Two chitin synthase genes from *Ustilago maydis*

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PCR was used to amplify fragments corresponding to *CHS* genes from *Ustilago maydis*, utilizing as primers oligonucleotides devised according to the conserved regions of fungal *CHS* genes. The PCR product was employed as a probe to screen a genomic library of the fungus. Two different *CHS* genes (*Umchs1* and *Umchs2*) were thus identified in the positive clones recovered. Their sequence revealed high similarity with the *CHS* genes previously cloned from other fungi, especially in their central region. Alignment with the deduced protein sequences of all *CHS* genes reported up to date showed the existence of seven conserved domains. Transcripts from both genes were detected in the yeast and mycelial forms. In general, the transcripts from the *Umchs1* gene appeared to be present at a higher level than the transcripts from the *Umchs2* gene; the transcripts from both genes appeared to be more abundant in the mycelial form. Gene replacement of either gene and analysis of the resulting phenotype demonstrated that they are non-essential. Nevertheless, growth, chitin synthase activity levels, and chitin content of mycelial cells induced by cultivation in acidic media were all reduced in *chs1* and *chs2* mutants. However, mating, virulence and dimorphic behaviour were unaffected. Overall, the results indicate that the *CHS1* and *CHS2* genes encode products with redundant functions in *U. maydis*.

Keywords: *Ustilago maydis*, chitin synthases, cell wall, gene replacement

INTRODUCTION

Chitin is the most important structural component of the cell wall of fungi (Ruiz-Herrera, 1992). It is not surprising then that great effort has been dedicated to the study of the mechanisms involved in its synthesis. Results obtained with different fungi have demonstrated that chitin synthase, the enzyme responsible for the synthesis of the polymer, is accumulated in specific microvesicles, called chitosomes, which convey the enzyme from its site of synthesis to the cell surface, where chitin formation takes place (Bracker *et al.*, 1976; Bartnicki-Garcia *et al.*, 1978; Ruiz-Herrera, 1984). More data exist on the genetic control of chitin synthase than on the enzyme itself. By use of genetic and molecular biology approaches, it has been concluded that two types of chitin synthases exist in fungi: those encoded by *CHS* genes contain a single

polypeptide chain and are activated by proteolysis *in vitro*; and those encoded by *CSD* (or *CAL*) genes which contain more than one polypeptide, and are not believed to be activated by proteolysis *in vitro* (Bulawa, 1993). In all systems studied thus far, more than one *CHS* gene has been detected (Bowen *et al.*, 1992; Bulawa, 1993). Gene disruption of one of these genes is not lethal in most fungi, suggesting that there is functional redundancy.

An attractive possibility for the multiplicity of genes encoding chitin synthase is that they are involved in wall synthesis at different stages during cell differentiation. Specific roles during the cell cycle of *Saccharomyces cerevisiae* have been suggested for the different chitin synthases (Shaw *et al.*, 1991), although it has been demonstrated that chitin synthase III encoded by *CSD2* (or *CAL1*) contributes to the synthesis of most of the chitin of the cell (Valdivieso *et al.*, 1991; Bulawa, 1992). This enzyme also appears to be responsible for the formation of the chitosan layer during sporulation (Pammer *et al.*, 1992). A study of the two *CHS* genes of *Candida albicans* demonstrated that *CHS2* was differentially expressed during the dimorphic

Abbreviation: GlcNAc, N-acetylglucosamine.

The GenBank/EMBL/DBJ accession numbers for the *Umchs1* and *Umchs2* sequences reported in this paper are X87748 and X87749, respectively.

transition of the fungus (Chen-Wu *et al.*, 1992). Nevertheless, disruption of this gene had no effect on its dimorphic behaviour (Gow *et al.*, 1993).

Ustilago maydis is a plant pathogen responsible for corn smut. During its haploid saprophytic phase, the fungus grows in the form of budding yeast-like sporidia. When two compatible strains mate, they invade the plant and the dikaryotic phase grows in mycelial form. Mycelial growth of *U. maydis* can also be obtained transiently on solid medium during mating of compatible strains, or by forced diploids (reviewed by Banuett, 1992). The existence of two *CHS* genes in the fungus was demonstrated by PCR amplification using as primers oligonucleotides designed for the more conserved region of *CHS* genes from *S. cerevisiae* (Bowen *et al.*, 1992). Gold & Kronstad (1994) analysed the expression of both genes using these PCR fragments as probes, and carried out gene disruption. They concluded that they were equally expressed, and that neither gene was essential for mating or pathogenicity. We have recently described conditions which permit the dimorphic transition of haploid strains of the fungus in liquid or solid media (Ruiz-Herrera *et al.*, 1995). We have now cloned two *CHS* genes of *U. maydis* to study their expression during the dimorphic transition, and the phenotypic alterations brought about in mutants carrying substitutions of these genes.

METHODS

Strains and culture media. Strains FB1 (a_1b_1) and FB2 (a_2b_2) of *U. maydis* were obtained from Flora Banuett (University of California, San Francisco, USA). They were maintained and propagated as previously described (Ruiz-Herrera *et al.*, 1995). Yeast growth in liquid media occurs at pH 7, whereas mycelial growth is induced at pH 3. Growth was measured as OD₆₀₀ (Pharmacia Ultrospec III spectrophotometer) and calculated as protein by use of a calibration curve. Fuzz reaction on solid charcoal-containing medium was performed as described by Holliday (1974). *Escherichia coli* strains JM103 and KW258 were used for transformation, and for plasmid and phage propagation, respectively; they were grown in LB medium. For plasmid propagation, the bacteria were grown in TB medium containing the necessary antibiotics for selection. For phage propagation, LB medium was supplemented with 10 mM MgSO₄ and 2% (w/v) maltose.

