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# *Candida albicans* CHT3 encodes the functional homolog of the Cts1 chitinase of *Saccharomyces cerevisiae*

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**Abstract**

Chitin synthesis and chitin degradation play an important role in cellular morphogenesis and influence the cell shape of fungal organisms. The *Candida albicans* genome contains four chitinase genes, *CHT1*, *CHT2*, and *CHT3*, which are homologous to the *Saccharomyces cerevisiae CTS1* gene and *C. albicans CHT4*, which is homologous to *S. cerevisiae CTS2*. To determine which of the *C. albicans CHT* genes represents the functional homolog of the *S. cerevisiae CTS1* gene we constructed mutants of these genes and characterized the resulting phenotypes using morphological assays such as in vivo time lapse microscopy and enzymatic assays to determine the chitinase activity. Deletion of *CaCHT1* and *CaCHT2* provided no phenotypic alterations in liquid culture but resulted in increased hyphal growth on solid media. Deletion of *CaCHT3* generated chains of unseparated cells in the yeast growth phase strongly resembling the *cts1* deletion phenotype of *S. cerevisiae* cells. Expression of *CHT3* under control of the regulatable *MAL2*-promoter in *C. albicans* resulted in the reversion of the cell separation defect when cells were grown in maltose. Cht3, but not Cht2 when expressed in *S. cerevisiae* was also able to reverse the cell separation defect of the *S. cerevisiae* c *ts1* deletion strain. Measurements of chitinase activity from yeast cells of *C. albicans* showed that Cht2 is bound to cells, consistent with it being GPI-anchored while Cht3 is secreted into growth medium; Cht3 is also the principal, observed activity.


*Keywords:* Chitin; Cell division; Cytokinesis; In vivo time lapse; Morphogenesis; Functional analysis

## 1. Introduction

The cell wall of yeasts and filamentous fungi is a complex three dimensional structure that is composed of β-glucan (β-1,3 and β-1,6 linkages) and mannoproteins (Klis et al., 2001; Munro and Gow, 2001; Roncero, 2002). Chitin in the cell wall is only a minor component, but is highly enriched at septal sites. Covalent association of glucan with chitin contributes to the mechanical strength of the wall (Hartland et al., 1994). Localized chitin concentrations are regulated by chitin synthesis and hydrolysis. These processes are performed by two sets of enzymes, chitin synthases and chitinases, respectively. In *Saccharomyces cerevisiae* most chitin synthesis (about 90%) is generated by Chs3. Isoenzymes, Chs1, and Chs2, act in a temporally and spatially controlled manner (Cabib et al., 2001). Genomic variability in the number of chitin synthases (and/or chitinases) may allow for a more complex regulation of chitin synthesis (and degradation) during morphogenetic development, since *Schizosaccharomyces pombe*, for example encodes only one chitin synthase whereas *Aspergillus fumigatus* encodes seven of these isoenzymes. Different models of cell wall growth have implicated either a concerted regulation of chitin synthesis and degradation as in the "unitary model" (Bartnicki-Garcia, 1973) or simply a

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regulation of chitin synthesis without the need to co-regulate chitin degradation in the cell wall for growth processes (Sietsma and Wessels, 1994). Recently, it was shown that chitin synthesis and chitin degradation in *S. cerevisiae* and *C. albicans* are regulated independently from each other (Selvaggini et al., 2004). Chitinase activity of Cts1 as well as β-1,3-glucanase activity by Eng1 in *S. cerevisiae* are specifically required for mother–daughter cell separation (Baladron et al., 2002; Colman-Lerner et al., 2001; Kuranda and Robbins, 1991). The *S. cerevisiae* genes coding for Cts1 and Eng1 are expressed in a daughter cell specific manner and are regulated by the Ace2 transcription factor in *S. cerevisiae*. Similar results for the *ENG1* and *ACE2* homologs were obtained in *S. pombe* (Martin-Cuadrado et al., 2003). In contrast to *S. cerevisiae*, *C. albicans* has four chitinase genes, *CHT1*, *CHT2*, and *CHT3* and a recently identified fourth chitinase gene, *CHT4* (McCreath et al., 1995, 1996; Selvaggini et al., 2004). No transcript has been detected for *CHT1* while the transcript levels of *CHT2* and *CHT3* were found to be expressed in the yeast growth phase, but down-regulated during the hyphal growth phase (McCreath et al., 1996). In contrast to these transcript levels, a recent report suggests that the specific activities of Cht2 and Cht3 were higher in the hyphal phase compared to the yeast phase (Selvaggini et al., 2004). The *C. albicans CHT1*, *CHT2*, and *CHT3* genes bear closer similarity with the *S. cerevisiae CTS1* gene. *C. albicans CHT4*, on the other hand, is more similar to the sporulation specific *S. cerevisiae CTS2* gene.

Loss of *CTS1* in *S. cerevisiae* leads to a cell separation defect. Mother and daughter cells remain attached at septal sites, continue budding and generate an aggregate of cells (Kuranda and Robbins, 1991). This aggregate can be dissolved by mechanical force indicating that cell division was completed. *Ace2* mutants in *Candida albicans* and *Candida glabrata* show a very similar phenotype of cell separation defects indicating conserved regulation of chitinases (Kamran et al., 2004; Kelly et al., 2004). In this study we analyzed the role of the *C. albicans* chitinase genes in cell division during yeast and hyphal growth stages. We demonstrate that the *C. albicans CHT3* gene encodes the functional homolog of the *S. cerevisiae CTS1* gene by heterologous complementation experiments. Expression of *CHT1*, *CHT2*, or chimeric constructs using *CHT2* and *CHT3* sequences in *S. cerevisiae* did not result in complementation of the *S. cerevisiae cts1* mutation.

## 2. Materials and methods

### 2.1. Strains and media

*Candida albicans* and *S. cerevisiae* strains used in this study are listed in Table 1. Cells were grown in YPD or complete supplement medium (CSM) at 30 °C. Induction of hyphal formation was done at 37 °C in CSM to which 10% serum (calf serum, Sigma) was added. For hyphal induction on plates CSM with 10–20% serum or Spider medium (Liu et al., 1994) was used. Plates were incubated 3–7 days at 37 °C prior to photography. To induce expression of *MAL2*-promoter or *GAL1*-promoter driven genes cultures were grown in CSM media containing 2% maltose or 2% galactose, respectively, as sole carbon source.

Table 1
Strains used in this study

| Strain[a] | Genotype | Source |
|---|---|---|
| SC5314 | *C. albicans* wild type | Gillum et al. (1984) |
| BWP17 | *ura3::λimm34/ ura3::λimm34 his1::hisG/his1::hisG arg4::hisG/arg4/hisG* | Wilson et al. (1999) |
| CAA1 | *CHT3/cht3::URA3* | This study |
| CAA2 | *CHT3/cht3::HIS1* | This study |
| CAA3 | *cht3::URA3/cht3::HIS1* | This study |
| CAA4 | *cht3::HIS1/cht3::URA3* | This study |
| CAA5 | *cht3::URA3/MAL2p-CHT3:HIS1* | This study |
| CAA7 | *CHT2/cht2::URA3* | This study |
| CAA8 | *CHT2/cht2::HIS1* | This study |
| CAA9 | *cht2::URA3/cht2::HIS1* | This study |
| CAA10 | *cht2::HIS1/MAL2p-CHT2:URA3* | This study |
| CAA11 | *CHT1/cht1::URA3* | This study |
| CAA12 | *CHT1/cht1::HIS1* | This study |
| CAA13 | *cht1::URA3/cht1::HIS1* | This study |
| CAA26 | *CHT4/cht4::HIS1* | This study |
| CAA29 | *cht4::HIS1/cht4::URA3* | This study |
| DSY1768 | *cht2::hisG-URA3-hisG/cht2::hisG* | Selvaggini et al. (2004) |
| SPY24 | *cht3::hisG-URA3-hisG/cht3::hisG* | Selvaggini et al. (2004) |
| DSY1741 | *cht2::hisG-URA3-hisG/cht2::hisG; cht3::hisG/cht3::hisG* | Selvaggini et al. (2004) |
| Y36947 | MATa/α *his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; LYS2/lys2Δ0; MET15/met15Δ0; ura3Δ0/ura3Δ0; YLR286c(cts1)::kanMX4/YLR286c(cts1)::kanMX4* | Euroscarf |
| Y16947 | MATα *BY4742; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0 YLR286c (cts1)::kanMX4* | Euroscarf |
| BY4742 | MATα *his3Δ1;leu2Δ0;lys2Δ0;ura3Δ* | Euroscarf |

[a] All CAA strains are derivates of BWP17 with the indicated genotypic alterations.

