CHS2, a chitin synthase gene from the oomycete *Saprolegnia monoica*

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PCR was used to amplify fragments corresponding to the chitin synthase (*CHS*) genes from the Oomycetes *Saprolegnia monoica*, *Phytophthora capsici* and *Achlya ambisexualis*, utilizing as primers, oligonucleotides designed from the conserved region of *CHS* genes of chitinous fungi. Chitin synthase homologues were found in the three cellulosic fungi. The chitin synthase 2 gene (*CHS2*) from *S. monoica* was cloned, sequenced and characterized. The amino acid sequence deduced from the *CHS2* genomic DNA revealed several domains, corresponding to the catalytic domains and polypeptide signatures, of high identity with *CHS* genes from chitinous fungi. Existence of a *CHS* gene family in *S. monoica* was supported by the identification of two *CHS* sequences among the PCR products, the localization of *CHS* homologues on two chromosomes, and the detection of two transcripts in mycelia and protoplasts. Polyclonal anti-chitin synthase antibodies raised against the N-terminal and the neutral fragments of the *CHS2* products revealed, respectively, two and four proteins in membrane fractions and a truncated active form in entrapped product. The overall comparison of the structure and organization of *CHS* genes indicates that in spite of their divergent evolution, Oomycetes and chitinous fungi have evolved with conserved chitin synthase systems.

Keywords: *Saprolegnia monoica*, Oomycetes, cell wall, chitin synthases, immunodetection

INTRODUCTION

In filamentous fungi, chitin is the supporting component of the cell wall while, in yeasts, it is a relatively minor component used to maintain the structure of the mother bud junction.

Chitin synthases have been characterized from a wide range of fungal species (Cabib, 1987; Bartnicki-Garcia *et al.*, 1978). Most preparations are zymogenic, that is, they are produced as inactive proenzymes that are activated by proteases, but so far, no chitin synthase polypeptides have been purified to homogeneity. The genetics and molecular biology of chitin synthase are providing increasing information (for a review see Bulawa, 1993). Three chitin synthase genes of *Saccharomyces cerevisiae* have been characterized. Two of them, *CHS1* and *CHS2* encode protease-activated zymogen

while *CHS3*, encoded by *CAL1/CDS2*, does not require proteolytic activation. Their roles have been studied in gene disruption experiments. Each chitin synthase has a different role in yeast morphogenesis: *CHS3* is involved in the formation of the chitin ring during budding, *CHS2* catalyses the formation of the central layer of the primary septum (Shaw *et al.*, 1991), and *CHS1* acts as a repair enzyme (Cabib *et al.*, 1989).

The analyses of chitin synthase genes of filamentous fungi have not been as comprehensive as in yeast. Conserved regions of *CHS* genes from several filamentous fungi have been sequenced, establishing the widespread distribution of the enzymes in chitinous fungi (Bowen, 1992; Mehmman *et al.*, 1994). Analysis of DNA fragments from taxonomically diverse fungal species have shown that most fungi have three to six chitin synthase genes (Mellado *et al.*, 1995; Din *et al.*, 1996; Karuppayil *et al.*, 1995). However, there is little information on the function of the gene products during growth of filamentous fungi. Inactivation of *Neurospora crassa chs1* and of *Aspergillus nidulans chsB* induced morphological abnormalities of the hyphae (Yarden &

Abbreviation: RT-PCR, reverse transcriptase polymerase chain reaction.
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Yanofsky, 1991; Yanai *et al.*, 1994). In contrast, there was no apparent morphological consequence of the inactivation of *N. crassa chs2*, *A. nidulans chsA* and *Ustilago maydis chs1* and *chs2* as the mutants retained the wild-type morphology (Din & Yarden, 1994; Yanai *et al.*, 1994; Gold & Kronstadt, 1994; Xoconostle-Cazares *et al.*, 1996). These results suggest that redundant genes must exist.

Fungi have been subdivided into categories based on dual combination of the principal cell wall polymers. Oomycetes were classified as a cellulose-glucan group while higher fungi were considered as chitin-glucan groups (Bartnicki-Garcia, 1968). The Oomycetes have been grouped with the true fungi but comparison of several biochemical and morphological characteristics and molecular analyses indicate that the fungi are polyphyletic (Cavalier-Smith, 1987; Gunderson *et al.*, 1987; Barr, 1992). Oomycetes can be considered as pseudofungi that have evolved from heterokont algae (Cavalier-Smith, 1986). *Saprolegnia monoica*, which belongs to the class Oomycetes, has been traditionally regarded as a cellulosic fungus. In a previous report, we demonstrated the presence of chitin in the cell wall of *S. monoica* by chemical extraction of the polymer and its identification by X-ray and electron diffraction patterns and infrared spectroscopy (Bulone *et al.*, 1992). Like enzymes from chitinous fungi, solubilized chitin synthase from *S. monoica* is zymogenic, activated by trypsin and catalyses *in vitro* synthesis of spindle-like crystals (Gay *et al.*, 1992, 1993).

