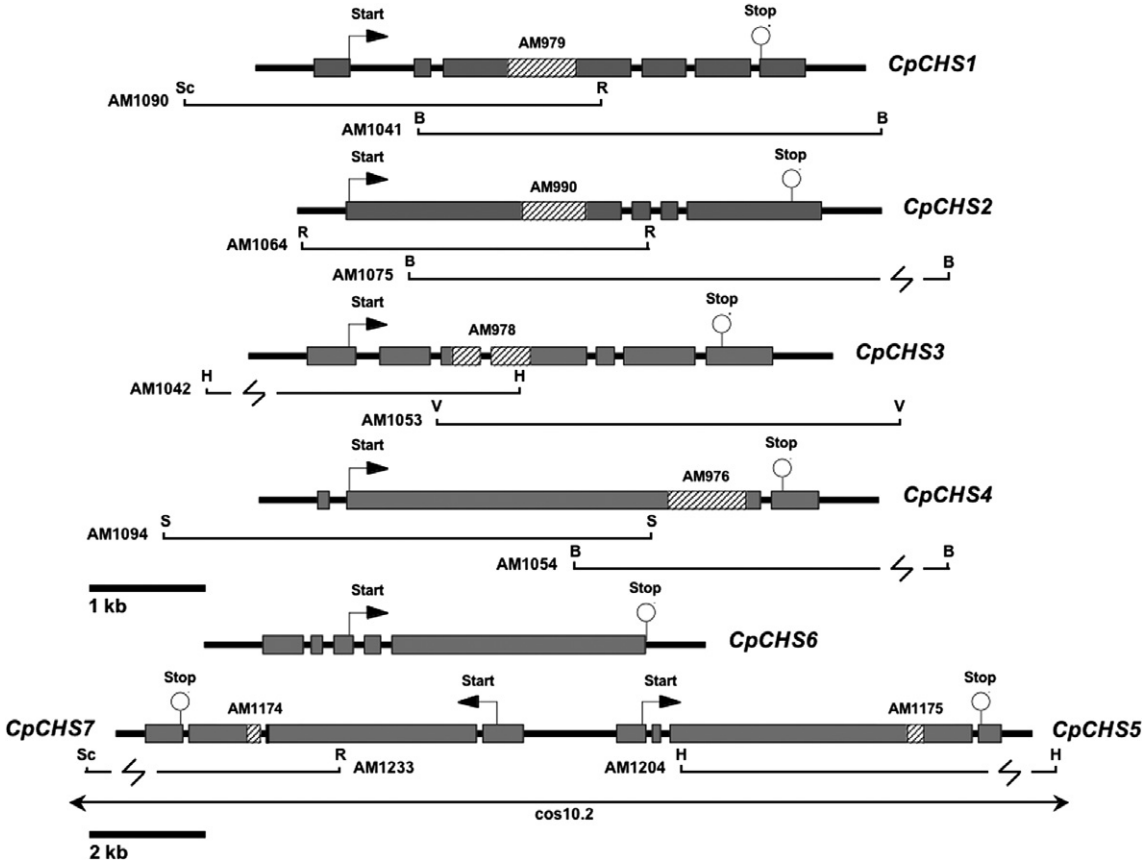

Fig. 1. Schematic representation of the gene structure of *C. posadasii CHS* genes and plasmid clones that contain these sequences. Full boxes represent exons, separated by introns. Hatched boxes represent amplified conserved regions used to screen libraries. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; S, *Sal*I; Sc, *Sac*I; and V, *Eco*RV. Note that the *CpCHS5*/*CpCHS7* contig is represented at a different scale.

one of which (λ6′) was selected. Two partially overlapping fragments, a 3 kb *Eco*RI fragment and a 6.4 kb *Bam*HI fragment (pAM1064 and pAM1075, respectively) were subcloned and sequenced to obtain the whole class II *CHS* gene of *C. posadasii*, which was named *CpCHS2* (Fig. 1). Class IV: primers OAM161 and OAM162 amplified a fragment of 700 bp, which generated clone pAM976. Sequence comparisons showed that this clone is homologous to class IV *CHS* genes. Clone λ4 was selected among several positive λ clones for subcloning and sequencing. Two overlapping fragments, a 5.6 kb *Bam*HI fragment and a 4.2 kb *Sal*I fragment (clones pAM1054 and pAM1094, respectively) revealed the complete class IV *CHS* gene of *C. posadasii*, which was named *CpCHS4* (Fig. 1). Classes V and VII: the primer pair OAM407 and OAM409 amplified two bands, which were cloned and sequenced. Clone pAM1175, with an insert of 348 bp, has sequences homologous to class V *CHS* genes. Clone pAM1174, with an insert of 408 bp, has homology to the recently re-defined class VII *CHS* genes (Chigira et al., 2002; Nino-Vega et al., 2004). Only two clones, λ6.1 and λ6.2, were obtained when the genomic library was screened with pAM1175, and they were almost identical. A 7.1 kb *Hin*dIII fragment from λ6.2 was cloned (clone pAM1204), and sequenced. Based on sequence similarities with other *CHS* genes from class V, one of the ends of the insert of pAM1204 was predicted to be about 640 bp downstream of the putative start codon. To obtain the 5′-end of the gene, a Silveira cosmid library was screened with a 2.5 kb *Hin*dIII/*Sma*I fragment from pAM1204, and several cosmid clones were isolated. Surprisingly, one of these cosmids, cos10.2, contained not only the whole class V *CHS* gene, but also the class VII *CHS* gene in a "head-to-head" configuration. The class V *CHS* gene was completely sequenced and named *CpCHS5* (Fig. 1). Several λ clones were obtained from the screen with clone pAM1174. A 5.1 kb *Eco*RI/*Sac*I fragment from clone λ5.3 was subcloned generating clone pAM1233, which was completely sequenced. The pAM1233 fragment contains a deduced ORF that represents about 50% of the class VII *CHS* gene, including the C-terminus. The rest of the sequence was obtained by PCR amplification and subcloning of clone cos10.2, using oligonucleotide primers based on contig sequences available from the TIGR *C. immitis* Gene Index (CiGI) (www.tigrblast.tigr.org/ufmg) (Fig. 1). The sequence of this *C. posadasii CHS* gene from strain C735 became available in GenBank (Accession No. AAQ10290), and since both sequences are nearly identical, we named this gene *CpCHS7* in accordance with the gene already submitted.