### 2.2. Disruption of Candida albicans genes

*C. albicans* genes analysed in this study were identified in the genomic sequence (http://www-sequence.stanford.edu/group/candida). Deletions of the complete open reading frames of both alleles of *CaCHT1*, *CaCHT2*, and *CaCHT3*, respectively, were performed by PCR-generated *FA-URA3* and *FA-HIS1* disruption cassettes as described previously (Gola et al., 2003) (see Tables 2 and 3 for the lists of primers and plasmids). All oligonucleotide primers were obtained from biomers (Ulm, Germany). Independent transformants were produced for each strain. The disruptions as well as the absence of the target ORF in homozygous mutant were verified by PCR on whole yeast cells as described previously (Gola et al., 2003). Phenotypic identity in the comparison of the independent homozygous transformants was used as a further indication that the correct mutant strains were constructed. To place *CHT*-genes under control of the regulatable *MAL2*-promoter heterozygous mutants (either *cht2::HIS1/MAL2p-CHT2:URA3* or *cht3::URA3/MAL2p-CHT3:HIS1*) were transformed with PCR-fragments amplified from either pFA-URA3-MAL2p or pFA-HIS1-MAL2p. Promoter shut-down experiments verified the correct construction of mutant strains indicating that the observed phenotypes were solely due to the desired genetic manipulations.

### 2.3. Transformation of C. albicans and S. cerevisiae

The lithium-acetate procedure was used for transformation of *S. cerevisiae* and with modifications for *C. albicans* as described previously (Gietz et al., 1995; Walther and Wendland, 2003).

### 2.4. Generation of plasmids and gene fusions

To generate gene fusions with the constitutively active *Ashbya gossypii TEF*-promoter or the regulatable *GAL*-promoter the *in vivo* recombination machinery of *S. cerevisiae* was used (Wendland, 2003). For *TEF*-fusions plasmid pRS415-kanMX was linearized using the *Nru*I restriction site which cleaves the plasmid once in the ORF of the kanMX resistance marker. The kanMX gene consists of the *A. gossypii TEF*-promoter and terminator and the ORF of the kanamycin resistance gene. PCR-fragments of the *CaCHT1*, *CaCHT2*, and *CaCHT3* ORFs were amplified with primers that contain additional flanking homology regions to the *TEF*-promoter and terminator. Template DNA was derived from plasmid libraries kindly provided by J. Ernst (Düsseldorf, Germany) or from fosmids kindly provided by P. Magee (Minneapolis, USA). Co-transformation of *S. cerevisiae* with the linearized plasmid and the PCR-fragment resulted in fusion of the respective ORFs with the *TEF*-promoter and terminator thus placing the *CHT1* and *CHT3* ORFs under control of *TEF* expression. Correct fusion was verified by PCR. Plasmids were isolated from yeast cells and transformed into *Escherichia. coli* for amplification. All newly generated fusion constructs were verified by sequencing (MWG-Biotech *A. gossypii*, Ebersberg, Germany). Fusion of the *CaCHT2* ORF with the *TEF* control regions could be achieved in *S. cerevisiae*. However, plasmids could not be recovered from *E. coli* suggesting that expression of *CaCHT2* from the *A. gossypii TEF* promoter is toxic for *E. coli*. Therefore, *CaCHT2* was placed under control of the yeast *GAL*-promoter, which is not active in *E. coli*. pRS415-TEF-CHT2($n-1$) containing a ($-1$) frame shift in the ATG region of the ORF (which was recovered in our unsuccessful attempts to amplify *TEF-CHT2* in *E. coli*) was used as plasmid and co-transformed with a kanMX- *GAL*p-cassette amplified from pFA6a-kanMX6-GAL1p-GFP which corrected the frameshift and placed *CHT2* under control of the *GAL*-promoter. The *S. cerevisiae CTS1* gene was amplified from a yeast plasmid library, subcloned into pDrive plasmid (Qiagen, Germany) and cloned as a *Bam*HI/*Xba*I fragment into pRS415 to generate pRS415-CTS1 in which the *S. cerevisiae CTS1* is controlled by its endogenous promoter.

To generate chimeric constructs between the *CaCHT2* and *CaCHT3* genes, *in vivo* recombination in *S. cerevisiae* was used. First, pRS415-TEF-CHT3 was linearized in the 5′-end of the *CHT3*-ORF and co-transformed with a *GALp- CHT2*-fragment amplified from pRS415-GAL-CHT2 designed to exchange the 5′-end of *CHT3* with sequences derived from *CaCHT2* yielding pCHT2-3 (in which the chimeric gene is under control of the yeast *GAL*-promoter. Vice versa, pRS415-TEF-CHT2($n-1$) was linearized in the 5′-end of the ORF and co-transformed with a corresponding *CHT3*-PCR-fragment to generate the pCaCHT3-2 chimera (under control of the *A. gossypii TEF*-promoter).

Table 2
Plasmids used in this study

| Plasmid number | Feature | Source |
|---|---|---|
| 150 | pRS415-kanMX | Wendland (2003) |
| 166 | pFA6a-kanMX 6-GAL1p-GFP | Longtine et al. (1998) |
| 186 | pRS415 | Sikorski and Hieter (1989) |
| 200 | pFA-URA3 | Gola et al. (2003) |
| 202 | pFA-HIS1 | Gola et al. (2003) |
| 230 | pFA-URA3-MAL2p | Gola et al. (2003) |
| 269 | pFA-HIS1-MAL2p | Gola et al. (2003) |
| 330 | pRS415-TEF-CHT3 | This study |
| 344 | pRS415-CTS1 | This study |
| 375 | pRS415-TEF-CHT2($n-1$) | This study |
| 376 | pRS415-TEF-CHT1 | This study |
| 455 | pRS415-GAL-CHT2 | This study |
| 501 | pRS415-GAL-CHT2-3 | This study |
| 502 | pRS415-TEF-CHT3-2 | This study |

### 2.5. Chitinase assays

To measure chitinase activity a fluorogenic assay was employed (McCreath and Gooday, 1992). In this assay the substrate 4-methylumbelliferyl-*b*-D-*N*-tetraacetylchitotetraoside (4-MU-[GlcNAc]$_4$) was used. Two samples from each