The aim of the present study was to undertake a detailed characterization of chitin-synthase-encoding genes from this cellulosic pseudofungus, to compare them to corresponding genes from true fungi and to extend the comparison of the chitin synthase system between cellulosic and chitinous fungi. We report the presence of two *CHS* genes in *S. monoica*, their localization on two chromosomes, the isolation of a *CHS* gene, its nucleotide sequence and the occurrence of *CHS* genes in two other Oomycetes, *Achlya ambisexualis* and *Phytophthora capsici*. Polyclonal antibodies, raised against a recombinant truncated Chs2 protein, revealed more than one protein of high molecular mass, confirming the presence of a *CHS* gene family in this cellulosic fungus.

METHODS

Strains. *Saprolegnia monoica* Pringsheim no. 53967 Dick, obtained from CBS Baarn, The Netherlands, and *Phytophthora capsici*, obtained from Dr P. Nodet (University of Brest, France), were routinely grown on potato dextrose agar (PDA). Liquid cultures were grown in Petri dishes containing 100 ml liquid medium (Machlis, 1953) inoculated with mycelial disks cut from the margins of 4-d-old colonies.

Cloning strategy. Oligonucleotides corresponding to the conserved regions of *CHS* from yeasts, designed by Bowen *et al.* (1992), were synthesized and used as primers for PCR (Table 1). PCR amplification of genomic DNA was carried out in 100 µl volumes, containing 1 U *Taq* DNA polymerase, 50 µM of each dNTP, 50 mM Tris/HCl pH 9.0, 16 mM (NH₄)₂SO₄, 50 mM KCl, 7 mM MgCl₂, 0.2 mg BSA ml⁻¹,

200 ng genomic DNA and 100 pmol of each primer. Thirty-five cycles of PCR were done on the genomic DNA with denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 3 min, followed by one final cycle at 72 °C for 10 min. The PCR products were analysed by electrophoresis on 1.5% (w/v) agarose gels and visualized by UV transillumination after staining with ethidium bromide. The PCR bands were excised, purified with glass milk and subcloned into the pUC18 vector using the *Hind*III and *Sal*I restriction sites engineered at the end of the primers. A genomic library of *S. monoica* constructed in the phage λEMBL3 (V. Bulone, unpublished) was screened using as a probe the *CHS2* PCR product labelled by random priming. Positive clones were purified and amplified. Fragments of the insert were subcloned in pUC18 for further analysis.

Nucleotide sequences were determined using the dideoxy chain-termination method (Sanger *et al.*, 1977) on double-stranded DNA using either universal primers or oligonucleotide primers to yield complete sequences from both strands.

Nucleic acid isolation and hybridization. DNA from *S. monoica* and from *P. capsici* was isolated according to the procedure of Raeder & Broda (1985). Southern hybridizations using Hybond-N membranes (Amersham) were as described by Sambrook *et al.* (1989). Total RNA was extracted by the acid guanidine thiocyanate procedure (Chirgwin *et al.*, 1979). RNA was separated in formaldehyde/agarose gels, transferred to Hybond-N+ membranes (Amersham) and hybridized with the radiolabelled probe (Sambrook *et al.*, 1989).

RT-PCR. Detection of RNA transcripts was carried out using reverse-transcriptase polymerase chain reaction (RT-PCR) (Shuldiner *et al.*, 1991). The first strand cDNA synthesis reactions (RT reaction) were performed on 1 µg denatured RNA which had been treated with DNase I (Boehringer Mannheim) in the presence of 1 M sodium acetate and 10 mM MgSO₄ for 4 h at 37 °C. The RNA was mixed with 1 mM of each dNTP, 20 U RNasin Ribonuclease Inhibitor (Promega), 50 mM KCl, 20 mM Tris/HCl pH 8.4, 2.5 mM MgCl₂, 1 mg BSA ml⁻¹, 100 pmol oligonucleotide-3' and 200 U Moloney Murine Leukemia Virus (MMLV)-Reverse Transcriptase (Promega), and incubated at 37 °C for 60 min followed by a 10 min incubation at 95 °C in order to inactivate the enzymes. Half of the RT reaction (10 µl) was used for PCR performed in the same buffer with 0.2 mM of each dNTP, 50 pmol of each oligonucleotide 5'-3' and 1 U *Taq* polymerase. Thirty-five cycles of PCR were done at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min. The primers used were derived from the coding sequences surrounding two introns, allowing cDNA-derived PCR products to be distinguished from those which could be derived from the genomic DNA (Table 1).

Chitin synthase extraction and glycerol gradient purification.

The mycelium, collected from liquid culture, was homogenized at 4 °C in extraction buffer (10 mM Tris/HCl pH 7.4, 500 mM Sorbitol) with a blade homogenizer (Virtis 45) for three 30 s periods. The homogenate was centrifuged at 2000 g for 10 min and the supernatant was then centrifuged at 48000 g for 30 min at 4 °C. The mixed-membrane fraction (48000 g pellet) was resuspended in extraction buffer and the membrane-bound enzymes were solubilized with digitonin (10 mg ml⁻¹) at 4 °C for 30 min. The detergent extract was centrifuged at 48000 g for 30 min and the supernatant was used as the solubilized enzyme preparation. The solubilized enzymes (2 ml sample) were layered onto a 30 ml 12–45% (v/v) glycerol gradient (in 10 mM Tris/HCl pH 7.4). The gradient was centrifuged for 4 h at 120000 g in a Beckman