PCR amplifications. Oligonucleotides corresponding to the conserved regions of *CHS* genes from *S. cerevisiae*, designed by Bowen *et al.* (1992) were synthesized, and used as primers for PCR. Their sequences are as follows: oligo 5', 5' CTG AAG CTT AC(TGCA) ATG TA(TC) AA(TC) GA(GA) GAC 3', and oligo 3', 5' GTT CTC GAG (TC)TT (GA)TA (TC)TC (GA)AA (GA)TT (TC)TG 3'. The following conditions were employed: 30 cycles; melting step, 1 min at 94 °C; oligonucleotide annealing, 1 min at 50 °C; polymerization, 2 min at 72 °C. The bands obtained were ligated to PCRII vector (Invitrogen) and transformed into *E. coli* JM103.

Transformation of *U. maydis*. The procedure described by Sanchez *et al.* (1993) for *Kluyveromyces lactis* was slightly modified. Cells were harvested by centrifugation after reaching OD₆₀₀ 1.5 and resuspended in fresh culture medium containing 10 mM DTT. After 30 min cells were recovered by centrifugation, washed four times with deionized water and resuspended in water to a final concentration of 10¹⁰ cells ml⁻¹. Cells (40 µl)

were electroporated with a Bio-Rad electroporator in 0.2 cm cuvettes under the following conditions: 1500 V, 25 mF and 400 Ω. Efficiency of transformation under these conditions using an autonomous replication vector was of the order of 8 × 10⁵–1 × 10⁶ transformants (µg DNA)⁻¹.

DNA techniques. Restriction endonucleases and modifying enzymes were obtained from BRL and used according to the supplier's recommendations. Oligonucleotides were synthesized using a DNA synthesizer (Applied Biosystems) by reversed-phase chromatography. Southern's techniques were carried out as described by Maniatis *et al.* (1982).

Hybridization. Double-stranded DNA probes were labelled with [α -³²P]dCTP by random priming according to the manufacturer's instructions (USB). Nylon membranes containing DNA samples were hybridized under high stringency conditions with the radiolabelled probe. Washing was carried out at 60 °C with 2 × SSC (Maniatis *et al.*, 1982).

Screening of the genomic library. The genomic library of *U. maydis* RK32 (a_2b_3) constructed in λEMBL-3 was obtained from Regine Kahmann (Institute für Genetik und Mikrobiologie, Universität München, Germany). The screening was carried out using as a probe the PCR product labelled by random priming. Positive clones were amplified in *E. coli* KW258. To identify by restriction analysis the phages containing sequences related to *CHS*, DNA was isolated, digested with *Bam*HI, *Eco*RI and *Sal*I, and probed by Southern hybridization with the radiolabelled PCR product. Fragments detected with the probe were subcloned in pBluescript for further analysis.

DNA sequencing. Sequencing of DNA was performed by the dideoxynucleotide chain-termination method of Sanger *et al.* (1977) using the Sequenase kit (version 2.0; USB). Single- or double-stranded DNA were used as templates. The terminal sequences of the cloned fragments were obtained using the forward and reverse universal primers. Additional primers were designed to extend these sequences and also to resolve ambiguous regions.

RNA extraction and Northern blotting. *U. maydis* yeast and mycelial cells were grown according to the method of Ruiz-Herrera *et al.* (1995) for 24 h. Cells were collected by centrifugation and washed with diethyl-pyrocyanate-treated water. The pelleted cells were ground with a mortar, cooling with liquid nitrogen. Total RNA was isolated using the acid guanidine thiocyanate procedure (Chomczynski & Sacchi, 1987). RNA was separated in formaldehyde/agarose gels, transferred to Hybond-N+ membranes (Amersham) and hybridized with the radiolabelled probe under stringent conditions (Maniatis *et al.*, 1982).

Strategy for gene replacement. A vector for *Umchs1* gene replacement was constructed as follows: a *Bam*HI fragment of *Umchs1* 1.7 kbp in length was subcloned in a pBluescript KS(–) vector, from which the *Sal*I site had been previously deleted. A *Hind*III linker was cloned in the *Nru*I site of the insert. Then the *Hind*III–*Sal*I fragment of *Umchs1* (30 codons in length) was removed and substituted by a 2.6 kbp *Hind*III–*Sal*I fragment containing the hygromycin-resistance cassette from pCM54 (Tsukuda *et al.*, 1988). For *Umchs2*, the strategy was as follows: a 1.5 kbp *Bam*HI fragment from *Umchs2* was cloned into pBluescript KS(–) from which the *Xho*I site had been previously deleted. A *Hind*III linker was cloned in the *Nru*I site of the insert. The internal *Hind*III–*Xho*I fragment, which contains 76 codons, was substituted by the 2.6 kbp *Hind*III–*Sal*I hygromycin-resistance cassette.

Additional methods. Cell-free extracts were obtained with a Braun homogenizer or by grinding in a mortar with liquid