Table 3
Oligonucleotide primers used in this study

| Primer number | Sequence of primer[a] |
|---|---|
| #599 U3 | GGAGTTGGATTAGATGATAAAGGTGATGG |
| #600 U2 | GTGTTACGAATCAATGGCACTACAGC |
| #601 H2 | CAACGAAATGGCCTCCCCTACCACAG |
| #602 H3 | GGACGAATTGAAGAAAGCTGGTGCAACCG |
| #656 ScCTS1-5′ | TGGGAATATGTGGGATATCCTC |
| #657 ScCTS1- 3′ | CATGATGGGGCACGAATTCGC |
| #670 S1-CHT2 | AGTAGCACAGTACATTTAACAATTACTCAAAAAACACGCTAAATCTCACACACCACTAAACTTTTTTAATAAT TTTTTCACAAACCAAGCATACATAACCgaagcttcgtacgctgcaggtc |
| #671 S2-CHT2 | ATATATCAATACAAAAAGAAAAAATATTAGAGTAAACAAGAGGTTAATTCATTAGAAGGCAAAGGCAGCCA ATAAGAATGGAACAGGAAACCAAACACTAtctgatatcatcgatgaattcgag |
| #674 G4-CHT2 | **tccccgcgg**CAATGTCTTAATAACTATTTGAGGG |
| #675 G1-CHT2 | GAATATTAGCCCGCTTTGCATTGG |
| #700 S1-CHT3 | GCTATCATTTATATCCGACCAACGTTCCCTAGATATCCTAGAAATTCTTATATTATACCTACCGCCTTTTTTAC CTGCTTTTCCGGGAATAATCATAAGAgaagcttcgtacgctgcaggtc |
| #701 S2-CHT3 | CCACTGTACTTGGTTCAGCAAAAACTGTTTCAATATGTGTTCTAGTTGTCAATATTTGATAAGTCGTTGTAAG GGTGCGAAGAGTAGTTGGAGCCCATACtctgatatcatcgatgaattcgag |
| #702 G1-CHT3 | CTTGGTTGTGTTTTTAAGGAC |
| #703 G4-CHT3 | CATACAGAATAGAACCGGGG |
| #736 S1-CHT1 | GTCCCTGGCAGGTGTTTTTCACACACCCCCCCTTTCAATGGGATCTTATAAAAGTTTTGATTTATGTCCTCCC CCAGTCCGTTTTTCTTCCAACAAACACgaagcttcgtacgctgcaggtc |
| #737 S2-CHT1 | GACAGTTGAGTACACAGTTTGTAGCGTCGTGGAGGTGGTTGTTCCATCAAACCAACCAAACCAATTGAACG CTGACGCCGTGGGTTTTGGAGCAGATGTAtctgatatcatcgatgaattcgag |
| #738 G1-CHT1 | CGCCACTAATTGCGATGATGG |
| #813 G4-CHT1 | CGAATAAGGATTGTAGCATTTGGCTG |
| #814 I2-CHT1 | CCGCTGCTGAATGTCCCAG |
| #815 I3-CHT1 | GGTGGAAGCACTGCTACTGC |
| #816 I2-CHT2 | CAGTTTCTTGACAGTATTGTGCC |
| #817 I3-CHT2 | GCCACTCCAGTTACTACTGG |
| #818 I2-CHT3 | GAAAGAATCACAATATCAACAGCATCGG |
| #819 I3-CHT3 | GCCACTTCTACCACCAGTGCC |
| #820 S2-MAL2p-CHT2 | GACCACCAGCACCATTTTGACCCCAGTAAAGAGCAACTTGATTAGAGGCACTGGCCAAAGCTGATGATGCT ACAACTGCAGCAGCTAATAATGATTTAAAAGACAAcattgtagttgattattagttaaaccac |
| #821 S2-MAL2p-CHT3 | GGGAACCACCAGAGTTTTGTCCCCAATAAACAGCAACATTGGAGTTACTTCTAGCATTAATAGCTAAAGCAG GTAATAATAATGAAAATATAGTTAACAAGTATAGcattgtagttgattattagttaaaccac |
| #861-CaCHT3 | cgcggatccCTGCTATTCACCAAGTGTTCTTTGC |
| #862-CaCHT3 | aaaatctagaCATACAGAATAGAACCGGGGATAC |
| #876 CHT3-XL1 | TCTTGCTAGGATACAGTTCTCACATCACATCCGAACATAAACAACCATGctatacttgttaactatattttcattattattacctg |
| #877 CHT3-XL2 | ATGACAAGTTCTTGAAAACAAGAATCTTTTTATTGTCAGTACTGActaattatagataaccactgtacttggttc |
| #879 CTS1-XL2 | ATGACAAGTTCTTGAAAACAAGAATCTTTTTATTGTCAGTACTGAttaaaagtaattgctttccaaataagagaaattac |
| #935 CHT1-XL1 | TCTTGCTAGGATACAGTTCTCACATCACATCCGAACATAAACAACCatgattctcaatcttataatcctacttgcc |
| #936 CHT1-XL2 | ATGACAAGTTCTTGAAAACAAGAATCTTTTTATTGTCAGTACTGActatgaacatataagagcaagtagtgg |
| #938 CHT2-XL2 | ATGACAAGTTCTTGAAAACAAGAATCTTTTTATTGTCAGTACTGAttagaaggcaaaggcagccaataagaatgg |
| #988 I2-CTS1 | AAACAAAGTTCTTAACTTGGTAGCT |
| #989 I3-CTS1 | CACCAAGACAAAATCTAGTCTAG |
| #996-CaCHT2 | CAGCAGCAGAAGTAGTGGTGGC |
| #997-CaCHT3 | GGAAGAACTGGTGGAAGAACTGC |
| #1000 CHT2-XL2-chim | AGTGGTGGTAGTGGTGGTAGAAGAAGAAGTTGTTTCTTCTCCTGGTGAAGCttggtttaaaacattcttgacttggacaac |
| #1001 CHT3-XL2-chim | AGTAGTGGTAGATTGTGTAGTAGCAGAAGAAGATGGGGCAACACAAGCATTtttcttaacaatggctttcatattttcaacaaag |
| #1013 CHT2-XL1 | TCTTGCTAGGATACAGTTCTCACATCACATCCGAACATAAACAACCatgttgtctttttaaatcattattagctgctgc |
| #1084 S1-CHT2-GAL | CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCcgagctcgaattcatcgatg |
| #1085 S2-CHT2-GAL | GAGGCACTGGCCAAAGCTGATGATGCTACAACTGCAGCAGCTAATAATGATTTAAAAGACAAcattataagatccgg ggtttttttctccttgacg |
| #1282 G1-CTS2/CHT4 | GGTCAGCGGGATATCCGAAGG |
| #1283 G4-CTS2/CHT4 | GAGTGTATTTGCCAGAATGG |
| #1284 I2-CTS2/CHT4 | GTGTGGTGTCCAATGATACC |
| #1285 I3-CTS2/CHT4 | CCACAACATCAGCTTCTATAC |
| #1299 S1-CTS2/CHT4 | GCGAACATACCAAACAAACAAACACGGTAATACTAAATTCGCTGTATAATTTGGTCGTGCTACAGTAATTAC GCCAGAACGGTTATGGTTATGCCACGATgaagcttcgtacgctgcaggtc |
| #1300 S2-CTS2/CHT4 | GTCACTGAAACATCACCAGCAGAATCCCACCACATCCCACCACCCAATTGTCTCAGTTGTATAAAGCTAGCT TTTATTCTAGCACACTGGAGATTATCGAtctgatatcatcgatgaattcgag |

[a] In long primers uppercase sequences correspond to DNA sequences used as homology regions for recombination whereas lowercase sequences correspond to 3′-terminal annealing regions for the amplification of transformation cassettes. Short primers were used for verification purposes. All sequences are written from 5′ to 3′.

culture were assayed to measure both the total chitinase activity as well as the chitinase activity found secreted into the growth medium (supernatant of 2 min centrifugation at 24,000*g*). Cultures were grown at 30 °C in YPD or selective medium. Chitinase activity was measured in 100 µl per well containing McIlvaine's buffer at pH4, 50 µM 4-MU-[GlcNAc]$_4$ and 5 µl sample. Excitation and emission wavelengths of 340 and 465 nm, respectively, were used to measure fluorescence (Genios microplate reader, Tecan). Initial rates of 4-MU release were used to calculate units of enzyme activity: 1 U is the amount of enzyme catalyzing the release of 1 pmol of 4-MU min$^{-1}$ at 30 °C.

### 2.6. Staining procedures

Chitin staining was done by adding calcofluor (1 µl of 1 mg/ml stock solution) to a 100 µl cell suspension followed by a short incubation at room temperature.