2.6. Fungal class VI CHS sequences

The sequence corresponding to a class VI *CHS* gene from *C. posadasii* strain C735 (*CpCHS6*) was deposited in GenBank (Accession No. AAP74955). From comparisons of this reported sequence to the *C. posadasii* genomic database (www.tigr.org/tdb/tgi/fungi.shtml), the *C. immitis* genomic database (www.broad.mit.edu/annotation/fungi/coccidioides_immitis) and six *C. posadasii* ESTs in the NCBI dbEST database (CO023798, CF816152, CF823254, CF825720, and CF816153), we were able to extend the *CpCHS6* locus 1017 bp upstream of the reported start codon. The *Neurospora crassa* hypothetical protein NCU05268.1 with similarity to CpCHS6 (GenBank Accession No. XP_324625) was predicted from genomic DNA (GenBank Accession No. XM_324624). This genomic sequence has two putative introns in the same position as those we predicted for *CpCHS6*. Based on sequence similarity, the *A. fumigatus AfChsD* deduced ORF (GenBank Accession No. U62614.1) was extended upstream from the predicted start codon at nucleotide 443 to a new predicted start codon at nucleotide 198. Two introns are predicted, one between nucleotides 259 and 301, and another between nucleotides 469 and 528. The sequence corresponding to the *F. graminearum* hypothetical protein FG01949.1 (GenBank Accession No. EAA68845) includes four predicted introns. Based on comparisons with the similar *CpCHS6* and *N. crassa* sequences, we believe the third and fourth of these introns, which are in frame with the ORF, actually encode part of the protein, and thus did not splice them from the ORF sequence in our analyses. The *M. grisea* predicted protein MG06064.4 (GenBank Accession No. EAA52936) is derived from a genomic sequence and includes two predicted introns in the same positions as those in *CpCHS6* and *N. crassa* NCU05268.1. For our analyses, the predicted splicing-acceptor site in the third exon of MG06064.4 at nucleotide 27,953 was switched to nucleotide 27,908, based on sequence similarities. The genomic sequence (GenBank Accession No. AACD01000016) corresponding to the *A. nidulans* hypothetical protein AN1046.2 (GenBank Accession No. EAA66164) was expanded from the reported nucleotides 5763-6708 to 4964–7421, with intron–exon boundaries and the ORF predictions based on similarities with the *N. crassa* and *CpCHS6* sequences.

2.7. Sequence analyses

All clones were sequenced at the GATC facility of the Arizona Research Laboratories, University of Arizona, using BigDye v3.0 chemistry (Applied Biosystems) with specifically designed primers in an ABI PRISM® 377 DNA Analyzer from Applied Biosystems. *CHS5* subclone pAM1204 and *CHS7* subclones pAM1201 and pAM1233 were sequenced using the Genome Priming System GPS-1 (New England BioLabs, Beverly, MA), where a TnsABC transposase was used in vitro to generate a population of clones with randomly interspersed primer-binding sites, according to the manufacturer's specifications. These clones were sequenced using a 96-well format in an ABI PRISM® 3730 xl DNA Analyzer from Applied Biosystems. Sequence analyses were performed using MacVector 7.2 software (Accelrys, San Diego, CA).

2.8. Phylogenetic analyses

The predicted amino acid sequences of *C. posadasii* CHS1-7 were used as queries in BLASTp searches. For each CHS, the top five hits available in GenBank were retained. CpCHS1-7 proteins were submitted to the Conserved Domain Database (CDD, NCBI) to determine their domain structures. This search identified a single domain that is common to all CHS proteins, which was used to produce a global alignment of CpCHS1-7 and each of their most closely related proteins using the ClustalW algorithm. This domain is represented by amino acids 399–562 in CpCHS1. The resulting alignment of 177 amino acid characters (of which 144 were parsimony-informative), was analyzed phylogenetically with maximum parsimony in PAUP4.0b8[10], with the following settings: gaps treated as "21st amino acid," 100 repetitions performed with random sequence addition, branch swapping by tree bisection reconnection (TBR), heuristic search performed with multiple trees saved and steepest descent invoked. Only the most parsimonious (MP) genealogies were retained and summarized in a strict consensus.

2.9. RNA expression analyses

For Real-Time reverse transcriptase-PCR (Real-Time RT-PCR) expression analyses, first strand cDNAs were synthesized as described above using 7.5 ng of poly(A)$^+$ RNA isolated from mycelia, 48, 72, and 96 h spherules. Serial (half-fold) dilutions of each first strand cDNA were tested in Real-Time RT-PCR experiments using a specific set of oligonucleotide primers for each gene and the SYBR® Green PCR Master mix in an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). For the data presented in this paper, three dilutions (0.125X, 0.0625X, and 0.03125X) from the original first strand cDNA were used in the Real-Time RT-PCR experiments. These experiments were repeated more than two times with different preparations of RNA each time (i.e. biological replicates). Following is a list of the oligonucleotide primers used for our Real-Time RT-PCR experiments. These primers were designed based on the 3′-end sequences of the isolated *C. posadasii CHS* genes and γ-actin (Peng et al., 1999).γ-actin: OAM637: 5′-AGC GTC TTG GGT CTC GAA A, OAM638: 5′-CCA GAC ATG ACG ATG TTT C; *CHS1*: OAM639: 5′-GCG ATC TAT GTA ACC GTG A, OAM640: 5′-GAC ATC AGC GAC ACA ATC A; *CHS2*: OAM695: 5′-CAT GTT CAC GTC ATC AGC CC, OAM696: 5′-GCC CCA GGT AAC GTC GTG TG; *CHS3*: OAM643: 5′-TTC GAG GCA ACT

GTG AAA C, OAM644: 5′-AAA TCC ATG AAG CTA CTA G; *CHS4*: OAM645: 5′-AAG CGT GGA AGC AGC CTG T, OAM646: 5′-TGA CCG GCT TTG TAC ATG G; *CHS5*: OAM710: 5′-CGA AGA AGA GCG TCA AAC AAG AAC T, OAM711: 5′-TGG CCT GAA AGA ACT GCT TCA GTC G; *CHS6*: OAM788: 5′-AGC AGC GCG GCT GAA CCA GA, OAM789: 5′-ACA GAT GCG TCT GCC GTC CT; *CHS7*: OAM683: 5′-TCA GCG ACA GAG TAT GCG TAG TC, OAM684: 5′-GAT TTG GTG GAT TTC TGA AAT.