GTCGCTGTATTCTTGTGTGATCGGCCAACGCAACATGGC(TATTA)TCGAGACGGGTCGGCCGGAGGTGCGGCACAGGACGATCAAGACCATATCC 100
 ATACTATCCAGATCCGCCCTGATTGTCGGCAGTGGTGCACACATCGTTTGTCAACCATACGAAGCATCCGGGGCAGCATCTTCGGCGTCTCATACG 200
 TCTCCCTCTCGGATGAGCATGCTGCATCTCTCGCCGGCATCCATTCTGCCCTCTCGCATCAGCAAGTGTCTGCTCATGCAGCAGCAGCAACACATG 300
 M S M L H L L A G I H S A P L A S A S V C S C T Q Q H M
 AGCATCAGCGTGGATCCGAGAGATGGGCGAGTCTCGCATGCCTGGGCTCAGCGAGAGTTTACAGTCAAGCAGCGTATGGCTCGCTACCCACCAGCCC 400
 S I S V D P R D G Q Q S R M P G L S E S F T V K Q R M A R Y P T A R
 GCGCAGGCGCTTTAAGCCCATCAGCTCACCTGCCACGCTACCCGAGCACTCCAGGCTCCCTGTGAGGCTCGGTGAGAGATATCATCTACAGCC 500
 A G A L S P S A H L A T L P E H S Q A P L S G S V D G E Y H L Q P
 AGAGCGCGCTGGACACTACTCATCGTAGCTCACGCCATTGGCCAGAGCATGAAGACGACGATGCCAGACAAATACTGCTAGCTCGGCGCAT 600
 E R R W T L L I A S S P P L A K T M K T T T M P R Q I L P S S A H
 GATGAAAGATGATATGATCGTCCGATTGAGCGGCGAGCGGAATTCTCCCTTTGGAAGAGCAGCTGGTATCGCGTACTTGAGAGCCCATATGCTCA 700
 D E K Y A Y D R P I Q R Q P E L L P L E E Q L V S R T C R A H M L K
 AGTTGCACGCAACGATGACGACGACGAGGATGAGGCGCGAAGATCCATACCGGTCTTGACGCGTATTCTGCTTTGAGGCGGACAAACGCGCAAGGTA 800
 L H A T M T T T R M R R R R S I P V L T R D S A F G G D N G Q G Y
 TGACCCAACTCTGCTATGGTGGCGCGGTTATGGCTGACTACCGGTGAGTTGGCGATAACCACTTGACACGCAACACTTTGGTCCCGCTCCACGA 900
 D P N S A Y G G A G Y G W T T G Q F G D N H F D T Q H F G P A P R
 CGCGGTCTCAGCTTCGTAGGCACAGCAAGCAAGAACGTACGCCCTCACAAGAAACCTCATTCTGGATTGCCCGTCCGCAAGTTCGACAGCTTC 1000
 R G A Q L R R H K T K K N V R L T K K P H S G L P V P T K L Q T F L
 TCACGCGACGAGCGAAGCAATTCACACGATGGTATTGGCGCGTCACTGCGATCCGAGGATTTGGAGCGATCCTTCACGCTTCGACAGCGCT 1100
 T R R A E D F T T M R Y S A V T C D P T D L E R S F T L P A L
 GTACGGTCGACACAGGAATCTTCATCGCCATCACCATGTACACGAAGACGAGTGCTTTTCTGTCGAATTTCCACGCGGTGATGAAAAACATTGGC 1200
 Y G R H T E L F I A I T M Y N E D E V L F C R T F H G V M K N I A
 CACCTCTGTTCCGCAACAGTCAGCAGATGGGCGAAGATGGCTGGAAAAAGTGGTGGTCCCATCATCTCGGACGGCGCAAGAGATTACCCCTA 1300
 H L C S R N K S R T W G K D G W K K V V V A I I S D G R K K I H P R
 GAGTGTCTGACTGCTCGCGCACTAGGAGTTTACCAAGACGAGTGGCGAAAAACATGGTGGTGGCAAGAGTACGTGCGCATCTTTACGAATACAC 1400
 V L D C L A A L G V Y Q D G V A K N M V D G K E V R A H L Y E Y T
 GACCCAACTTTGATGATTCACCTGCAGTTCAAGGCGCGAAGAGGGCTGGTCCAATGCAGATCATCTTCTGCTTGAAGGAGAAGAACGCGAAA 1500
 T Q L S I D S N L Q F K G A E R G L V P M Q I I F C L K E K N A K
 AAGATCAATTCGATGATGTTCTTCAACGCGTCTGCCCTATCTGCAACCAACGTCACCATCTTGTGGAGGTAGGTACACGACCGGAGACAAGT 1600
 K I N S H R W F F N A F C P I L Q P N V T I L L E V G T R P G D K S
 CGATTACTACCTGTGAAATCGTTGATGTGAATCGAACGTTGCCCGCAATGTGGAGAGATCTGCCCGAGACCAAGGCAATGGGAGTGGGACC 1700
 I Y Y L W K S F D V N S N V A A Q C G E I C A E T K G K W G V G P
 TTTGCTCTAATCCACTCGTAGCAGCGCAGAACTTTGAGTACAGATCAGCAACATCTCGACAAGACTACCGAGAGTGTATGGGATACATCTCGGTG 1800
 L L L N P L V A A Q N F E Y K I S N I L D K T T E S V M G Y I S V
 CTTCCCGGTGATTCCTCGCTTACCGCTACATCGCGCTGCAGACGACGAATTTGCCATGGACCTCGGCTTCTACTTCAAGGCGAAACCTGTTGG 1900
 L P G A F S A Y R Y I A L Q N D E F G H G P L A S Y F K G E N L L G
 GACGTGACGCTGACGCTTTCACATCCAACATGTACTCGCCGAAGATCGAATCTTGTGCTTCGAGTTGGCGCGAAGCTGGGATGGATGGGTGTTGAA 2000
 R D A D V F T S N M Y L A E D R I L C F E L A A K R G H G W V L K
 GTACGTGAATCAGCTCGTGGAGTGACCGATGTTCCCGAGGACTCCCGAATTCATCAGTCAGCGACGTGTTGGTTAAACGGATCCTCACCCAGCTCT 2100
 Y V K S A R G V T D V P E G L P E F I S Q R R R W L N G S S P T S
 TTGGAAGACCCGAGTTCAAGCTCCCGGCTTGGCGTATTCAATGTGTTATGCAGTACATCTATCTCGGTACTGTGGTATCGAGCTTCTCTCGGA 2200
 L E D P T F K L R G I G V F N V F M Q Y I Y L G T V V S S F I F A M
 TGGGTAACCGACCGCAGGGAAGCAAGTGGAGTACTGGGCTGCCGTGCTGTTGCTCTGCTTACCGTGTACATGATGGTCCGCCGCTTGTGTGTT 2300
 G N R P Q G S K W K Y W A A V V V F A L L T V Y M M V A A V L C L
 GAGCAAGTGTGCTCGCTCGAGTAGAACGATGCCATCTATGCTCAGATGGTGGTCTCGCTTCTGGCCAGTACGGAGTGTATCTGATCTCGTGGCTCTC 2400
 S K V V A R V E H D A I Y A Q M V V S L L A T Y G V Y L I S S L L
 GCGTGCATCCTTTGCACCTGATCACGTCGTTCTCAGTACCTTCTGCTTGCACCGACCTACATCAACATCCTCAACATCTACAGCCTTCTGTAACGG 2500
 A C D P L H L I T S F L Q Y L L L A P T Y I N I L N I Y S L L *
 CACGATATGTCCTGGGTACGAAGGGGAAGGCCGAGTGCAACATCTCCGTGACCATGGACTTCGATAAGCGTCCAAAGGGCCATCCAAACTGCCTCGG 2600
 ACGCTAACCATTCGCTCCCGCTGGTGTGATGACGCTCCCTTGTCTTCTGGGTACTAACTCCTGGGTTCTACCGTTTTCATGACCGCTCCAAGGA 2700
 GATCCCTGGCTGTGGAATGTGCGTTGGCACTAGCGGTGGATCTGTGATTATGAAGTGAAGCAGATTAAAGTAACTATAAACGCTGTAACAT 2800
 TATAAACTCTTTTCATCTTATAT 2824