### 2.7. Time lapse microscopy

Strains were processed for time lapse microscopy as described (Walther and Wendland, 2004). For time lapse imaging every 3 min a stack of 10 focal planes was recorded. Instrumentation consists of a Zeiss AxioplanII-imaging microscope (Zeiss, Jena, Göttingen) operated via Metamorph 4.6 software (Universal Imaging Corporation) and a digital imaging system (MicroMax1024, Princeton Instruments). Image acquisition, processing of images and image stacks and video clip generation (frame rate of 10 images/s) was done with Metamorph as described (Walther and Wendland, 2004). Movie files will be curated at our website (http://pinguin.biologie.uni-jena.de/phytopathologie/pathogenepilze/index.html).

## 3. Results

### 3.1. Comparison of chitinase genes

The four *C. albicans* chitinases Cht1, Cht2, Cht3, and Cht4 were compared with the *S. cerevisiae* Cts1 and Cts2 chitinases using phylogenetic tree comparisons. These comparisons revealed a higher level of amino acids sequence identity between the *C. albicans* Cht1, Cht2, and Cht3 with *S. cerevisiae* Cts1 and placed *C. albicans* Cht4 and *S. cerevisiae* Cts2 in a second group (Table 4). This may be consistent with a functional difference of chitinases in *S. cerevisiae*. Cts1 is required during budding growth for mother–daughter cell separation while Cts2 has been described as a sporulation specific chitinase (*Saccharomyces* Genome Database entry by Bogengruber et al., 2001; C. Specht unpublished data). A higher degree of similarity between the chitinases was found in the N-terminal region containing the catalytic domain with conserved cysteine residues as well as aspartic and glutamic amino acids. For Cts1 and Cht1-3 variable degrees of conservation were found in the C-terminal half which has a Ser/Thr-rich region followed by a C-terminal domain (Fig. 1A). The C-terminal sequence of Cts1 encodes a type-2 chitin binding domain that is not present in the Candida chitinases (Colussi et al., 2005; Kuranda and Robbins, 1991). Located in the C-terminal sequence of Cht2 is a potential GPI-anchor site that is not found in the other *S. cerevisiae* or *C. albicans* chitinases (analysis with DGPI software at http://129.194.185.165/dgpi/index_en.html). Attachment of a GPI-anchor would sequester Cht2 to the plasma membrane or possibly cross-link it to β-1,6 glucan in the cell wall (Frieman and Cormack, 2004).

Table 4
Percent amino acids sequence identity of chitinases[a]

| | CaCht2p | CaCht3p | CaCht4p | ScCts1p | ScCts2p |
|---|---|---|---|---|---|
| CaCht1p | 37.6 | 36.1 | 11,3 | 40.0 | 10.0 |
| CaCht2p | | 35.9 | 11.1 | 36,1 | 10.6 |
| CaCht3p | | | 12.9 | 38.7 | 10.0 |
| CaCht4p | | | | 10.3 | 22.2 |
| ScCts1p | | | | | 10.4 |

[a] Abbreviations used: *Sc*: *Saccharomyces cerevisiae*; *Ca*: *Candida albicans*.

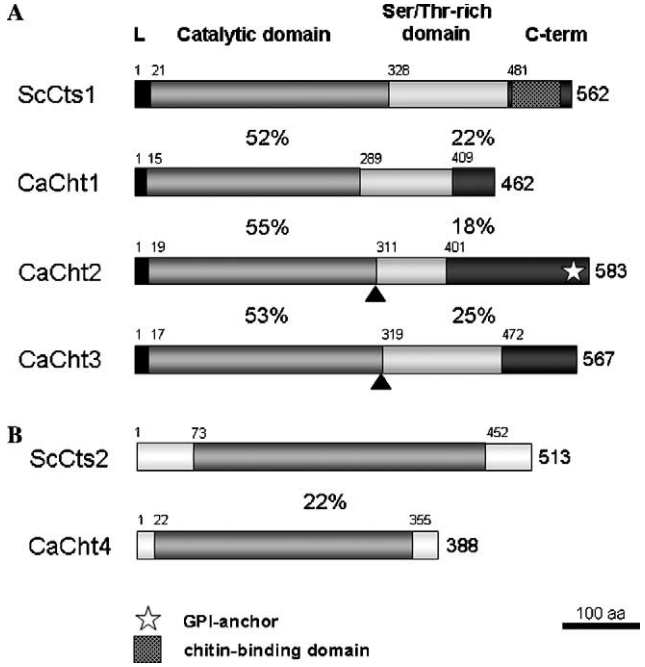


Fig. 1. Domain structure of chitinases. Chitinases are composed of several domains as indicated (L: leader/signal sequence; GPI: glycosylphosphatidylinositol anchor). Percent amino acids sequence identity of the catalytic domains and of the C-termini of *C. albicans* Cht1-3 chitinases in comparison to the *S. cerevisiae* Cts1 (A) and *C. albicans* Cht4 in comparison to *S. cerevisiae* Cts2 (B) are shown. Black triangles mark the corresponding positions in Cht2 and Cht3 which were used to generate chimerical genes between *CHT2* and *CHT3*. *C. albicans* protein sequences were derived from the Stanford database and are in GenBank: Cht1 (EAK99759), Cht2 (EAL03025), Cht3 (EAL00460), and Cht4 (EAL03671). Cht1p, Cht2p, Cht3p, and Cht4 correspond to assembly 19 annotated ORFs 7517, 3895, 7586, and 1515 respectively. Accession numbers for *S. cerevisiae* Cts1 and Cts2 are AAB67331 and NP_010659, respectively. Note the close similarity both on the amino acids level and in protein domain composition between *C. albicans* Cht4 and *S. cerevisiae Cts2*.

Interestingly, the differences observed in the protein alignments show that CaCht4 lacks a Ser/Thr-rich domain as does its *S. cerevisiae* homolg Cts2 further contributing to the separation of *C. albicans* chitinases into two classes (Fig. 1B). In the comparisons of full lengths protein sequences the *C. albicans* Cht3 showed a slightly higher percentage of identity with *S. cerevisiae* Cts1 than the other *C. albicans* chitinases. Based on this initial sequence analysis no further conclusions on the functional properties of the *C. albicans* chitinases could be drawn. Also, expression data on the chitinases suggested that *CHT1* is not expressed, whereas *CHT2* and *CHT3* appeared to be regulated in the same manner (McCreath et al., 1995). This created the basis for our experimental approach to explore whether one of the *C. albicans* chitinases might represent the functional homolog of Cts1p. To this end we decided to proceed with the generation of single deletions of the *C. albicans* chitinase genes.

### 3.2. Deletion of C. albicans chitinase genes

To be able to study the role of chitinases on cell morphology and growth during the yeast and hyphal growth stages of *C. albicans* we generated independent homozygous complete ORF deletion strains by sequential disruption via PCR-based gene targeting methods in all four *C. albicans* chitinase genes. As marker genes *FA-HIS1* and *FA-URA3* cassettes were employed using BWP17 as progenitor strain. Correct gene targeting was verified by diagnostic PCR that established (i) correct marker integrations and (ii) absence of the target ORF in the homozygous mutants. All single deletion strains were found to be viable. Deletion of *CaCHT4* did not produce any obvious phenotypes and was not studied further.

## 4. Analysis of chitin distribution

To analyze chitin distribution in the different strains calcofluor staining was used. Both diploid *S. cerevisiae* and *C. albicans* produce bipolar budding patterns and form buds on both cell poles which results in the accumulation of bud scars as remnants of budding events at the poles. *C. albicans cht1* and *cht2* mutant strains did not exhibit defects in cytokinesis and the budding pattern in these strains was bipolar as in the wild type. The *C. albicans cht3* and the *S. cerevisiae cts1* mutant cells showed an identical phenotype of interconnected cells that failed to separate at the end of the cell cycle (Fig. 2). Budding pattern showed a preference to unipolar budding in the *cht3* mutant generating tree-like arrays of cells. Using mechanical force these cell arrays could be dissolved into single cells demonstrating that the cytoplasms of mother and daughter cells were separated.