Table 1. Synthetic oligonucleotides used for PCR experiments

Oligo position*	Use	Sequence†
2230 } 2804 }	CHS fragments for polyclonal antibodies	5' CTGCCATGGCGTATATGTGCAAGGAG 3' <i>NcoI</i>
		5' CGCGGATCCCTTGACAGCACGGATGGC 3' <i>BamHI</i>
1330 } 1715 }	CHS fragments for polyclonal antibodies	5' CGCGGATCCGCACGCCTCCGCGCCCTG 3' <i>BamHI</i>
		5' CGCGGATCCGTTGGCTTGCCGTCC 3' <i>BamHI</i>
1610	RT-PCR	5' ACACCTTGGTCCAGC 3'
1977	RT-PCR	5' GCGATCTTGAGCTGC 3'
		T G C C C A
2163	PCR	5' CTGAAGCTTACAATGTATAATGAG 3' <i>HindIII</i>
		C A C A A T
2697	PCR	5' GTTCTCGAGTTTGTATTCTGAAGTTCTG 3' <i>XhoI</i>

* Position of primers was based on the genomic nucleotide sequence presented in Fig. 3.

† Multiple lettering indicates oligonucleotide mixtures. Restriction sites for cloning PCR products are underlined.

SW27 rotor at 4 °C. Fractions (1.5 ml) were collected and assayed for chitin synthase activity.

Chitin synthase assay. Membrane-bound or solubilized enzymes (100 µg protein) were assayed in 10 mM Tris/HCl pH 7.4 with 0.5 mM UDP-GlcNAc, 0.5 nCi (18.5 Bq) UDP-[¹⁴C]GlcNAc (Amersham, 8.7 GBq mmol⁻¹), 20 mM GlcNAc, 10 mM MgCl₂ and 2.5 µg trypsin in a total volume of 200 µl. The assay mixture was incubated at 25 °C for 30 min and the reaction was stopped with 2 ml 95% (v/v) ethanol. The reaction mixture was filtered through a 2.5 cm diameter glass fibre filter (GF/C, Whatman) and washed successively with 5 ml 0.5 M NaOH, 2 × 10 ml 95% (v/v) ethanol/1 M acetic acid (2:8, v/v) and 2 × 5 ml 95% (v/v) ethanol. The discs were dried and their radioactivity was measured by scintillation spectrometry (Gay *et al.*, 1993). Protein content was determined according to Bradford (1976).

Chitin synthase entrapment. Product entrapment was carried out according to the method of Duran & Cabib (1978) using as the enzyme source the most active Chs fraction isolated following glycerol-gradient centrifugation (Gay *et al.*, 1993). The reaction mixture scaled up to 2 ml was incubated as above. After 30 min of incubation, the chitin-enzyme complexes were sedimented by centrifugation (10000 g for 30 min) and the pellet was resuspended in extraction buffer for further analysis.

Production of anti-Chs antibodies. Polyclonal antibodies were raised against part of an amino-terminal region (amino acid positions 8–137) and part of the neutral domain (amino acid positions 270–460) of the product of the *CHS2* gene of *S. monoica*. These peptides were expressed in *Escherichia coli* as fusion proteins with a six-histidine tag (Qiagen). The 375 and 574 bp fragments, encoding 124 and 191 amino acids re-

spectively, were amplified by PCR using *S. monoica* *CHS2* as a template and primers containing restriction sites (Table 1). The PCR products were digested and ligated into a pQE expression vector (Qiagen). The plasmids, pQE60 and pQE30, were introduced into *E. coli* M15 cells. Expression of fusion proteins was induced by adding 2 mM IPTG and after 2.5 h, cells were harvested and lysed in 6 M guanidine hydrochloride. The recombinant proteins were purified by nickel-chelating chromatography, then analysed by SDS-PAGE. The protein bands were excised, lyophilized and used to immunize rabbits.

Immunodetection of Chs. For immunodetection of Chs2, protein samples from *S. monoica* were separated on a slab gel (5–12.5%, w/v polyacrylamide) (Laemmli, 1970) and transferred to a Hybond-ECL nitrocellulose membrane (Amersham) according to Towbin *et al.* (1979). The membranes were blocked for 1 h with 5% (w/v) non-fat dry milk in Tris-buffered saline solution (TBS), pH 7.4. After three washes in TBS, blots were incubated for 1 h at room temperature with polyclonal rabbit antibodies diluted in TBS/milk and washed three times in TBS. Horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G (dilution 1:10000) was used as a second antibody. After three washes in TBS, reactive bands were revealed with the kit 'ECL Western blotting protocols' (Amersham).