Fig. 1. DNA and deduced amino acid sequence of *Umchs1*. The ORF (2280 nt) is shown with the predicted amino acid sequence (760 amino acids). The putative TATA and polyadenylation signal sequences are boxed. Oligonucleotides used to generate the PCR probe are indicated by arrows.

nitrogen. No significant difference in chitin synthase activity was observed when either method was used. Cell walls were recovered by centrifugation at 2000 *g*, washed successively with

2 M NaCl and water, and freeze-dried. The initial supernatant was centrifuged at 165000 *g* (Rav), and the pellet (mixed membrane fraction) was used as source of chitin synthase. Chitin

TCAAGCTCGATGACGAGCACTAAGTGGACGGTCCAACGCAATATAAAGTACGACCTGACAAAGTTGACGACATCGTTCAATCTGCCCAACAGACCT 100
 CGTACATCGGATCCTTCGATGAGCATGACGGTGATGCGCAACTGATATGCGCCGCTCGACTTTGACGAGCACGGCAACATGTGCGCCCTGTC 200
 M R P P R T L T S T A T M S A L S
 GACAAGCATGCCGATGGAGATTGACGATGTGATGGACGAAGACGCTGTCAACGGCCAGGCGAGAGGCGACTGCTCCACACGCAAGTTCCCAAGATGAC 300
 T S M P M E I D D V M D E D A V N G Q A R G R L L H T Q V P H D D
 GATTACATGCCCGGTGGCTTCGATCCCAACGTGCTCGAAAATGGCCAGGCTGGAGGTGTGCGCTTCGGCAAGATCCCAACGTGTGCCAAGACGATACA 400
 D Y M P G G F D P N V L E N G Q A G G V R F G K I P Q R V P R R Y K
 AGACGCTCAAGAGGGTGAACCTTACCATGGCAACCTCGTCTCGATTGTCCCGTCCCTCCAAAGTGTGACAAGCTCAACGACCGAGAGTCCCGAGA 500
 T L K R V E L Y H G N L V L D C P V P S K L L D K L N D R E S R E
 ATTCACCCATATGCGATACCTGCTGCCACTTGGCCAGGCGAGGTTCAAAACCGAGGTTACACATTGCGACAGGTTCTTTGACCTCTCTGTCGC 600
 F T H M R Y T A A T C D P D E F K T E R Y T L R Q V L F D P P R R
 ACAGAGCTGTTATAGTTCTCACCATTGTATAACGAGGACGAAGAGCTTTTACGCGCACCATGACGGAGTCATGACCAACATCGCCCATCTTTGTACTC 700
 T E L F I V L T M Y N E D E E L F T R T M H G V M T N I A H L C T R
 GAGAGCGCTCAAGACCTGGGGCAAGGCTGGAAGAAAGTCGTCTGCTGCATGCTCGGACGGCGCTCTCAAGATCAACTCGCGACCTTGGCGTG 800
 E R S K T W G K E G W K K V V V C I V S D G R L K I N S R T L A C
 CTTGCTCGGATGGGAGTGTACCAAGAGGGCGGTGGGAGAACGTCGTCAATGGCAACCCGCTCACTGCTCACATCTACGAATACACAGCTCAGCTCTCG 900
 L A A M G V Y Q E G G G Q N V V N G K P V T A H I Y E Y T A Q L S
 ATCGATCTAGCATGCACTTCAAGGGTGGGAGGACATGACCCGTCAAATTTCTCTTCTGCTCAAGGAGCGCAACGAAGAAGATCAACTCGCACCC 1000
 I D P S M H F K G R E G I M P V Q I L F C L K E R N Q K K I N S H R
 GTTGGTTCTTCAACGCTTTCGGCCAGATCTTGACGCCCAACATCTGCTACTGCTTGTGATGAGGAACCATGCCAAGACCTCGCTCCATTACCATTCTG 1100
 W F F N A F G Q I L Q P N I C V L L D V G T M P R P R S I Y H L W
 GAAAGCCTTGACATCAACTCCAACGTCCGGGTTCTGTGGTGAGATTGTGCACTCAAGGGCAAAATTTGGGGGGCACTGCTCAATCCACTGTCGCG 1200
 K A F D I N S N V A G S C G E I V A L K G K F W G A L L N P L V A
 GCGCAAAATTTGAGTACAAGATGTCCAACATTCTCGACAAGCGGTGGAGTCGCTTGGTACATCACGGTGCTTCAGGTGCCTTCCCGCGTACC 1300
 A Q N F E Y K M S N I L D K P L E S V F G Y I T V L P G A F S A Y R
 GCTACATTGCGTTACAAAACGATGCTCAGCGTCAAGGGCCCTTGTGCTCGTACTTTAAGGGTGAACATTACACGGTGGCCAGTGGACGACGACGCTT 1400
 Y I A L Q N D A H G Q G P L C S Y F K G E T L H G G Q S D A D V F
 TACGTGCAACATGTATCTTGGCCAGGATCGCATCTTGTGTTGGGAGTAGTGTGGAAGCAGACAGCGCTGGATCTTGCAATTGTCAAGTGTGCCCAA 1500
 T S N M Y L A E D R I L C W E L V S K R D S A W I L H Y V K S A Q
 GCAGTCACGGATGTGCCGACCAAGTTCCCGAATGATCTCGCAACGCCGTGTTGGCTTAACAGGATCCATTGCGCGCGCTCTACGCCCAACTTCACT 1600
 A V T D V P D I S Q R R W L N Q D P F A A L Y A Q L H F
 TCTAAGGTCTGGACAGCAAGCACTGTTGCGCCGAAAGATATTCTCCAGGTGCAATTCCTACAGTTTATACAACTGCTATTCTCTGGTCTC 1700
 Y K V W T S K H S F G R K I F L Q V E F L Y Q F I Q L L F S W F S
 GATCGCAAGCTTCGTCTGACATTGAGAATCATCAGCACTCTTCGACCCAGGCGCAAGTGGACGAAGTGGCTCTTTCAACCTGAATCCTAACAAG 1800
 I A S F V L T F R I I T T S S T P G P K C D E A G S F N L N P N K
 GAGACTCTTTCGCTCTTCTCGAGCTCTACGAGACATTGATCCCATCAATCGCGGTTGCGTCTATCTTGGTAGTCTGTCCATGCTCTCAAGGCGCAAA 1900
 E T S S L L L E R L R D I D P I N R R C V Y L G S L S M L L K A Q S
 GCAAGACGCTCGAGCTGAGCCTCTTCTCAGGCGGGGCGTCTGCTCGGCTCGACATTGACTCGGCGGCGAAGTTGTTGATTTGATGATGGGAGAG 2000
 K T L E L Q P L L R P G R L V S A S T L T R P A K L F V L V W E S
 CGAGAACCCAGCTCAACGGTGGCCCGCAAGCTGCTCATCTCTGGTTTGACGCTCGGAGGCGACCTTGTGATTTCTCAACCAATTGACGCTGG 2100
 E N P A S T V P P R S S S S P G L T P A E A T L L Y S S T N S T W
 TCGCTTCAAGCACATGAAGCAGCACTTCTCAGCATCTTGAGACGCGTCCGGCTCTGCTCGCTCAGGTCTCGTTGATTCCGACGAATCAAGTCGCC 2200
 S L P S T S K Q H F S A S W R R V R L S S L R S R S I P T N S S R P
 CGATAAGTCGAGGACTTTCAGGTGAGCAAGCAAAAGTACTGGCGTCCAATCGAAGTCCCCAACGACGAGAAACCACTGGGTCAATTTTCATC 2300
 I K S R T F R S A R N K R Y W R P N R R S P T T R K P R G S F S S
 TTGGTCGCAACTGGTCAATGGGAACTCTCACCTTCATGTCTGCTGGCTCTCGCCGCTCCATAGGCTCGATCTACCTCATGAAGACCGCGTAACGAC 2400
 W S Q L V N G K L S P S C P G A L S P A P *
 GTTGAACCATGGACTTAGCATAGCACATATGCACAGTGGCTCGGTAATAACACTGACTAAGCCGT 2468