### 4.1. Regulatable expression of CHT3

To validate the mutant phenotype of *cht3* cells a heterozygous *CHT3*/*cht3* strain was used and the sole copy of *CHT3* was placed under control of the regulatable *MAL2* promoter (Fig. 3). Upon growth in glucose as carbon source, which shuts off the expression of *MAL2p-CHT3*, cells of this strain exhibited the characteristic cell separation defects identical to the homozygous mutant strain. Growth in maltose permitted the expression of *MAL2p-CHT3* and reversed the mutant phenotype back to wild type. Growth in different carbon sources did not affect the cell morphology of either wild type or the *cht3*/*cht3* mutant demonstrating that loss of *CHT3* was solely responsible for the observed cytokinesis defect.

### 4.2. Heterologous complementation of S. cerevisiae cts1

Our results demonstrated that in *C. albicans* Cht3 fulfils similar functions as Cts1 in *S. cerevisiae*. To analyze whether Cht3 is the sole functional homolog of Cts1 heterologous complementation studies were initiated. To this end, a plasmid based approach was used. The *S. cerevisiae CTS1* gene was used under transcriptional control of the endogenous promoter and cloned into a CEN-ARS-vector. The open reading frames of the *C. albicans CHT*-genes were placed either under the control of the *A. gossypii TEF*-promoter (*CHT1* and *CHT3*) or under control of the regulatable *S. cerevisiae GAL*-promoter (in case of *CHT2*, for details see Materials and Methods) and cloned into the same vector backbone using an in vivo recombination approach (Wendland, 2003). Both the *S. cerevisiae* haploid and diploid *cts1* strains were used in these studies. Diploid *S. cerevisiae* cells were imaged and analyzed after an overnight growth period in liquid culture (Fig. 4). For these analyses all plasmids were transformed into a diploid *S. cerevisiae cts1*/*cts1* strain. Additionally, in vivo time lapse microscopy was employed to monitor cytokinesis during growth on solid medium using the haploid *Sccts1* strain as a host for the plasmids (see supplemental movie files that are curated at the author's website). The *ScCTS1* and the *CaCHT3* genes were able to complement the cell separation defect of the *Sccts1*/*cts1* strain but neither the *CaCHT1* nor *CaCHT2* genes (*CaCHT2* expression was induced by galactose) showed complementing ability demonstrating that *CaCHT3* is the only functional homolog of *CTS1*.

Since chitinases are composed of several domains, we generated two chimerical constructs in which the 5′-half of *CHT2* or *CHT3* (containing the leader and the catalytic domain) was fused to the 3′-end of *CHT3* or *CHT2* (containing the Ser/Thr-rich domain and C-term), respectively. These constructs were tested for their ability to complement the *Sccts1* defect. None of these constructs resulted in full complementation but the CaCHT2-3 chimera at least partially reduced the cell separation defect (Fig. 4).

### 4.3. Time lapse analysis of chitinase mutants reveals a cytokinesis defect in cht3 cells

In vivo time lapse microscopy was performed for a duration of >10 h which ensures imaging of more than

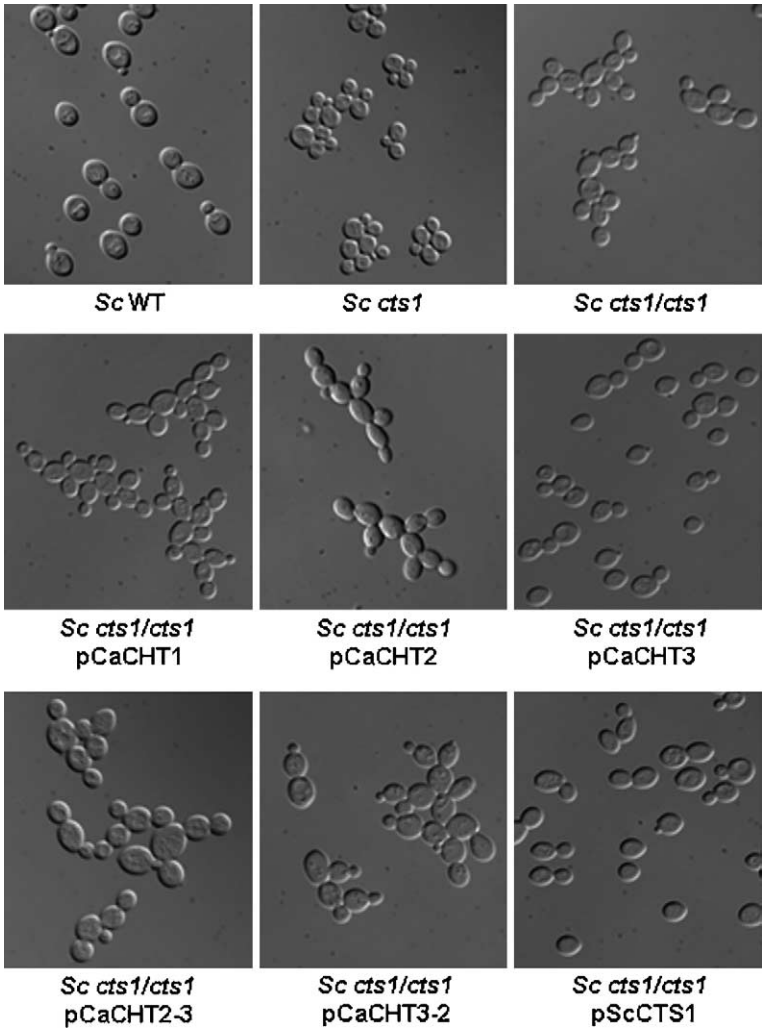


Fig. 2. Heterologous complementation of the *S. cerevisiae cts1* defect. Different *S. cerevisiae* (*Sc*) strains were grown overnight in minimal medium (lacking leucine for the selection of plasmids where applicable). The wild type strain was (BY4742). Both haploid and diploid *cts1* mutant strains which differ in their budding pattern are shown. The diploid *cts1*/*cts1* strain was used for complementation experiments shown here. Plasmid transformants bearing the indicated plasmids were analyzed for cell separation defects. Representative images of cells are shown. Note the complementing activity of plasmids carrying either *CTS1* or *CHT3*. Additionally time lapse analyses showing heterologous complementation in the haploid *Sccts1* strain were carried out which can be viewed from our web site at http://pinguin.biologie.uni-jena.de/phytopathologie/pathogenepilze/index.html.

seven cell cycles to record cytokinesis events in the wild type and the chitinase mutants. Defective cytokinesis was observed in *cht3* cells as compared to the wild type (Fig. 5A). Cytokinesis, i.e. the complete separation of the daughter cell from its mother, became evident as a lateral movement of mother and daughter cell, which alters both axes of polarity. As a result, in the wild type, cells are pushed outwards in the thin-layered microscopy chamber and a round colony consisting of a single cell layer of cells is formed. Failure of cytokinesis in *cht3* cells was visible by the lack of such a lateral movement of mother and daughter cells (Fig. 5B). Furthermore, a high frequency of unipolar budding generated linear tree-like arrays of cells. In the end *cht3* colonies formed uneven colony edges and multi-cell layers in the centre of the colonies.