RESULTS

Presence of chitin synthase genes in Oomycetes

Degenerate oligonucleotides corresponding to the conserved regions of *CHS* from *Sacch. cerevisiae* (Bowen *et al.*, 1992) were used for priming PCR amplification of *S. monoica*, *A. ambisexualis* and *P. capsici* genomic DNAs

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S.m CHS1 MYNEDENE-LRS-TNRKVCNNVLYL-KQQLPGYEGDDAWKQVLVIVSDGRTKANKGTLEN
S.m CHS2 MYNEEGSEILGTLTG-LAKGLG-YMCKE-----Y-GQDFWQEVAVIVSDGRTKASKTCLCY
P.c CHS1 MYNEDENE-LRS-TLRKVCNNVLYL-KQQLPGYEGDDAWKQVLVIVSDGRTKANKGTLEW
A.a CHS1 MYNEDQVE-MYS-TLKKVANNIAYI-KSQGLPGYEGDDAWRNILVIVSDGRTKANKGTLAF

      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
S.m CHS1 LSNVGNVDEDV-MTVTSLGVDTPA--GEN--Q-----TFE-----NSIRFPPLQVIFALKE
S.m CHS2 LNLGAFDEEIIIMTVTSLGVDTPA--GEN--Q-----TFE-----NYY-FPPLQVIYALKE
P.c CHS1 LSNVGLYDEDV-MNITSTGVKQCHLFEHSLQM---TKE-----NSIRFPPLQVTFALKE
A.a CHS1 LRDVGAFDEDV-MNIMMVGEVVMCHVFEFCVQLKAKTIEQSATSNE-RSPPTQVVFALKE

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Fig. 1. Comparison of the peptide sequences deduced from partial sequencing of PCR-amplified fragments from genomic DNA of Oomycetes. A.a, sequence deduced from the 600 bp fragment from *Achlya ambisexualis*; P.c, sequence deduced from the 450 bp fragment from *Phytophthora capsici*; S.m, sequences deduced from the 600 bp fragment from *Saprolegnia monoica*. Asterisks indicate identical residues. Dashes indicate gaps created in order to obtain alignment.

(Table 1). Amplified products derived from the two primers were detected, their size ranging from 450 to 950 bp. The PCR fragments of 600 bp from *S. monoica* and *A. ambisexualis* and of 450 bp from *P. capsici* were cloned into pUC18 and partial DNA sequences were determined. Analysis of several clones from *S. monoica* PCR products revealed two ORFs whose predicted amino acid sequences exhibited high similarities to Chs1 and to Chs2 from *Sacch. cerevisiae*. Comparison of the deduced amino acid sequences of the fragments from *A. ambisexualis* and *P. capsici* revealed homology to the *CHS* genes from *S. monoica* (Fig. 1). An overall identity of 35% was found among the *CHS* fragments of the three Oomycetes. These results indicate that Oomycetes can also be considered as chitinous organisms. The two *CHS* PCR-derived fragments from *S. monoica* have a similar size, resulting in a single band on the gels. The different sizes of the PCR fragments from the other Oomycetes may be due to a different number and/or length of introns in the amplified DNA region.

Evidence for a *CHS* gene family in *S. monoica*

Alignment of the predicted amino acid sequences of the PCR fragments revealed an identity of 50%, indicating the presence of two *CHS* genes in *S. monoica*. This was confirmed by Southern analysis of *S. monoica* DNA digested with different restriction enzymes. Hybridization to *SacI*-cut DNA with radioactive PCR fragments revealed two bands, of 0.6 and 1 kb (not shown). From the restriction map of the *CHS2* nucleotide sequence, one can predict that the 600 bp *CHS2* probe should hybridize to the 1 kb *SacI* fragment. Therefore, the remaining band on the blot must be due to hybridization with the *CHS1* probe.

S. monoica chromosomes were separated by CHEF electrophoresis and probed with the cloned PCR products. Electrophoretic conditions were such as to allow the separation of the largest chromosomes (i.e. higher than 2 Mb) whereas the smallest chromosomes appeared as broad bands (Mort-Bontemps & Fèvre, 1995). Using stringent hybridization conditions, the probe hybridized to chromosomal bands of 4.6 and 3.4 Mb, confirming

Fig. 2. Chromosomal localization of *S. monoica* *CHS* by Southern blot hybridization. (a) Electrophoretic karyotype of *S. monoica* by CHEF gel electrophoresis stained with ethidium bromide. The electrophoretic conditions for separating the chromosomes were 96 h ramped pulse from 180 s to 2700 s at 50 V in 0.6% (w/v) agarose gels. Chromosomes of *S. monoica* (lanes 4) were run along with chromosomes of *Sacch. cerevisiae* (lane 1), *Hansenula wingei* (lane 2) and *Schizosaccharomyces pombe* (lane 3). The numbers on the left indicate the size of the chromosome markers in megabase pairs. (b) Southern hybridization of the electrophoretic karyotype transfer. The 600 bp PCR fragment was used to probe the separated chromosomes. The arrows indicate the chromosomal bands 2 (4.6 Mb) and 3 (3.4 Mb).

the presence of two genes and of a chitin synthase gene family in *S. monoica* (Fig. 2).

Nucleotide sequence of *CHS2* from *S. monoica*

The PCR fragment identified as *CHS2* by its homology to the *Sacch. cerevisiae* *CHS2* gene was used as a probe to screen a genomic library constructed in the phage λ EMBL3. Three positive signals were obtained out of 18000 recombinant phages screened. The phages were isolated and amplified, and their DNA was digested with different restriction enzymes and subjected to Southern analysis. The three clones were overlapping and contained the entire *CHS2* gene. A 4.5 kb segment was sequenced completely on both strands using sub-clones and oligo primers. The *CHS* coding region was located by comparison of the predicted amino acid sequence with that of other *CHS* gene products. Fig. 3 shows the *CHS2* nucleotide and deduced amino acid sequences. The sequence revealed an ORF of 2854 bp interrupted by three introns. These intervening sequences were identified on the bases of consensus 5' and 3' splice junction sequences. Two introns are situated at the 5' region of the sequence as in other fungal *CHS* genes. The third intron is located in the 3' region as in

chs1 of *N. crassa* and *chsB* of *A. nidulans* (Yarden & Yanofsky, 1991; Yanai *et al.*, 1994). The non-translated 5' and 3' regions were analysed for consensus regulatory sequences. The ORF shows a low bias for codon usage, as 60 out of the 61 codons possible are used.