Fig. 2. DNA and deduced amino acid sequence of *Umchs2*. The ORF (2214 nt) is shown with the predicted amino acid sequence (738 amino acids). The putative TATA and polyadenylation signal sequences are boxed. Oligonucleotides to generate the PCR probe are indicated by arrows.

synthase activity was measured by the filtration method originally described by Ruiz-Herrera & Bartnicki-Garcia (1976) in 50 μ l 50 mM MES or potassium phosphate buffers of pH 6.5, containing 0.25 M sucrose, 10 mM $MgCl_2$ or $MnCl_2$, 40 mM *N*-acetylglucosamine (GlcNAc), 0.5 mM $UDP[^{14}C]$ GlcNAc (sp. act. 18 mCi $mmol^{-1}/6.66 \times 10^8$ Bq $mmol^{-1}$), and where indicated

40 μ g trypsin ml^{-1} . Activity was expressed as nmol GlcNAc incorporated by 1 mg protein in 1 min. For chitin measurement, cell walls were hydrolysed with 6 M HCl, and glucosamine was measured by the Elson-Morgan method as described by Dische (1962), after HCl evaporation at room temperature under vacuum. Glucosamine content was related to wall neutral sugars

Fig. 3. Multiple amino acid sequence alignment of CHS genes deposited in GenBank as derived by maximal homology with GeneWorks software. Comparison of the sequences at that position among all sequences, gaps are indicated by hyphens. Roman numerals indicate conserved domains (see Table 1). Abbreviations: NcCHS1, *N. crassa* CHS1; NcCHS2, *N. crassa* CHS2; AnCHSA, *A. nidulans* CHSA; AnCHSB, *A. nidulans* CHSB; ScCHS1, *S. cerevisiae* CHS1; ScCHS2, *S. cerevisiae* CHS2; CaCHS1, *C. albicans* CHS1; CaCHS2, *C. albicans* CHS2; RoCHS1, *R. oligosporus* CHS1; RoCHS2, *R. oligosporus* CHS2; UmCHS1, *U. maydis* CHS1; UmCHS2, *U. maydis* CHS2.

measured in 2 M HCl hydrolysates with anthrone (Dimler *et al.*, 1952), to have an internal standard to compare the samples.

For the determination of virulence, 10-d-old corn plantlets were injected with 0.2 ml of a suspension of a mixture of *U. maydis*-compatible mating strains (10^8 cells ml^{-1} ; five plants per mixture). Plants were incubated in a greenhouse and observed at intervals for the appearance of the galls, characteristic of the disease, which normally occurred after 10–20 d.