2.10. Statistical analyses of Real-Time RT-PCR data

The levels of expression of each *CHS* gene for each developmental time point (i.e. mycelia, 48, 72, and 96 h spherules) were normalized relative to the levels of expression of γ-actin at the same time point. Then, the levels of expression of specific *CHS* genes at different time points of spherulation were compared to their levels of expression of normalized mycelia. Relative quantification calculations were performed using the algorithm $X = 2^{-\Delta\Delta C_t \pm \mathrm{SPE}}$ (Livak and Schmittgen, 2001). "*X*" is the factor by which the amount of each *CHS* gene at a certain time point in spherule development has changed relative to the expression of the same *CHS* in mycelia, $\Delta\Delta C_t = (C_{t_{CHS}} - C_{t_{actin}})_{spherule} - (C_{t_{CHS}} - C_{t_{actin}})_{mycelia}$, where "*CHS*" refers to *CpCHS1-7*, and "spherule" refers to spherules at 48, 72, or 96 h of development and SPE is the standard propagation error determined as $\pm\sqrt{(\sigma^2_{CHS} + \sigma^2_{actin})}$, with σ being the standard deviation error. γ-Actin was used as the internal-control gene. Differences in relative expression values for *CpCHS1 – CpCHS7* were tested by ANOVA in pair-wise analyses.

3. Results

3.1. Coccidioides posadasii classes I–VII CHS genes

Our goal was to isolate and characterize *CHS* genes from *C. posadasii*. Based on conserved sequences among different *CHS* genes, degenerate oligonucleotide primers were designed and used to PCR-amplify DNA fragments corresponding to *CHS* classes I to V and class VII. All PCR fragments were cloned and several clones per fragment were sequenced to confirm their identity. For each PCR fragment, only a single sequence was obtained, suggesting single representatives of each class in the *C. posadasii* genome. Each fragment gave a unique pattern of hybridization when DNA blot analyses were performed under high stringency conditions (data not shown). BLAST searches of the subsequently available genome of *C. posadasii* strain C735 (TIGR) and the annotated genome of *C. immitis* strain RS (Broad Institute) did not reveal any additional *CHS* genes. Based on sequence similarity to other *CHS* genes, they were classified as shown in Table 1.

CpCHS1 belongs to class I *CHS* genes, contains a deduced ORF of 2745 nucleotides separated by five introns, and encodes a putative protein of 914 amino acids (Fig. 1).*CpCHS2* is a class II gene, contains a deduced ORF of 3618 nucleotides separated by three introns, and encodes a deduced protein of 1205 amino acids (Fig. 1). *CpCHS3* is a class III gene, contains a deduced ORF of 2712 nucleotides that is interrupted by six introns, and encodes a putative protein of 903 amino acids (Fig. 1). *CpCHS4* has similarity to class IV *CHS* genes, has a deduced ORF of 3651 nucleotides that contains one intron, and encodes a putative protein of 1216 amino acids (Fig. 1).*CpCHS5* (class V) has a deduced ORF of 5574 nucleotides interrupted by three introns and encodes a putative protein of 1857 amino acids. *CpCHS7* (class VII) has a predicted ORF of 5322 nucleotides, encoding a putative protein of 1773 amino acids, and is interrupted by three introns. CHSes from classes V and VII are similar to each other by the presence of myosin motor-like domains at their amino termini (Fujiwara et al., 1997), whereas other classes of CHSes lack this domain. The genomic DNA sequence corresponding to *CpCHS6* deposited in GenBank (Accession No. AAP74955) was compared to six *C. posadasii* ESTs in the NCBI dbEST database, and to *C. posadasii* and *C. immitis* genomic regions from the TIGR and Broad Institute databases, respectively. From the results of these comparisons, we extended the *CpCHS6* sequence 1017 nucleotides upstream of the reported start codon (see Section 2). The *CpCHS6* sequence that we propose has a 5′ leader 746 nucleotides long with two introns and five uORFs ranging in size from 12 to 72 nucleotides, and a start codon 271 nucleotides upstream of the previously reported ATG. *CpCHS6* has a deduced ORF of 2,448 nucleotides, contains two introns and encodes a putative protein of 815 amino acids (Figs. 1 and 2). All the predicted intron-exon boundaries of the *C. posadasii CHS* genes were confirmed by sequencing cDNA clones obtained by RT-PCR as described in Section 2 (data not shown), with the exception of *CpCHS6*, where ESTs were used to establish these boundaries. Start and stop codons were deduced from existing ESTs in the NCBI dbEST database, with the exception of *CpCHS6*, where no 3′ ESTs were available. The deduced amino acid sequences of all the CpCHSes have similarities to fungal CHSes, with greatest similarity to other members of the Onygenales, as shown in Table 2.

3.2. Conserved regions of CpCHSes

Con1 (conserved region 1) is a highly conserved amino acid region that is present in all CHS enzymes of yeast as well as filamentous fungi (Nagahashi et al., 1995). *Con1* is defined by comparison of the amino acid sequence of *S. cerevisiae* ScChs2p from positions 490–607 to other fungal CHSes. It is divided into three conserved subdomains (I–III), and it also contains two putative catalytic sites, EDR and QR/GRRW (Nagahashi et al., 1995) (Fig. 3). We compared the sequences corresponding to *con1* in all the *C. posadasii* CHSes. We found the conserved sequences