### *4.4. Growth of C. albicans cht-mutants on hyphal inducing solid media*

Growth of the mutant strains was compared with the wildtype on solid media with or without induction of hyphal growth via the addition of serum (Fig. 6). Wild type yeast colonies (at 30 °C) had a shiny appearance and showed smooth colony edges consisting solely of yeast cells. In contrast, colonies of *cht1* and *cht2* mutant strains displayed the ability of filament formation under these conditions. Differing from both of the former phenotypes, *cht3* colonies displayed a more wrinkled appearance after three days of growth without the filamentation seen in the *cht1* and *cht2* mutants (Fig. 6). Under serum induction, the wild type and all chitinase mutants were able to filament demonstrating that single deletions in any of the *CHT* genes did

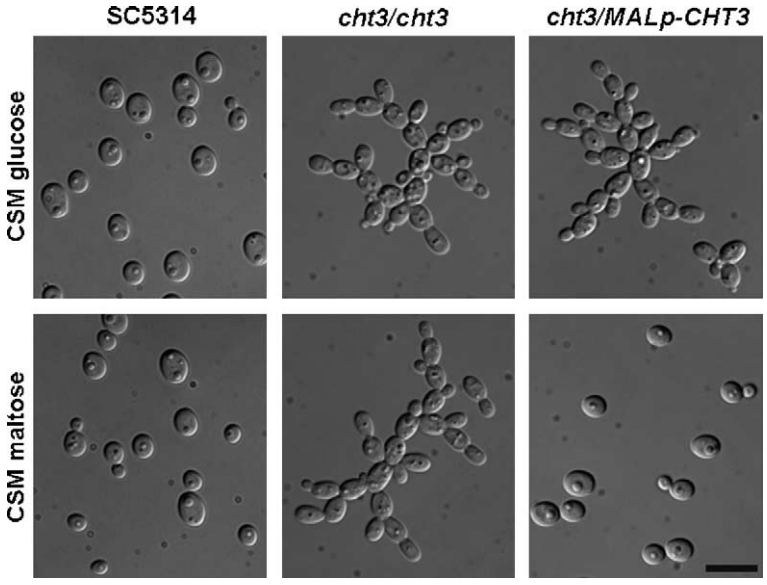


Fig. 3. Regulatable expression of *CHT3*. The wild type (SC5314), the homozygous *cht3*/*cht3* mutant strain as well as a heterozygous strain in which one copy of *CHT3* was deleted and the remaining copy of *CHT3* was placed under the control of the *MAL2*-promoter were grown in minimal media containing either glucose (which inhibits expression of *MAL2p-CHT3*) or maltose (which induces expression of *MAL2p-CHT3*) as the sole carbon source for 20 h at 30 °C. Representative images of cells are displayed. Bar is 10 μm.

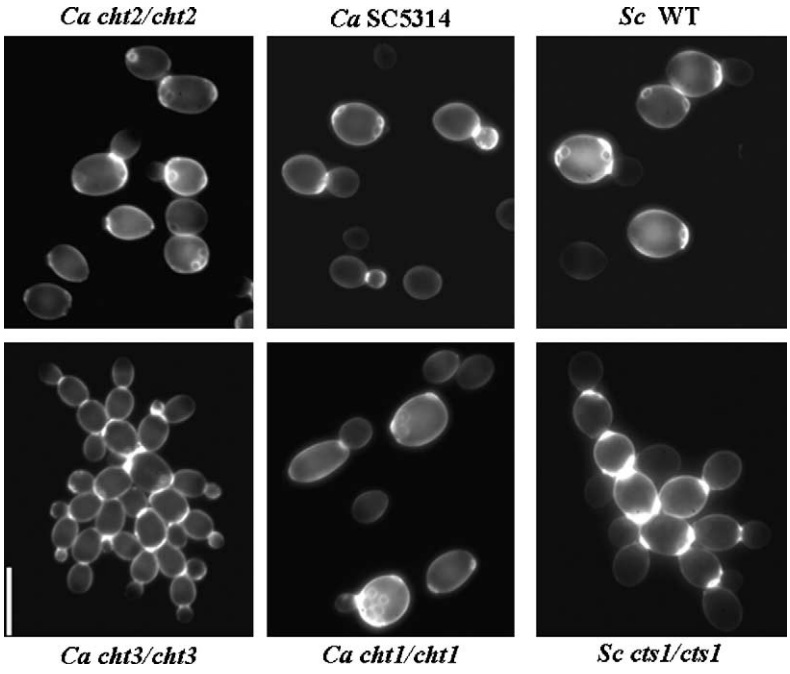


Fig. 4. Analysis of chitin distribution. The indicated *C. albicans* and *S. cerevisiae* strains were grown overnight in complete medium at 30 °C, stained by calcofluor and imaged using fluorescence microscopy. Note the comparable mutant phenotypes of *cht3* and *cts1* mutants. Bar is 10 μm.

not interfere with the ability to perform the yeast to hypha transition (Fig. 6). The mutant phenotype of *cht2* cells, which produced filaments under non-inducing growth conditions, was due to lack of *CHT2* expression since a heterozygous strain in which the remaining *CHT2* allele was placed under regulatable expression controlled by the *MAL2*-promoter did not produce elongated cells under a maltose growth regime (our unpublished results).

### *4.5. Chitinase activities in cht and cts1 mutants of C. albicans and S. cerevisiae*

The *C. albicans* chitinase genes were shown to be transcribed at higher levels during yeast growth phases (McCreath et al., 1995). Therefore we measured chitinase activity of yeast cells and compared the total measurable chitinase activity with chitinase activity of the supernatant (Fig. 7).

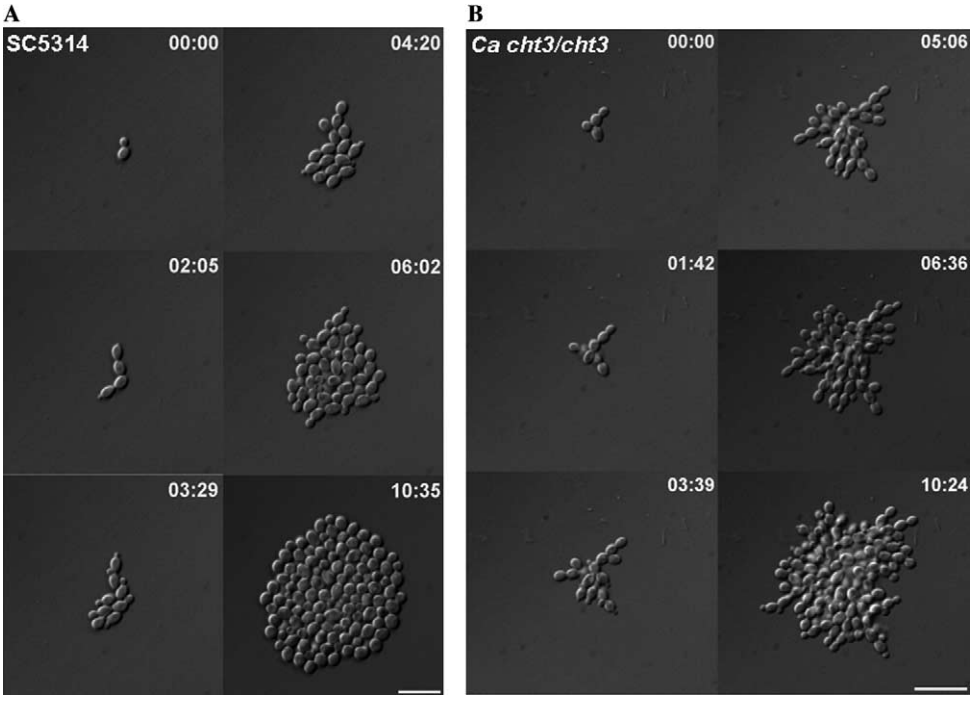


Fig. 5. *In vivo* time lapse analyses of yeast phase growth of the wild type SC5314 and *cht3* strains. Representative frames of movies of the wild type (A) and *cht3* cells (B) are shown over a growth period of more than 10 h. Note the cell separation defect in *cht3* cells which alters colony morphology in comparison to the wild type. Time is in hh:min. Bars represent 10 μm. Movies are available at http://pinguin.biologie.uni-jena.de/phytopathologie/pathogenepilze/index.html.