RESULTS

Isolation of PCR products

Using the procedure and primers described in Methods, a major band of about 600 bp was obtained and cloned. Restriction analysis of different clones containing the fragment showed three different restriction patterns,

suggesting that they corresponded to more than one PCR product. This was confirmed by Southern analysis of *U. maydis* DNA digested with different restriction enzymes probed with one of the PCR fragments; in all cases two bands were observed. Sequencing of the two putative different PCR products showed ORFs whose predicted amino acid sequences displayed high similarity with both *CHS1* and *CHS2* gene products of *S. cerevisiae* (not shown), but displayed differences with those reported by Bowen *et al.* (1992) for *U. maydis* (see below).

Cloning and sequencing of two *CHS* genes

Using one of the inserts as a probe, we screened a genomic library of the fungus. Twenty positive signals were obtained out of 40 000 recombinant phages screened. The phages were isolated, amplified, and their DNA was isolated, digested with different restriction enzymes and subjected to Southern analysis. Phages 7 and 12 were selected as representatives of the two different *CHS* genes, and their restriction fragments were subcloned for further analysis.

Umchs1 gene

The structural gene and the regulatory sequences were contained in plasmids pi6, p7-1 and pii6. Fig. 1 shows the *Umchs1* nucleotide and amino acid deduced sequences. The sequence shows a single continuous ORF, encoding a polypeptide of 760 amino acids and a molecular mass of 85 106 Da. The predicted amino acid sequence of *Umchs1* is 26 and 30% similar to *S. cerevisiae* *CHS1* and *CHS2*, respectively. No consensus splicing sequences occur in this gene. The non-translated 5' and 3' regions were analysed for consensus regulatory sequences. A putative TATA box is present at position 43. A consensus polyadenylation signal was also identified.

Umchs2 gene

The *Umchs2* gene was contained in p12, pb5 and pa7 plasmids. The complete DNA and deduced amino acid sequence appears in Fig. 2. A continuous ORF of 738 amino acids defining a polypeptide with a theoretical molecular mass of 83 520 Da is shown. The predicted amino acid sequence of *Umchs2* is 27 and 35% similar to *S. cerevisiae* *CHS1* and *CHS2*, respectively. In the 5' non-translated region a putative TATA box is present, whereas a polyadenylation signal at the 3' end was identified.

Sequence comparison of reported chitin synthase genes

As indicated above, we noticed differences in the sequence of the PCR products synthesized by Bowen *et al.* (1992) and the corresponding fragments of *Umchs* genes reported here. At the amino acid level, similarities are of the following order: *chs1* PCR product vs *Umchs1*, 58%; and *chs2* PCR product vs *Umchs2*, 61%.

Table 1. Conserved domains in chitin synthase gene products

Domain*	Sequence
I	T(M/Y)YNED
II	FCLK(Q/E)XNXKK(I/L)NSHRW
III	NPLV(A/Y)XQNFYKXSNILDK
IV	VLPGA(F/L)SAYR
V	NM(F/Y)LAEDRILC
VI	TDVP
VII	QRRRW(L/I)N(G/Q)

* Domains are numbered starting at the amino terminus.

Using GeneWorks software (Intelligenetics, release 2.3) we made a comparison of the deduced amino acid sequences of *U. maydis* genes with those previously reported (Fig. 3). The amino acid sequences of the *Umchs1* and *Umchs2* gene products show homology with products of the following genes: *Rhizopus oligosporus* *CHS* (unpublished results from T. Motoyama, GenBank accession nos D10159 and D10160); *C. albicans* *CHS1* (Au-Young & Robbins, 1990); *C. albicans* *CHS2* (Chen-Wu *et al.*, 1992); *S. cerevisiae* *CHS1* (Bulawa *et al.*, 1986), *S. cerevisiae* *CHS2* (Silverman *et al.*, 1988); *Neurospora crassa* *CHS1* (Yarden & Yanofsky, 1991), *N. crassa* *CHS2* (Beth Din & Yarden, 1994); and *Aspergillus nidulans* *ChsA* and *ChsB* (Yanai *et al.*, 1994). This similarity is located in the central region of the genes, whereas the amino and carboxy termini showed only isolated conserved amino acids (not shown). Both proteins appear to be hydrophobic, mainly towards their carboxy termini. Analysis of possible modification motifs revealed several putative phosphorylation sites in both *Umchs1* and *Umchs2*; and two putative sequons in *Umchs1* (data not shown).

According with the homology displayed, we have identified seven domains comprising at least four amino acids in all the sequences (Table 1). At least part of domain III and whole of domain VII are shared by *CSD2* genes that have been cloned (Ruiz-Herrera & Xoconostle-Cázares, 1995). With the sequence data a dendrogram of grouping of fungal chitin synthases was constructed (Fig. 4).

Expression of *Umchs1* and *Umchs2* genes during dimorphism

We determined the abundance of *Umchs1* and *Umchs2* transcripts as a measure of the expression of both genes. Accordingly, Northern blot hybridization of total RNA obtained from the yeast or mycelial phases of the fungus was performed. Two non-homologous fragments, a 232 bp *Bam*HI–*Xho*I fragment, and a 263 bp *Bam*HI–*Xho*I fragment, located towards the 3' region of *Umchs1* and *Umchs2*, respectively (see Fig. 6), were used as specific probes. A *Bgl*II fragment corresponding to the actin gene of *Trichoderma reesei* was used as a control. Our data (Fig. 5) indicate that *Umchs1* transcripts are more abundant than *Umchs2*, and that the transcript levels from both

Fig. 4. Maximal parsimony dendrogram showing fungal chitin synthase grouping. The tree was calculated with GeneWorks software. Numbers in the branches are related to the differences between groups; numbers in parentheses show the SEM in the branching. Abbreviations are as in the legend to Fig. 3.

Fig. 5. Northern blot analysis. Total RNA was isolated from 24-h-old yeast or mycelium forms. (a) Blot was probed with the 232 bp *Bam*HI-*Xho*I fragment of *Umchs1*. (b) Blot probed with the 263 bp *Bam*HI-*Xho*I fragment of *Umchs2*. (c) Blot was re-probed with the *T. reesei* actin gene. Y, Yeast RNA; M, mycelium RNA.

genes (but especially *Umchs2*), are higher in the mycelial stage of *U. maydis*.