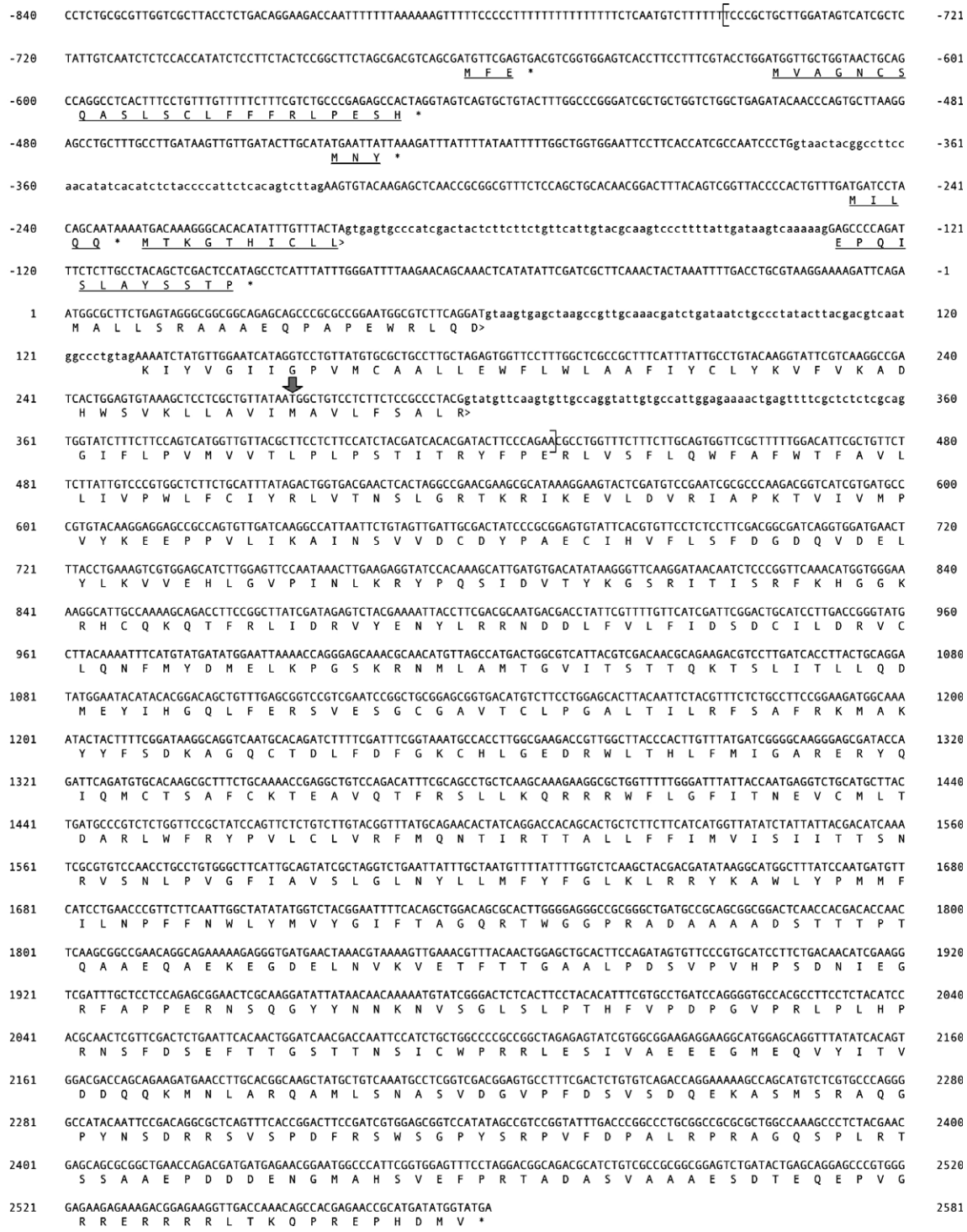

Fig. 2. *CpCHS6* sequence. Our proposed start codon is at position +1, the arrow indicates the previously reported start codon (GenBank Accession No. AAP74955). Between brackets is the sequence corresponding to the longest 5′-EST available, along with the four introns which are indicated in lower case. The five putative uORFs in the 5′-UTR are underlined.

QXXEY and ESXXGX$_4$LPGX$_5$R in domain I, L(A/G)EDRXL in domain II, and TX$_{11}$QRRRW in domain III (Fig. 3). There are other conserved regions found between subsets of the CHSes, including transmembrane domains, but none that are conserved among all of the classes.

3.3. Expression of *C. posadasii CHS* genes

The expression of *C. posadasii CHS* genes during different stages of spherule development and during mycelial growth was analyzed using Real-Time RT-PCR. γ-Actin was chosen as the reference gene instead of *GAPDH*

Table 2
Comparison of the deduced amino acid sequences of *C. posadasii* CHS enzymes with other chitin synthases

Coccidioides posadasii CHS	*Class*	Accession No.	*Closest homologues*	Accession No.	% Identity/% similarity
*Cp*CHS1	I	AAF82801	*Aspergillus oryzae* chsC	BAB85684	77/86
			Arthroderma benhamiae CHS1	BAB17766	74/81
			Wangiella dermatitidis WdChs2p	AAC34496	73/82
			Aspergillus nidulans CHS1	A59054	72/80
			Blumeria graminis BgChs1	AAF05595	65/72
*Cp*CHS2	II	AAK07645	*Paracoccidioides brasiliensis* CHS2	CAA70433	61/69
			Aspergillus nidulans chsA	JC2314	59/70
			Ericoid mycorrhizal fungus *PSIV* chs	CAC95227	58/69
			Fusarium oxysporum f. sp. lycopersici *chs2*	AAT77182	57/67
			Phaeosphaeria nodorum chs2	CAB41508	57/65
*Cp*CHS3	III	AF298189	*Aspergillus fumigatus* chsG	AAB07678	81/88
			Aspergillus oryzae chsB	AAK31732	80/89
			Penicillium chrysogenum CHS4	AAF04828	80/87
			Aspergillus nidulans CHSB	BAA11845	79/87
			Botryotinia fuckeliana BCCHSIII	AAM14606	74/83
*Cp*CHS4	IV	AAK72391	*Paracoccidioides brasiliensis* PbrCHS3	AAD19614	76/84
			Wangiella dermatitidis WdChs4p	AAD28744	67/88
			Aspergillus nidulans CHSD	EAA64262	66/78
			Tuber magnatum chs4	CAB41410	65/75
			Botryotinia fuckeliana BcchsV	AAF19527	64/75
*Cp*CHS5	V	AAR88368	*Aspergillus nidulans* CsmA	BAA21714	75/86
			Aspergillus oryzae chsY	BAB88128	74/85
			Wangiella dermatitidis WdChs5p	AAL79830	72/83
			Blumeria graminis chs2	AAF04279	69/82
			Glomerella graminicola chsC	AAL23719	68/81
CpCHS6	VI	AAP74955	*Aspergillus fumigatus AfchsD*	AAB60781	61/72
			Aspergillus nidulans hypothetical protein	EAA66164	59/71
			Magnaporthe grisea hypothetical protein	EAA52936	58/69
			Fusarium graminearum hypothetical protein	EAA68845	56/68
			Neurospora crassa hypothetical protein	XP324625	53/66
*Cp*CHS7	VII	AAQ10290	*Paracoccidioides brasiliensis* CHS4	AAD19613	71/82
			Aspergillus oryzae chsZ	BAB88127	67/79
			Glomerella graminicola ChsA	AAL55424	61/74
			Neurospora crassa hypothetical chs	XP323703	57/71
			Botryotinia fuckeliana BcchsVI	AAS21657	57/68