Predicted amino acid sequence of *S. monoica* CHS2 and comparison with other chitin synthase sequences

The *CHS2* gene encodes a predicted polypeptide of 886 amino acids with a calculated molecular mass of 99 kDa and a pI of 9.2. These values are similar to the calculated pI values and molecular masses of other Chs enzymes.

The amino acid sequence deduced from the intron-deleted nucleotide sequence was compared with that of other *CHS* genes (Fig. 4) by dot matrix computer analysis, revealing a structurally similar region. Comparison of the deduced amino acid sequence with those previously reported showed that similarity was located in the central region of the genes. The amino and carboxy termini showed only isolated conserved amino acids. The most pronounced regions of predicted amino acid identity (spanning amino acids 208–740 in *S. monoica* *CHS2* gene) were aligned; 110 residues were conserved among all the sequences and 154 conservative amino acid substitutions were detected. The longest stretches of identical amino acids occurred from residue 207–596 based on the *S. monoica* Chs2 sequence. In this region, the predicted amino acid sequence of *S. monoica* Chs2 exhibits 28–44% identity with the predicted polypeptides of chitinous fungi. The identity is higher, ranging from 41 to 77% among the predicted Chs amino acid sequences of the chitinous fungi (Fig. 4).

An extensive search using the PROSITE database revealed the presence of a motif FEYK(I/L/M)SNIXDK that is specific for enzymes of classes I and II (Bowen *et al.*, 1992). When comparison was extended to class III, this motif was reduced to FEYKXXXXXXXXX as noticed by Xoconostle-Cazares *et al.* (1996). Another motif SWGTKG was also found in all proteins of classes I and II except in those of *Ustilago maydis* (Xoconostle-Cazares *et al.*, 1996), whose sequences appeared to be truncated in comparison to those of other fungal genes. Genes of the classes III, IV and V (Specht *et al.*, 1996) contained the motif SWG. These motifs could be considered as signatures of the Chs enzyme families (Fig. 5).

Chitin synthases are processive β -glycosyltransferases that interactively transfer sugar residues to an acceptor forming the carbohydrate chain (Saxena *et al.*, 1995). From hydrophobic cluster analysis of the sequences of several types of glycosyltransferases, we have deduced that two domains are responsible for processivity and have identified the putative catalytic amino acids in each domain (Saxena *et al.*, 1995). The A domain is characterized by two aspartates and they are conserved among all the sequences (corresponding to the residues 291 and 376 of *S. monoica* Chs2). An aspartate and the motif

QXRRW, which are the catalytic residues of the B domain (corresponding to residues 494 and 531–535 of *S. monoica* Chs2), are also conserved in all the sequences (Fig. 5). These amino acids have also been shown to be essential for the catalytic activity of *Sacch. cerevisiae* Chs2 (Nagahashi *et al.*, 1995).

Hydrophobicity analysis (Kyte & Doolittle, 1982) of *S. monoica* Chs2 predicts a protein with three domains: a hydrophilic region at the amino terminus, a central neutral region and a hydrophobic region at the carboxy terminus containing five potential hydrophobic transmembrane domains. Prediction of the orientation of these transmembrane proteins indicates that the hydrophilic and the neutral domains are in the cytosol. The hydrophobicity plots of the Chs proteins (Fig. 6) were compared following an alignment of the sequences at the residues FEY (at position 420 in the *S. monoica* Chs2 sequence). This analysis confirmed the great structural similarity of the different proteins in spite of the weak homology of the deduced amino acid sequences. Regions corresponding to the catalytic domains A and B and to the Chs signatures exhibited extensive homologous properties. The large hydrophobic region following the B domain consists of multiple hydrophobic peaks, indicating that these proteins have a related membrane topology in common. The amino terminus region of all the proteins is different, indicating that this hydrophilic domain is probably not essential for enzymic activity.

Expression of *CHS*

Northern hybridizations of total RNA and poly(A)⁺-enriched RNA were done. Unreproducible (or undetectable) signals were obtained, indicating that the *CHS* transcripts were not abundant. RT-PCR was used to show that the genes are expressed during hyphal growth and protoplast regeneration. Primers (Table 1) were designed to amplify sequences situated on each side of the two introns; these primers allowed the target cDNA and genomic DNA to be size fractionated on agarose gels. Two PCR products, of 234 and 150 bp, were revealed whereas no fragment corresponding to the size of the amplified genomic DNA was detected (Fig. 7). To confirm the identity of the ethidium-bromide-stained bands, gels were blotted and probed with a *CHS* fragment. Signals on the autoradiograms confirmed the results obtained on the stained gels (Fig. 7). These results indicate that two homologous *CHS* genes are expressed during the vegetative phase of growth and the cell wall regeneration of the protoplasts. However, one cannot exclude that alternate splicing of introns produces two *CHS* mRNAs.