Gene replacement of *Umchs* genes

The method for construction of the vectors for gene replacement is shown in Fig. 6. Cell transformation with the linear *Bam*HI fragments containing the replacement cassettes was performed by electroporation as described in Methods. Transformants were recovered in complete

medium containing 1 M sorbitol and 400 μ g hygromycin B ml⁻¹. Gene replacement for both *chs1* and *chs2* mutants was confirmed by Southern analysis. Fig. 7 shows the results for FB2 strains. Identical results were obtained with FB1 (not shown).

Phenotypic analysis of interrupted mutants

In synthetic medium of pH 3, which induces mycelial development, growth of both mutants was reduced as compared to wild-type (Table 2). Addition of 1 M sorbitol did not restore normal growth (not shown). At pH 7, growth was not affected. Cultivation in complete medium did not restore normal growth at pH 3.0. All mutants inoculated in synthetic or complex media of pH 7 grew as normal yeasts; whereas in synthetic or complex medium of pH 3 they grew in mycelial form (Ruiz-Herrera *et al.*, 1995). All *chs* mutants displayed Fuzz colonial phenotype on charcoal-containing synthetic medium of pH 3.0.

Gene replacement had no effect on mating or virulence. Mating, as determined by the Fuzz reaction, was normal when the following pairs of strains (one *a*₁ *b*₁, the other *a*₂ *b*₂) were mixed: *chs1* × wild-type; *chs1* × *chs1*; *chs1* × *chs2*; *chs2* × wild-type; and *chs2* × *chs2*. All these mixtures were able to induce tumour formation in corn plants.

Determination of chitin synthase activity of wild-type and *chs* mutants grown in the yeast form at pH 7, or in the mycelial stage at pH 3 was performed as described in Methods. Two metal cations, Mg²⁺ or Mn²⁺, and two buffers, MES or phosphate, were used. Higher activity was obtained with MES buffer in the presence of Mg²⁺,

UmCHS1 *UmCHS2*

Fig. 6. Strategy for *Umchs* genes replacement. An internal conserved *Umchs* fragment was replaced by the hygromycin-resistance gene. For details see text. Thin line is the pBluescript KS(–) vector. Restriction enzyme abbreviations: B, *Bam*HI; H, *Hind*III; N, *Nru*I; S, *Sal*I; X, *Xho*I.

but only when extracts from the yeast forms were tested; activity of mycelial extracts was very low (not shown). When phosphate and Mg^{2+} were employed, activity in yeast or mycelial extracts was rather similar. Activity in the *chs* mutants was lower than wild-type, but only when the extracts from the mycelial stage were analysed in the absence of trypsin; activity of the yeast cells with or without trypsin was about equal (Table 3). Addition of trypsin to the reaction mixtures decreased activity. This result contrasts with that for most fungi (Ruiz-Herrera, 1992).

Chitin levels in the cell wall were reduced in the yeast and mycelial forms of *chs2* mutants, and slightly reduced in the mycelial form of *chs1* strains (Table 4).

Fig. 7. Southern blot hybridization of *chs* mutants. (a) *chs1* mutants. Lane 1, DNA from wild-type strain FB2; lanes 2–4, DNA from mutants. (b) *chs2* mutants. Lane 1, DNA from wild-type strain FB2; lanes 2–5, DNA from mutants. DNA was digested with *Bam*HI, electrophoresed and blotted onto a nylon membrane. Blot was probed with the fragment of 1.7 kb *Bam*HI from p7-1 for *Umchs1*, and with the 1.5 kb fragment from p12-2 for *Umchs2*.

Table 2. Comparison of growth of *chs1* and *chs2* mutants with wild-type in synthetic and complete medium

Inoculum was prepared in hygromycin-free medium. Cells were washed four times in water and inoculated into fresh medium as described in Methods to obtain yeast or mycelial growth in media of pH 7.0 or 3.0, respectively. Inoculum was of the order $15\text{--}20\ \mu\text{g protein ml}^{-1}$ for all strains. Data are expressed as the ratio of growth between each mutant and wild-type, and are means $\pm$ SEM of four experiments.

Medium	pH	Time (h)	Growth ratio	
			FB2 <i>chs1</i>	FB2 <i>chs2</i>
Synthetic	7.0	18	1.02 ± 0.30	1.21 ± 0.09
		24	1.01 ± 0.06	0.90 ± 0.09
		48	1.06 ± 0.06	1.05 ± 0.06
	3.0	18	1.02 ± 0.10	0.96 ± 0.06
		24	0.66 ± 0.08	0.68 ± 0.08
		48	0.54 ± 0.04	0.69 ± 0.10
Complete	7.0	18	2.19 ± 0.50	0.95 ± 0.03
		24	1.10 ± 0.08	0.95 ± 0.02
		48	1.00 ± 0.06	1.05 ± 0.03
	3.0	18	0.96 ± 0.05	1.18 ± 0.05
		24	0.55 ± 0.13	0.48 ± 0.10
		48	0.60 ± 0.03	0.66 ± 0.04

DISCUSSION

We have cloned and characterized two *chs* genes from *U. maydis*, which by their analogy to *S. cerevisiae* genes we have designated *Umchs1* and *Umchs2*. They probably

Table 3. Chitin synthase activity levels in *U. maydis* wild-type and *chs* mutants

Mixed membrane fraction was obtained from cells grown in complete medium at pH 7.0 (yeasts) or pH 3.0 (mycelium). Chitin synthase activity was measured as described in Methods in phosphate buffer in the presence of Mg^{2+} . Data are expressed as nmol GlcNAc incorporated min^{-1} (mg protein) $^{-1}$. Data are means $\pm$ SEM of two determinations from three different extracts.