because it was more evenly expressed at all developmental stages that were tested (data not shown). As seen in Fig. 4, *CpCHS2*, *CpCHS3*, and *CpCHS6* are more highly expressed during mycelial growth than at the stages of spherule development tested. *CpCHS1* and *CpCHS4* show increased expression at 48, 72, and 96 h of spherule development compared to their levels during mycelial growth. *CpCHS5* and *CpCHS7* expression do not show significant changes during mycelial growth or spherule differentiation. Pair-wise analyses by ANOVA corroborated these apparent differences. These data demonstrate that all of the *C. posadasii CHS* genes are expressed to some degree during mycelial and spherule growth, with none appearing to be absolutely stage-specific.

3.4. Phylogenetic relationships of different C. posadasii CHS

The entire predicted amino acid sequences corresponding to each isolated *CHS* gene, together with the putative protein product of the *CpCHS6* gene, were used to perform BLASTP searches on the NCBI databases. Five CHS proteins with the greatest similarity from each class were retained, and only the homologous portion of all CHS proteins (the latter portion of CS2 domain, pfam03142) was aligned using the ClustalW algorithm. The gene genealogy (Fig. 5) depicts the same relationships of the seven classes of CHSes as presented in Choquer et al. (2004). However, because we are following the nomenclature as proposed by Nino-Vega et al. (2004), our clade VI corresponds to Choquer's clade VII, and vice versa. Each CpCHS sequence is placed in the same class predicted by comparisons of the conserved PCR-amplified fragments (data not shown). Phylogenetic analyses of this alignment resulted in most parsimonious trees showing two well defined families, as previously reported (Roncero, 2002; Ruiz-Herrera et al., 2002). Within Family I are CpCHSes from classes I, II, III; and within Family II are CHSes from classes IV, V, and VII.

4. Discussion

In this paper, we show that the pathogenic fungus *C. posadasii* contains seven *CHS* genes, one corresponding to each CHS class that has been described (Munro and Gow, 2001; Roncero, 2002; Martin-Garcia et al., 2003; Nino-Vega et al., 2004). These are *CpCHS1* (class I),

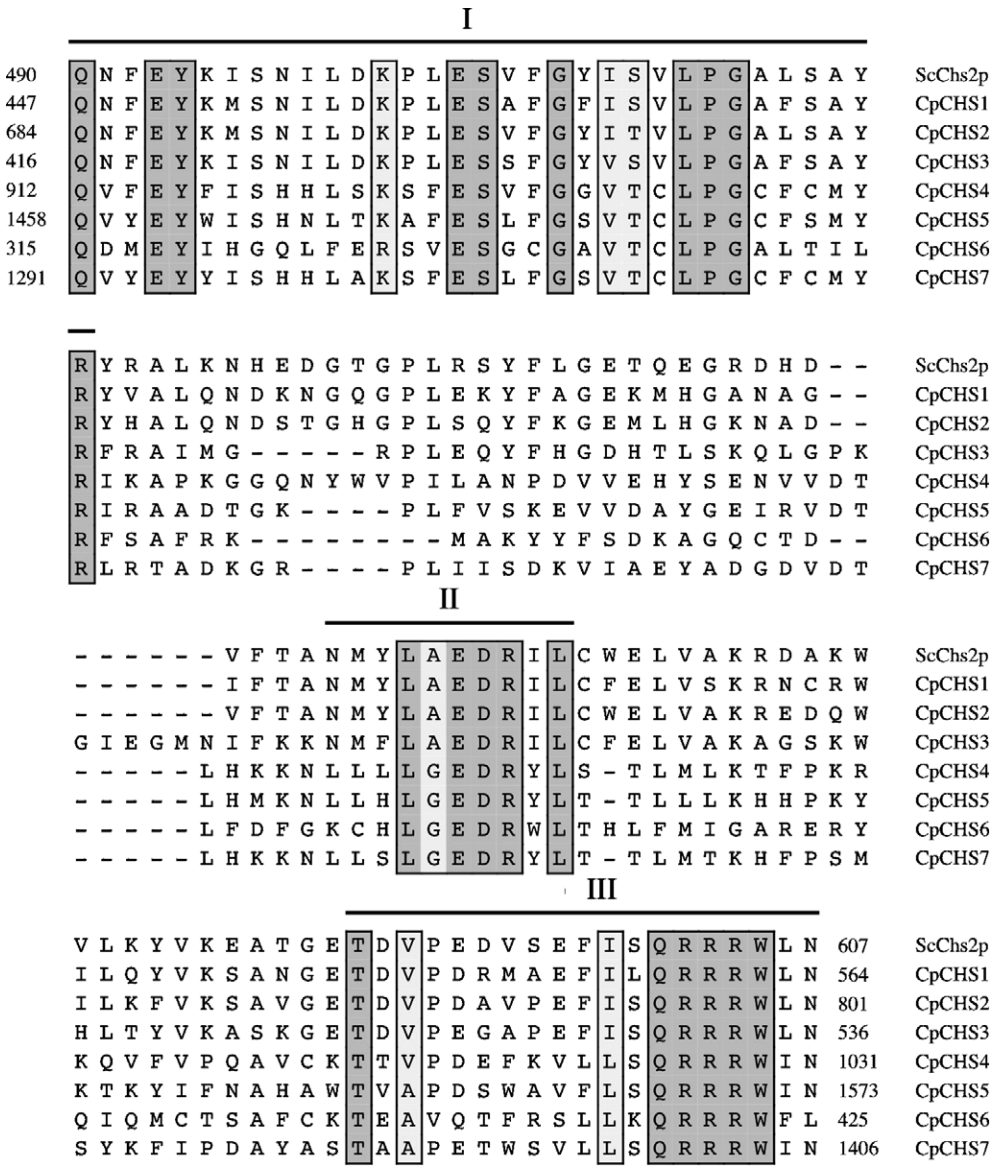

Fig. 3. Comparison of the catalytic region *con1* among *S. cerevisiae* ScChsp2 (Accession No. P14180), and the seven CpCHSes. Identical and similar conserved amino acid sequences are in dark and light boxes, respectively. Roman numerals above the sequences represent the three conserved subdomains I, II, and III. Numbers indicate the position of the sequences in the whole protein.