Immunodetection of Chs2

As the entire *CHS2* gene or fragments encoding the hydrophobic carboxy terminus could not be expressed in bacterial cells, segments of *CHS2* encoding parts of the hydrophilic and neutral domains were PCR amplified (Table 1), and the resulting products Chs2 8–137

tgtaacgcgctataaagcgtagtagagtaggtatgcgttaaactgagacggtacatgttcgccatttacctc	72
tgccgctgcaaacccgaacctgagtcgaataattaaggagcagcagcgagtggaacaccacgacgaagaatctg	144
tcgctgtgggtgcgcgctgctgcgtgctcgacgcgcgcaagcaaacgcgatcctttgcttcggctagcctgtac	216
tctctgtgaaccactattaggtataacgcgcgctttaaaagttttaacgtgaaaaataaacctcacttaagt	288
caacttattttatgtcctctttggctccaccatcaatgaaaaaatcgagatttcggtatgtaatacacaggc	360
gaggaagaaacgcgtggagattcggcgacgaaccgcgggacgaggttcgctcatggcatgatgggcttctctg	432
caggtagatgggctggaaactgtccatggctcgtgaggtgatgccgctggctgcgtgagctggcgcgaaaca	504
tcgctgcttgatccattcctggaaggggccatgattggactgctaagcgcgtgcaccctctctctcatggca	576
cgagagccctcacaagaatgacaagatgggtatcaatgatattctggtactttgctaaccattaaatgaaggaa	648
tctattgtaggagctctgcacatcccccgatttttgtcagcctatcgagagcccaataagtcacaaaaagcg	720
taacgctgaggggtgggttcgtccagggcaaaaaaagagtgggttcttgtgttgaaaagcgtctacgtccagc	792
ttctctcttttgagcacattttctcttgcgtgaccattttgtgttaccgcgtatccaccatcgtgtccacctcgt	864
ggacacaccatttgggttcttgtgttcttcaaaagtgtccttacgggcatcgatagatctctagaagcggcg	936
caagtgaggactgcagtaacaattcagaagaatccggtaggagcttccacgattacatgcaagggatga	1008
cagcaacagcaagatgtcccgctcgattgatcgatgctgatctgtgtccttcgccttgcatgtgaagcagtc	1080
tgcttgcgttcctcgacagggtttttggcgctgtttttggataaaacccaagatttctccgtaataccaaac	1152
ggcgcccttacttcagtgatatacaaccaagattggcaaaagctttatcccccaagcttttgcccaattaaatcta	1224
tcgaaaaaaattgaagtacatacacttgagcgtgtctcacatttatattgtttccatttcggtacatttgccaa	1296
gccgacagc ATG AGT GAC CAG CTC GAC CTC GCG GCA CGC CTC CGC GCC CTG CGT	1350
M S D Q L D L A A R L R A L R	15
GAG GGC AAC GCC GCG CCA GCC GAC CCC GAG ACC GCC ACC CAC ACA GCC TGG CCC	1404
E G N A A P A D P E T A T H T A W P	33
GCG CCG CAG TAC CAT CCA CAC TGC CGC CGC TGT ATA CAC AAG AGT CGC TCG AGT	1458
A P Q Y H P H C R R C I H K S R S S	51
TGG GAG GGA CGT ATG CCA CGG GCA GCC CCG TGC GCC GAG GCC GAG GGG TCC TAC	1512
W E G R M P R A A P C A E A E G S Y	69
TCG CAA GTA CCT GTG TGG AAG GAC TCC AAG GAG ACG CGA AGA AGT TAC CTG GAC	1566
S Q V P V W K D S K E T R R S Y L D	87
GAT GAG CCG ACG CCG CAG CCC CAA TCG CTC ATT AAC ATG GCT AAC ACC TTG GTC	1620
D E P T P Q P Q S L I N M A N T L V	105
CAG CGC CAA CGT CGA ATC AAT CTT CCG GCG GCA GCA TAC AGC GAA CTT CCG ACC	1674
Q R Q R R I N L P A A A Y S E L P T	123
GCT GCC CAA CAC CGT CGA GGA GCT TCT GGA CGG CAA GCC AAC gtacgaaggagcgtt	1731
A A Q H R R G A S G R Q A N	137
tcgcctcgtgcagctcgcggtgcagatggagcaag T ACG GCG ACC CAG GTG CTG CAA TTA ACT	1793
T A T Q V L Q L T	146
TGT ACG TGG ACG CTG GCA CGA CGC TCG TCG AAG TC ggtaagcgtgaggtcgaccctcttc	1853
C T W T L A R R S S K S	157
tgcaagaaggcgcacagcaaaaggcctttgagctcctgcaagT GCC GAG GAG CTC GGC ACG TGG	1917
A E E L G T W	165
ATG AAC ACG GTG GCC GAG GAA GCG CGG AAA GCT GCG TTG CCA CCG CAG CTC AAG	1971
M N T V A E E A R K A A L P P Q L K	183
ATC GCG GCG ACC AAC GTC CCG ACG GTC GAG CAA GCG TGG AAG GGT CGC ACT CCG	2025
I A R T N V P T V E Q A W K G R T P	201
CCT TTC CAT GAC GCC GAC GAG TTC CGA CTT ATG CGG TAC ACG GCC GTG GCA ACG	2079
P F H D A D E F R L M R Y T A V A T	219
AAA GAC CCG ATC CAG TTC TCG AAC GAC GGA TAC GTG CTC CGG GTG CAC CAG CTA	2133
K D P I Q F S N D G Y V L R V H Q L	237
CAT CGG CGC ATC AAG GTC TTC ATC ACA ATC ACT ATG TAC AAC GAA GAA GGC TCA	2187
H R R I K V F I T I T M Y N E E G S	255
GAG ATC TTG GGC ACG CTC ACT GGT CTC GCC AAG GGC CTC GGG TAT ATG TGC AAG	2241
E I L G T L T G L A K G L G Y M C K	273
GAG TAT GGC CAG GAT TTT TGG CAA GAA GTT GCT GTG GCT ATC GTC TCA GAC GGC	2295
E Y G Q D F W Q E V A V A I V S D G	291
CGC ACC AAA GCT AGC AAG ACG TGT CTC GAG TAC CTC AAC GGC CTC GGC GCA TTT	2349
R T K A S K T C L E Y L N G L G A F	309
GAC GAA GAG ATC ATG ACG GTC ACG AGC CTC GGT GTC GAC ACT CCA GCT GGT GAG	2403
D E E I M T V T S L G V D T P A G E	327
AAC CAG ACG TTT GAA AAC TAC TTT CCG CCG CTC CAA GTG ATC TAC GCG CTC AAA	2457
N Q T F E N Y F P P L Q V I Y A L K	345
GAG AAC AAC GGT GGC AAG CTC AAC TCG CAT CTG TGG TTC TTC AAC GCC TTC AGC	2511
E N N G G K L N S H L W F F N A F S	363
GAG CAA TTG AAC CCC AAG TAC ACT GTA CTC GTG GAC GTC GGC ACC ATT CCC GCC	2565
E Q L N P K Y T V L V D V G T I P A	381
GAA ACG TCC GTT TTC CGC TTG ATC CGC AGC ATG GAG CGC AAC TAC CAG ATC GGC	2619
E T S V F R L I R S M E R N Y Q I G	399
GGC GTT GCG GGG GAG ATT GCA GTC GAA GCA CCT AAC TAC TTC AAT CCT GTC ATT	2673
G V A G E I A V E A P N Y F N P V I	417
GCC GCA CAG CAC TTC GAG TAC AAG ATC TCA AAT ATC ATG GAC AAG TCG CTT GAG	2727
A A Q H F E Y K I S N I M D K S L E	435