Stage	Trypsin	Specific activity		
		FB2	CHS1	CHS2
Yeast	No	21.1 $\pm$ 2.6	20.7 $\pm$ 1.5	20.3 $\pm$ 0.8
	Yes	11.9 $\pm$ 1.4	14.2 $\pm$ 1.2	13.6 $\pm$ 1.7
Mycelium	No	21.6 $\pm$ 3.2	12.9 $\pm$ 1.3	14.2 $\pm$ 1.0
	Yes	12.6 $\pm$ 1.3	11.0 $\pm$ 1.3	13.0 $\pm$ 1.0

Table 4. Relative content of hexosamines and neutral sugars in the cell walls of *U. maydis* wild-type and *chs* mutants

Sugars were measured in walls obtained from yeast or mycelial cells obtained as described in Methods. Data are means $\pm$ SEM of four different batches of cell walls.

Strain	Ratio hexosamines/neutral sugars	
	Yeast	Mycelium
FB1	0.19 $\pm$ 0.011	0.29 $\pm$ 0.028
FB1 <i>chs1</i>	0.18 $\pm$ 0.017	0.19 $\pm$ 0.020
FB1 <i>chs2</i>	0.13 $\pm$ 0.022	0.14 $\pm$ 0.008
FB2	0.23 $\pm$ 0.020	0.21 $\pm$ 0.002
FB2 <i>chs1</i>	0.23 $\pm$ 0.023	0.19 $\pm$ 0.003
FB2 <i>chs2</i>	0.13 $\pm$ 0.013	0.15 $\pm$ 0.015

correspond to the PCR fragments previously isolated by Bowen *et al.* (1992) from this fungus, and designated *chs1* and *chs2* by Gold & Kronstad (1994), who used them for gene disruption. The differences observed in the sequence of the PCR products obtained by those authors and the whole genes is probably due to the different strains employed. Further work (unpublished) has demonstrated the existence of a *CSD2* gene in *U. maydis*. Accordingly, *U. maydis* follows the rule of other fungi, *S. cerevisiae* (Bulawa *et al.*, 1986; Silverman *et al.*, 1988; Bulawa, 1992, 1993; Valdivieso *et al.*, 1991) and *C. albicans* (Au-Young & Robbins, 1990; Chen-Wu *et al.*, 1992; Sudoh *et al.*, 1993), where the presence of both kinds of chitin synthase genes has been reported.

Comparison of the deduced amino acid sequences corresponding to both *CHS* genes of *U. maydis*, with those cloned from other sources revealed that they have significant homologies in the central part of the molecules

where seven highly conserved domains were identified. However, the amino and carboxy termini of *CHS* genes have no homology at all. It may be anticipated that the central region of the protein contains the domains corresponding to the active site of the enzyme, and possibly other regulatory domains. Our data on the conserved domains and the comparison dendrogram add further information to data described by Bowen *et al.* (1992) and Beth Din & Yarden (1994). The most important difference between the analysis made with short PCR products (Bowen *et al.*, 1992) and with the whole protein sequence (this study) is the disappearance of the originally described three *CHS* classes, and the early divergence of two branches. As described here, *CHS1* from *S. cerevisiae* appears as the least homologous *CHS* protein. Homology of many *CHS* gene products does not appear to coincide with the accepted phylogenetic relationships of the fungi analysed. This inconsistency is difficult to explain unless ancient evolution of *CHS* genes and convergence are invoked. It is interesting to note the high number of putative phosphorylation sites existing in both enzymes, mainly *CHS1* product. Whether they are involved in regulation of enzymic activity, remains to be determined. As described above, *CHS* gene products are activated *in vitro* by proteolysis (Bulawa, 1993).

Both genes were expressed in the yeast or mycelial forms of the fungus. We found a higher level of transcript for *Umchs1*, as compared to *Umchs2* in both mycelial and yeast-like stages, and increased levels of both transcripts in the mycelial phase, mainly for *Umchs2*. Gold & Kronstad (1994) did not observe significant differences in the expression of either gene between haploids and mating cells which grow in filamentous form. This is probably due to the different protocols employed for mycelial growth induction, and the fact that we used haploid cells.

The observation that *Umchs1* and *Umchs2* are not essential agrees with the earlier report from Gold & Kronstad (1994), who interrupted either gene with the corresponding PCR product. Our results on the null effect of *chs* gene substitution on mating and virulence also agree with the data obtained by those authors. These results are similar to the observation that both *CHS1* and *CHS2* from *S. cerevisiae* are dispensable genes (Bulawa *et al.*, 1986; Bulawa & Osmond, 1990). The levels of transcripts of *Umchs1* and *Umchs2* were different in the yeast or mycelial forms of *U. maydis*; similar (although more pronounced) differences were reported for *C. albicans* (Chen-Wu *et al.*, 1992). But as reported in this human pathogen (Gow *et al.*, 1993), gene replacement in *U. maydis* did not affect the dimorphic transition induced by incubation at acid pH. All these data suggest that either the function of both genes is interchangeable, or that a different chitin synthase, possibly the one encoded by the *CSD2*-like gene, is responsible for the synthesis of most of the chitin present in the cell wall, as is the case in *S. cerevisiae* (Valdivieso *et al.*, 1991; Bulawa, 1992).

Although neither gene is essential, their substitution brought about some minor changes in the physiology of the null mutants, especially in the case of mycelial cells. Substitution of either *Umchs1* or *Umchs2* provoked a

reduction in growth rate of both mutants at pH 3. Growth alteration does not appear to be due to an additional auxotrophy, since it was observed in synthetic or complex media. Besides, growth at pH 7 in synthetic medium was almost unaffected and different isolates behaved similarly. In the case of *S. cerevisiae*, disruption of *CHS1* did not affect cell growth or morphology, but growth alterations in synthetic media were recorded (Bulawa, 1993). *U. maydis* mutants carrying either gene substitution displayed a reduction in the levels of chitin synthase activity in mycelial cells, as compared to wild-type. An additional change in *chs2* mutants was a reduction in the chitin content of the cell wall of both mycelium and yeast cells. Although minor, these changes suggest that some of the roles played by either gene product in cell wall construction can not be completely compensated by the non-affected gene products.

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