CpCHS2 (class II), *CpCHS3* (class III), *CpCHS4* (class IV), *CpCHS5* (class V), *CpCHS6* (class VI) and *CpCHS7* (class VII). They have high similarity with members of their corresponding class, ranging from 53 to 81% amino acid identity over the whole deduced amino acid sequences. The highest similarity of the CpCHSes is with other ascomycetes in class Eurotiomycetes, including *A. nidulans*, *A. oryzae*, and the human pathogens *A. fumigatus*, *W. dermatitidis*, and *P. brasiliensis* (See Table 2 and Fig. 5). Within each CHS class, CpCHSes from classes I and III are more closely related to their homologues, whereas CpCHSes from classes II and VI are more divergent (See Table 2). The number of CHS isoenzymes in fungal species range from two in *Schizosaccharomyces pombe* (Martin-Garcia et al., 2003; Matsuo et al., 2004) to up to 10 in *Phycomyces blakesleeanus* (Miyazaki and Ootaki, 1997). Some fungal species have more than one CHS of a specific class (Munro and Gow, 2001; Roncero, 2002; Ruiz-Herrera et al., 2002), as is the case for zygomycetes, which usually have multiple members from class II (Motoyama et al., 1994; Thomsen and Beauvais, 1995). Classes V and VII CHSes are longer than CHS members of classes I–IV and class VI due to the presence of myosin motor like-domains at their N-termini (Fujiwara et al., 1997). Class VI CHSes, defined by AfChsD from *A. fumigatus* (Mellado et al., 1996b), lack myosin motor-domains, and until recently this class was only represented by that single member. Disruption of the *AfCHSD* gene resulted in a 20% reduction in the total mycelial chitin content, but no other obvious phenotype (Mellado et al., 1996b). With the sequencing of several

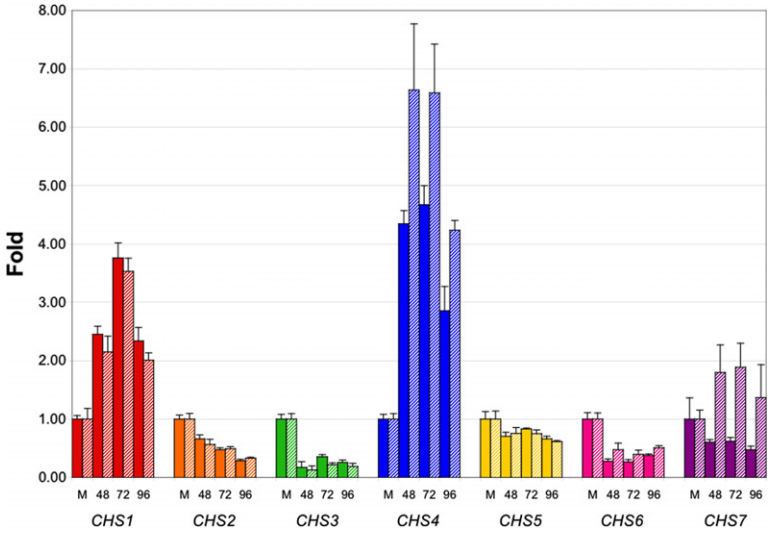

Fig. 4. Schematic representation of *CpCHS* expression data. Real-Time RT-PCR was performed with mycelial RNA (M), and spherules harvested at 48, 72, and 96 h of development, as described in the text. Fold refers to fold increase of each *CHS* gene during spherule differentiation normalized against the expression of the same *CHS* gene in the saprobic phase. The line above each bar represent the standard propagation error as determined in the text. The solid and hatched bars correspond to experiments performed with two independently isolated RNA preparations, each one carried out in triplicate.

fungal genomes, class VI homologues were identified in *N. crassa* (Munro and Gow, 2001; Munro et al., 2003), *M. grisea*, *F. graminearum* (classified as class VII by Choquer et al., 2004), *A. nidulans* (this paper), and *C. posadasii* strain C735 (TIGR). Chigira et al. (2002) reported the cloning of two chitin synthases from *A. oryzae*, AoChsY and AoChsZ. AoChsY is a class V CHS, and has a myosin motor-domain at its N-terminus with the characteristic P-loop, switches I and II, and the ATP domain (Hasson and Mooseker, 1995; Mooseker and Cheney, 1995; Rayment, 1996). AoChsZ also has homology to myosin motor-domains, but this region at the N-terminus is shorter and lacks most of the important features of this type of domain. These differences were enough to separate these two *A. oryzae* CHSes into two phylogenetically distinct classes. The authors proposed that AoChsZ should be in a new class, named class VI, which they group together with Umchs6 from *Ustilago maydis* and PbrChs4, in conflict with the class VI earlier defined by the *A. fumigatus* AfChsD (Mellado et al., 1996b; Chigira et al., 2002). Recently, the full-length cloning of *PbrChs4* was reported from *P. brasiliensis* (Nino-Vega et al., 2004) and it was proposed that this gene represents a new class of PbrCHS protein, which clades together with AoChs*Z* and UmChs6. They name this group class VII because of the precedence of the class VI originally defined by AfChsD. *C. posadasii* CpCHS7 is a member of this class, thus, following the classification of Nino-Vega et al., we propose that CpCHS7 belongs to class VII (Fig. 5 and Table 2). It should be noted that the analyses of *Botrytinia fuckeliana* (anamorph: *Botrytis cinerea*) CHS sequences do not follow this classification (Choquer et al., 2004). Choquer et al., identify a gene as *BcCHSVI* which they define as being in class VI, but should be classified as a class VII gene, based on precedence of class VI of Mellado et al. (1996) and the definition of class VII by Nino-Vega et al. (2004). Conversely, the *B. fuckeliana* gene identified as *BcCHSVII* belongs to the class VI proposed by Mellado et al. (1996).