Fig. 3. For legend see facing page.

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TCC GTC TTT GGT TTT ATC TCG GTG CTC CCG GGG GCC TTC TCA GCC TAC CGG TAC 2781
S V F G F I S V L P G A F S A Y R Y 453
GAA GCC ATC CGT GCT GTC AAG GGT GTG GGG CCG CTG CCA GAG TAC TTT AAG AGC 2835
E A I R A V K G V G P L P E Y F K S 471
CTC ACG TCG ACG ACC AAA GAG CTC GGG CCA TTT CAG GGC AAT ATG TAC CTC GCC 2889
L T S T T K E L G P F Q G N M Y L A 489
GAA GAT CGT ATT TTA TGT TTT GAA TTG CTG GCG CGC AAA CAT AAA CAG TGG ACA 2943
E D R I L C F E L L A R K H K Q W T 507
ATG CAC TAT GTC AAG GAC GCG ATC GCC CGC ACT GAC GTT CCC GAG ACG CTC GTA 2997
M H Y V K D A I A R T D V P E T L V 525
GAC CTG ATC AAG CAG GCG CGG CGT TGG CTC AAC GGG TCT TTC TTT GCC GGC CTC 3051
D L I K Q A R R W L N G S F F A G L 543
TTT GCC ATC GGG ACT TTG GCT GTG AGC CAG AGC TCC CAC ACC ATG TCC CGG 3105
F A I G T L A V W S Q S S H T M S R 561
AAG CTT GTG TTC ACG TTT CAG TTC TTT TAC CTT GCC CTA CAG AAC CTG CTC AGT 3159
K L V F T F Q F F Y L A L Q N L L S 579
TGG TTC CTC TTG AGC AAT TTG TTC CTT ACA TTC TAC TTT GTT TTG ACG CTC GCC 3213
W F L S N L F L T F Y F V L T L A 597
TTT ACG GAC TCA GCA CCG GCC CTT CTC CAA GCG ATG CTG ACG CTG TAT CTG GCC 3267
F T D S A P A L L Q A M L T L Y L A 615
ATT GTC GGT GGC TTG ATT GTC TTT GCG CTC GGG AAC AAG CCC GAA CCT CGG ACG 3321
I V G G L I V F A L G N K P E P R T 633
GCC AGC TTT TAC CTC TTC AGC TGC CTT TAC ATG GGC ATC ATC ATG ATG CTT GTG 3375
A S F Y L F S C L Y M G I I M M L V 651
ACC GGC ATT TCC ATC TAC GGC CTT GTC GGC AAG GGC ACA AGC GCT GTG AAA GAC 3429
T G I S I Y G L V G K G T S A V K D 669
CCA CGG GTG ATC ACG GGG CCC CTT GCA ACT GTA CTG TCT CTG AAG GGG AGC TTG 3483
P R V I T G P L A T V L S L K G S L 687
TCG GTG GTG TCG TCA CCT CGG CTT GGC TTG ATC TTC CTC TCT GCC TTC GTC CAT 3537
S V V S S P R L G L I F L S A F V H 705
GGC GAG TTT AGC ATC CTC CTC AGC GTC ATC CAG TAC TTC TTC ATG CTC CCA ACG 3591
G E F S I L L S V I Q Y F F M L P T 723
TTT GTG gtatgctatctccctttctctaccttatagtcctataaattttccattagaatgtgctgggc 3660
F V 725
atctatgcctacag CAA TTT GCA CAC TTG AGC TGG GGT ACC AAG GGC CTG GAG TCT 3716
Q F A H L S W G T K G L E S 739
GGG GGC CGC CAC GGA CCA ACG AAA ACC GGC GGC GGT AAA GTC AAG GAA GTC GTC 3770
G G R H G P T K T G G G K V K E V V 757
GAG CAG CAG AAG AAG CTC GAA GCC CAA CGC CAG GCC GCG GCT AAG GAG AAA GAA 3824
E Q Q K K L E A Q R Q A A A K E K E 775
GAT GTG GAC AAC AGC TTC CGG GCG TTC CGG TCG ACA CTG CTC GCT GTC GTG GCT 3878
D V D N S F R A F R S T L L A V V A 793
CAC GAC CAA CGG CAT TTG GCT CTA CGT TGT GAC GGA CTA CAT GTC GAG CGG GTG 3932
H D Q R H L A L R C D G L H V E R V 811
CTA CTT GAA GGG CCT CAG CTT GTC GTC GGC TTC TTT AAC GTC ATC GTT TCA CGG 3986
L L E G P Q L V V G F F N V I V S R 829
GCT GCG TTG TAT TTA TTA TCT TGC GCA TCT TTA AGC TAT TCG GCC TTA ACT GCT 4040