Deletion of either *CHT1* or *CHT2* did not drastically alter the chitinase activity compared to the BWP17 progenitor strain in our assays. In contrast, deletion of *CHT3* had a strong impact on total chitinase activity and almost eliminated chitinase activity in the supernatant. A double mutant strain bearing deletions in the *cht2*/*cht3* genes did not show detectable levels of chitinase activity (Fig. 7). The *S. cerevisiae cts1* mutant is chitinase negative in our assays (not shown). To provide evidence that the functional complementation of *cts1* by *CHT3* is based on restoration of secreted chitinase we also measured chitinase activity in the yeast strains used for heterologous complementation assays. Cells were grown in galactose media to ensure similar growth conditions and to fully activate *CHT2* expression. Reintroduction of *CTS1* into the *cts1* mutant on a plasmid restored chitinase activity and resulted in full complementation. Expression of *CHT3* in *S. cerevisiae* reached the highest levels of both total and secreted chitinase activity providing formal proof that *CHT3* expression in *S. cerevisiae* results in the production of functional chitinase. Expression of either *CHT2* or the *CHT2-3* chimera also produced chitinase activity (Fig. 8). However, our time lapse recordings demonstrate that this activity was not sufficient to complement the cell separation defect. Expression of *CHT1* and of the *CHT3-2* chimera did not result in measurable chitinase activity.

## 5. Discussion

The maintenance of cell wall integrity is important for fungal growth, development and survival to counteract environmental stresses. Chemical compounds that attack the cell wall may be particularly useful as antibiotics against human fungal pathogens. Although chitin is only a minor component of the fungal cell wall it is providing mechanical strength and an increase in the chitin content may compensate for defects in cell wall structure (Kapteyn et al., 2000; Popolo et al., 1997). Chitin turnover requires chitin synthesis and degradation. Both processes can be inhibited by specific compounds. Chitin synthases of *C. albicans* responsible for chitin synthesis can be inhibited by nikkomycin Z and CaChs1 can be specifically inhibited by RO-09-3143 (Kim et al., 2002; Sudoh et al., 2000). In contrast to *S. cerevisiae* the *C. albicans* genome contains two isoenzymes for class I chitin synthases encoded by the *CHS2* and *CHS8* genes (Munro et al., 2003).

Chitinases that degrade chitin can be inhibited by allosamidin (Dickinson et al., 1989). Defects in chitin synthesis affect the virulence of *C. albicans* and chitinases are specifically involved in the pathogenesis of insect pathogens, for example, in *Bacillus thuringiensis* (Bulawa et al., 1995; Sampson and Gooday, 1998).

### *Candida albicans CHT3 encodes the functional homolog of S. cerevisiae CTS1*

*C. albicans* encodes four chitinase genes. Three of these chitinase genes bear similarity to the *S. cerevisiae CTS1* gene. The fourth is more similar to *S. cerevisiae CTS2*. In *S. cerevisiae cts2* mutants were reported to be defective in sporulation. Therefore, we decided to attempt heterologous complementation of the *S. cerevisiae cts2* with this gene.

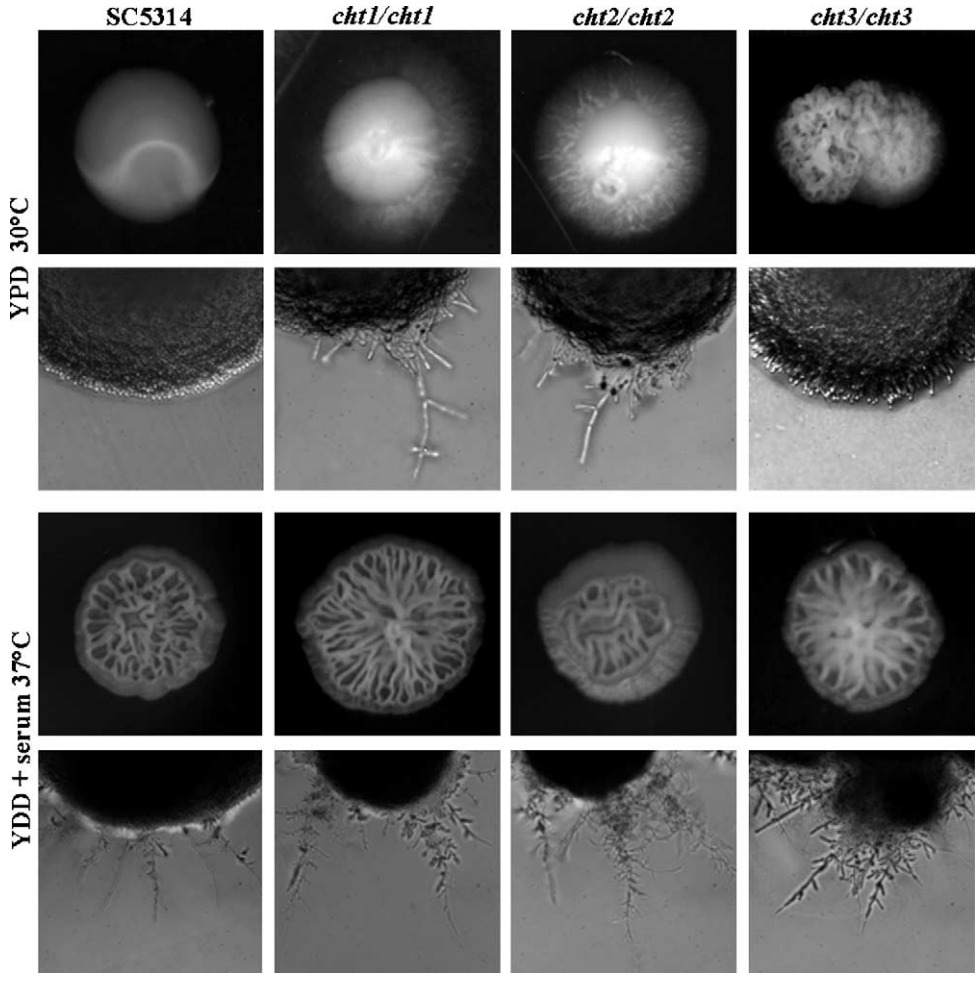


Fig. 6. Deletion of chitinases alters colony morphology on solid media but does not interfere with hyphal formation. Cells of the indicated strains were plated on YPD-plates with or without 10% serum and incubated at 30 °C or 37 °C (in the case of hyphal inducing conditions). Colonies were imaged after 3 d (YPD-30 °C) or 7 d (YPD + serum-37 °C) of growth. Microscopic images were taken after 1 d (second and fourth row) using 50× magnification.

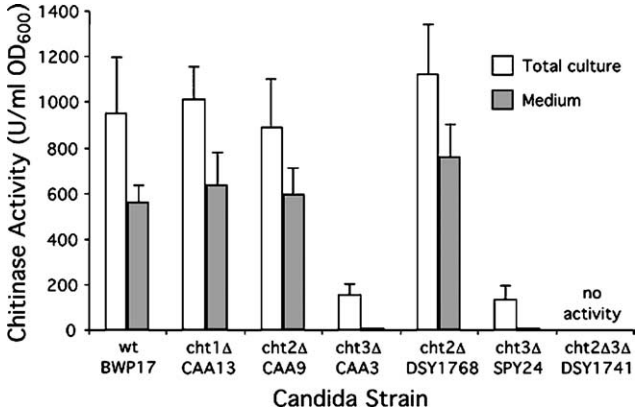


Fig. 7. Measurement of chitinase activity of *C. albicans* strains. The indicated strains were used for chitinase activity assays using 4-MU-$[GlcNAc]_4$. Cells were grown in YPD for 19 h. Light bars represent total chitinase activity; grey bars represent the chitinase activity of the culture supernatant. Error bars are standard deviations of three independent experiments.