The deduced amino acid sequences corresponding to all the newly isolated *C. posadasii* CHS sequences, and to our revised CpCHS6 were initially aligned with each other and with their corresponding closest homologues. Due to the varying domain structures among the seven classes of CHSes and the high level of sequence divergence among the classes, the final alignment was performed only with the one core domain shared by all classes of CHSes, the CS 2 domain (pfam03142). The genealogy was rooted with class VI as determined previously (Choquer et al., 2004). Interestingly, class VI has the simplest domain structure (only as CS 2 domain), which likely represents the ancestral state for all chitin synthases. The remaining classes form two sister clades, one composed of classes I, II, and III, and the other clade composed of classes IV, V, and VII. Classes I, II, and III all have lost the first portion of the CS 2 domain, and have replaced it with a CS 1 domain (pfam01644). This event is mapped onto the genealogy as a single evolutionary event. Likewise, classes IV, V, and VII all share the gain of a cytb5-like binding domain (pfam0073). Sister clades V and VII share the gain of a myosin-motor domain (cd00124). All domain gains and losses map onto the tree with no homoplasy, supporting the inferred relationships of these classes. The alternative hypothesis is that the ancestral CHS included all domains, and that as new classes evolved, various domains were lost, resulting in gene families with simpler domain structures. However, this scenario would require the homoplasious loss of the same domain in more than one class, including 2 losses of the CS 1 domain, 2 losses of the cyt-b5-like

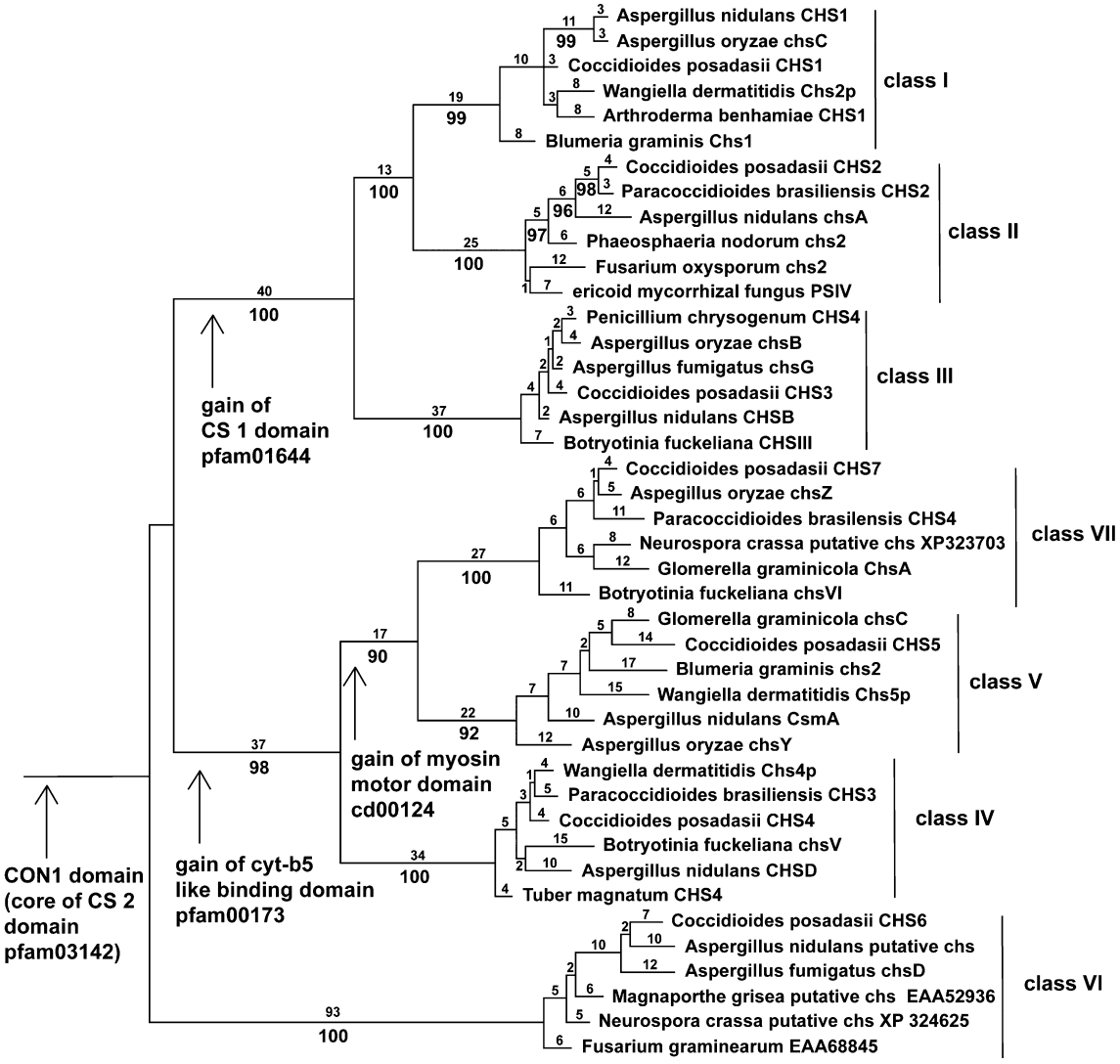

Fig. 5. Gene genealogy of CHS genes, inferred from the conserved CS2 amino acid domain found in all CHSes. The tree is rooted as determined by Choquer et al. (2004) with class VI, which has the simplest domain structure. Gains of additional domains (CS 1 domain, cyt-b5 like domain, and myosin motor domain) are mapped on the topology. Topology shown is one of 2098 most parsimonious trees. Branch lengths are given above the branches, and bootstrap values are given under the branches. Branches that receive significant bootstrap support (>90%) are present in the consensus of all most parsimonious trees. Refer to Table 2 for accession numbers to the sequences used for the analysis.

domain, and 3 losses of the myosin motor domain. *C. posadasii* does not have paralogous CHSes in the same class, as has been found in zygomycetes (Motoyama et al., 1994; Thomsen and Beauvais, 1995; Miyazaki and Ootaki, 1997), but rather has one representative gene from each class.