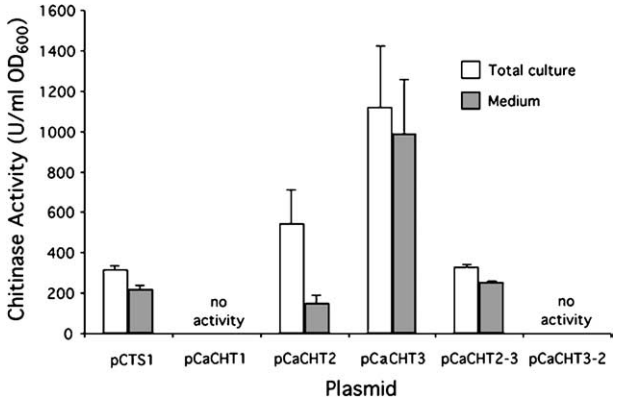


Fig. 8. Measurement of chitinase activity of *S. cerevisiae* strains. The *cts1* mutant harboring the indicated plasmids was used for measuring the activity of chitinase as described in the legend to Fig. 7. Cells were grown for 22 h in minimal medium lacking leucine but containing galactose as carbon source.

However, in our hands, using standard strains obtained from EUROSCARF, *cts2*/*cts2* mutant diploid yeast strains were able to produce zymolyase resistant viable spores and showed no other obvious phenotype that could be used for complementation. Deletion of *C. albicans CHT4* did not provide any phenotype either. Therefore, any involvement

of Cht4 in a potential sporulation process in *C. albicans* needs to be examined in more detail.

We have shown in this report that of three related *C. albicans* chitinase genes *CHT3* encodes the functional homolog of the *S. cerevisiae* chitinase Cts1p. This was based on several lines of evidence: (i) The full length protein of Cht3 bears closest sequence similarity with Cts1 and groups closest to ScCts1 in phylogenetic tree analyses; (ii) disruption of *CHT3* provided a highly similar phenotype compared to *S. cerevisiae cts1* strains whereas *cht1* and *cht2* deletion strains did not; (iii) heterologous complementation of the *S. cerevisiae cts1* defect was fully accomplished by *CHT3* but not by *CHT1* or *CHT2* and also not by chimeric constructs between *CHT2* and *CHT3*; and finally, (iv) measurement of chitinase activity demonstrated the expression of chitinase activity in the *S. cerevisiae cts1* mutant containing pCaCHT3.

In these chitinase assays lower amounts of chitinase activity was also found in *CHT2* and *CHT2-3* constructs which, however, did not result in complete reversion of the *cts1* defect.

To proof that the cell separation defect in a *C. albicans cht3* mutant was solely due to the deletion of *CHT3* we placed *CHT3* under control of the regulatable *MAL2*-promoter. We also analyzed the chitinase activity of a *MAL2-CHT3* strain grown in glucose, which represents repressed conditions. We noticed that after prolonged incubation (in this assay after 42 h) the chitinase activity of the *MAL2p-CHT3* strain went up which may be attributing to the fact that the cytokinesis defect of *MAL2-CHT3* cells in liquid culture was not as pronounced as in a *cht3* strain. This indicates some leakiness of the *MAL2*-promoter.

### 5.1. Chitinases may be developmentally regulated specifically in filamentous fungi

Chitin synthesis may not be linked with chitin degradation as was recently shown for *C. albicans* (Selvaggini et al., 2004). We found that the genome of the filamentous fungus *A. gossypii* does not encode a Cts1 chitinase homolog and may thus lack any chitinase activity during vegetative growth. This is intriguing when comparing yeast-like and filamentous growth: the *S. cerevisiae* Cts1 is required for mother–daughter cell separation, thus promoting cellularization. Filamentous fungi do not require such an activity during the hyphal growth phase, which, in fact, could destroy the hyphal tube. This is in line with evidence from *Aspergillus nidulans* in which the chitinase encoding gene *chiA* was shown to be down-regulated during the hyphal growth stage but up-regulated during conidiophore development which requires cellularization to produce metulae, philaides and conidia (Takaya et al., 1998).

Expression of *CTS1* is under specific regulation of Ace2 in *S. cerevisiae*. This ensures daughter cell specific expression and, therefore, the process of cell separation is solely carried out by the daughter cell (Colman-Lerner et al., 2001). In *C. albicans* it was shown that the *C. albicans* Ace2 homolog is required for *CHT3* expression. Deletion of *ACE2* resulted in a reduced expression of *CHT3*, however, the transcript level of *CHT2,* however, appeared to be unaltered (Kelly et al., 2004). In this report loss of *ACE2* also resulted in avirulence in a mouse model. In contrast, a report on the *C. albicans* forkhead homolog Fkh2 indicated that this transcription factor may contribute a level of control to the expression of *CHT2* (Bensen et al., 2002). Deletion of *ACE2* in *C. albicans* resulted in a similar cell separation defect as was observed in *CHT3* (Kelly et al., 2004). Interestingly, while deletion of *ACE2* in *C. albicans* led to avirulence, disruption of the *C. glabrata ACE2* homolog yielded hypervirulent strains (Kamran et al., 2004). How this relates to chitinase expression or other effects is unknown. However, as is the case in *S. cerevisiae* and *C. albicans*, deletion of *ACE2* in *C. glabrata* leads to a cell separation defect, thus pointing out a conserved regulon in which Ace2 controls the expression of chitinase genes in fungi.

In *S. cerevisiae ACE2* is a paralog of *SWI5* which was generated during the genome duplication in the *S. cerevisiae* lineage. *A. gossypii* represents an unduplicated genome and related to the fact that *A. gossypii* lacks a chitinase homolog of either Cts1 or Cht3 *A. gossypii* does also not contain a homolog of the Ace2 transcription factor and only possesses a homolog of *SWI5* (Dietrich et al., 2004). Transformation of *A. gossypii* with plasmids bearing either the *S. cerevisiae CTS1* gene or the *C. albicans CHT3* gene under control of the *A. gossypii TEF*-promoter yielded viable transformants (our unpublished results). However, the expression level of the constructs may be relatively low due to the copy number of the plasmids. On the other hand, expression of a chitinase gene in *A. gossypii* may not provide functional protein or the protein may not be delivered to septal sites. Analysis of ectopic chitinase expression in *A. gossypii*, therefore, requires refined experiments such as the genomic integration of a chitinase gene, preferably under a regulatable promoter. With these experiments one may be able to analyze whether untimely expression of chitinases in filamentous fungi may be detrimental to hyphal integrity.

### 5.2. The function of Cht1 and Cht2 in C. albicans?

One of the open questions is the role of Cht1 and Cht2 in *C. albicans*. Initially it was shown that *CHT1* is not or only weakly expressed during the yeast or hyphal growth stage. We have also not been able to detect its enzymatic activity in *C. albicans* or when heterologously expressed in *S. cerevisiae*. Both *CHT2* and *CHT3* are expressed in the yeast stage but down-regulated during hyphal growth (McCreath et al., 1996). Recently it was found that the activities of Cht2 and Cht3 were higher in the hyphal form than in the yeast form (Selvaggini et al., 2004). It was also shown that Cht2 is covalently linked to the cell wall which is consistent with the fact that it carries a C-terminal GPI-anchor motif (Iranzo et al., 2002). In addition *CHT2* and *CHT3* seem to be under differential regulation of the Fkh2 and Ace2

transcription factors (Bensen et al., 2002; Kelly et al., 2004). Our chitinase assays did not reveal a drastic decrease in chitinase activity in *C. albicans cht1* and *cht2* strains during yeast growth. Heterologous expression of the *CHT2* gene in *S. cerevisiae* showed an activity of Cht2 which was, however, not sufficient to complement the *S. cerevisiae cts1* defect.

Growth on solid media was found to lead to increased filament formation in *cht1* and *cht2* strains. An interesting hypothesis is, that this hyphal promoting activity may be due to the content of *N*-acetylglucosamine which is a polymer of chitin and a known hyphal inducing substance in *C. albicans* in yeast extract containing YPD medium. Reduced capabilities of degradation of this substance could lead to an increased stimulus for hyphal formation, which will be explored in more detail in the future.

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