The relatedness of chitin synthase classes and their divergent domain structures should serve as a guide to the functional characterization of the seven classes of CHSes, which, so far, appear to be partially redundant, in that individual gene knockouts are frequently rescued to some degree by the other CHSes. Each of the seven CHS classes is likely to be functionally divergent at some phenotypic level, dependent on both changes in domain structure and overall sequence divergence. However, these differences are difficult to summarize, due both to the various overlapping expression patterns of different classes of CHSes within one fungal system, and to lineage-specific differences in the observed phenotypes of mutants of the same class of CHS among different fungal systems.

Expression analyses indicate that none of the *C. posadasii CpCHS* genes show stage-specific gene expression, with each gene expressed at some level in the different developmental stages analyzed. Relative changes in stage-induced expression were observed for *CpCHS1-4* and *CpCHS6*. Expression analyses show that *CpCHS2*, *CpCHS3*, and *CpCHS6* are more highly expressed in mycelia than in developing spherules (Fig. 4). Class II CHSes are known to play an important role in yeasts as they synthesize chitin in the primary septum and are essential for septum formation in *S. cerevisiae* (Shaw et al., 1991). In *C. albicans*, the class II CaChs1p is the only reported essential CHS activity

and is involved not only in primary septum synthesis, but also in lateral cell wall integrity (Munro et al., 2001). The class II ChsA from *A. nidulans* has a minor role in the synthesis of chitin in the hyphae and is involved in conidium formation (Culp et al., 2000), while the *A. fumigatus* chsB and *N. crassa* chs2 have no known function (Din and Yarden, 1994; Munro and Gow, 2001). Recently, it has been reported that the combined activities of the class I ChsC and the class II ChsA share overlapping roles in septum formation in *A. nidulans* (Ichinomiya et al., 2005). Thus, the significance of the increased expression of the *CpCHS2* gene during mycelial growth of *C. posadasii* remains to be determined. The greater *CpCHS3* expression during mycelial growth correlates with the fact that class III CHSes are found exclusively in filamentous fungi. These genes are required for normal hyphal morphology in *N. crassa*, *A. nidulans* and *A. fumigatus* and strains with deletions of genes from this class show diminished radial growth rate, poor or no conidiation, and increased hyphal branching (Yarden and Yanofsky, 1991; Borgia et al., 1996; Mellado et al., 1996a). Preliminary results suggest that in *C. posadasii* the *CpCHS3* gene is also necessary for normal hyphal growth; its absence appears to result in a loss of polar growth, reduced cell wall integrity, and a reduction in conidiation ((Mandel et al., 2003); Mandel, Galgiani and Orbach, in preparation). *AfchsD*, the only class VI *CHS* gene that has been analyzed, is expressed during hyphal growth (Mellado et al., 1996b), but no other expression data has been reported. *CpCHS6* contains an unusually long 5′ leader that contains five uORFs, which may have the potential to play a post-transcriptional regulatory role (for reviews see Geballe and Sachs, 2000; Vilela and McCarthy, 2003). *CpCHS1* and *CpCHS4* show the greatest increases in expression during all the stages of spherule differentiation studied. The class I ScChs1p of *S. cerevisiae* repairs damaged chitin during cell separation (Cabib et al., 1992), but both class I CHSes from *C. albicans*, CaChs2p and CaChs8p, have no known function (Gow et al., 1994; Munro et al., 2003). In most filamentous fungi deletion of class I genes produce no apparent phenotypic defects (Fujiwara et al., 2000; Munro and Gow, 2001; Martin-Udiroz et al., 2004). Class IV CHSes in *S. cerevisiae* and *C. albicans* synthesize the bulk of chitin in vivo, although deletion of this gene showed that it is not essential (Valdivieso et al., 1991; Bulawa et al., 1995; Mio et al., 1996). The class IV CHSes of the filamentous fungi *A. nidulans* (chsE) and *N. crassa* (chs4) play an important role in synthesizing the bulk of chitin in vivo (Din et al., 1996; Specht et al., 1996). But mutational analysis of the *A. fumigatus* class IV *chsF* resulted in no apparent defect (Munro and Gow, 2001). Preliminary results suggest that *CpCHS1* and *CpCHS4* show no critical role during hyphal growth, but their roles during spherulation have not yet been tested ((Mandel et al., 2003); Mandel, Galgiani and Orbach, unpublished). *CpCHS5* and *CpCHS7* show no significant differences in expression between hyphal growth and spherule development. CHSes from classes V and VII are only present in filamentous fungi. Class V enzymes provide a major CHS activity in vivo, and when mutated show reduced chitin levels in *A. fumigatus* and *A. nidulans* (Specht et al., 1996; Aufauvre-Brown et al., 1997). Class V CHS6 from *U. maydis* not only provides a major activity in vivo, but is also required for virulence Garcera-Teruel et al. (2004). WdChs5p, a class V CHS from *W. dermatitidis*, is required for sustained growth at the temperature of infection and is essential for virulence (Liu et al., 2004). The function of the myosin motor-like domain (MMD) has only been reported in detail for the class V CsmA of *A. nidulans*. This MMD binds actin and anchors CsmA at the hyphal tips and septation sites (Takeshita et al., 2005).

Due to the complexity of roles of chitin synthases in fungal development, we expect that there may be some stages of growth where activities of different *CHS* genes will overlap, while there may be other growth stages where an individual gene will play a more critical role in cell wall development. Data suggests that the parasitic phase of the *Coccidioides* life cycle is more sensitive to inhibition by the chitin synthase inhibitor Nikkomycin Z (Hector et al., 1990; R. Hector, personal communication), which indicates that different CHSes are important for those different phases of growth. It is our interest to determine the function of each CpCHS during development. Presumably since these enzymes produce chitin, they should be necessary for the integrity of the cell wall, during both the saprobic and parasitic phases of *C. posadasii*. We plan to examine potential stage-specific roles for the CpCHSes by gene replacement. We have created deletion mutants of each *CHS* gene individually and are in the process of analyzing their phenotypes.

Acknowledgments

We thank Kris Orsborn for isolating the HMW DNA, and Ellen Kellner for technical assistance in the RNA preparations. We also thank all the members of the Galgiani and Orbach labs for their assistance and advice with techniques. In particular, we thank Brian Coullahan and other members of the ARL GATC facility at the University of Arizona for their help with Real-Time PCR experiments, Paul Szaniszlo for sharing results previous to publication, and Matt Sachs for helpful discussions. This work was supported in part by the US. Department of Veterans Affairs and NIH-NIAD Grant 1PO 1AI061310-01.

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