A A L Y L L S C A S L S Y S A L T A 847
GTG CAA TGG GGG CCA CCC ACG ATA CCT ATG AGC GCA ACC TGC CGG CCG ACT GGG 4094
V Q W G P P T I P M S A T C R P T G 865
AGA CTC ATT ACA ATG TAC AGA ACC AAG GCG ACG GTC GGG TTG TGG TTG CCC GTG 4148
R L I T M Y R T K A T V G L W L P V 883
CAG AGA GCA TAA acccagcaagcccgcgcgccctaccaagaagtcataactgcataatacacttgt 4215
Q R A * 887
acgacctcttccattttacgtatcccgattcgctcatgtcaataatttccttgatttacctcgagacggatga 4287
ctcgacatcacattagtagcagcattcatagaacttcaacctctgctccattcatgaaggcgtcggaatg 4359
gagcttgaccttcacaggaggtccctcgcgaaattcggaagggtttgtcttaaaacctagttttgcgcaat 4431
tcgactatcgcgccatacaaccgcggttcaaatagcagatcctctaa 4478

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Fig. 3. Complete nucleotide sequence of the *S. monoica* CHS2 gene and flanking regions, and the deduced amino acid sequence of CHS2. Introns are in lower case. The TAA translational stop codon is indicated by an asterisk.

and Chs2 270–460 were cloned into pQE expression vectors. SDS-PAGE analysis of the bacterial lysates revealed that IPTG-induced protein bands were the obviously dominant ones. The molecular masses of the expressed proteins Chs2 8–137 and Chs2 270–460 were 14 and 21 kDa, respectively. The Chs2-His tag recom-

binant proteins purified with Ni-affinity columns were used to raise rabbit polyclonal antibodies.

The antisera were used for Western blot analysis of total proteins extracted from mycelia. The anti-Chs2 8–137 serum detected two bands of approximately 100 kDa

Fig. 4. Comparison of the deduced amino acid sequences of the conserved domains of chitin synthase genes. The percentage of amino acid identity was calculated following alignment of the different sequences to the *S. monoica* sequence from amino acids 208 to 740. S.m, *Saprolegnia monoica*; N.c, *Neurospora crassa*; S.c, *Saccharomyces cerevisiae*; C.a, *Candida albicans*; A.n, *Aspergillus nidulans*; R.o, *Rhizopus oligosporus* and U.m, *Ustilago maydis*. (GenBank accession numbers U19946; NEUCHT; YSCCHSYNA and YSCCHIS; CACHS1 and CACS2A; ASNCGSA and ASNCHSB; RCHCHS1 and RCHCHS2; UMCHS1GEN and UMCHS2GEN, respectively).

but also showed cross-reaction with other proteins and was not used for further analysis. The anti-CHS2 270–460 serum recognized the doublet of 100 kDa and two other bands of about 120 kDa. These results

confirmed that *S. monoica* contains several chitin synthases (Fig. 8). As the doublet of 100 kDa was recognized by both antisera these proteins may correspond to the products of the two CHS identified following PCR amplification. Using Chs2 270–460 recombinant protein purified from bacterial cells as a standard, the concentration of Chs2 homologues in the total mycelial extract was estimated to be less than 10 pg per µg of membrane proteins.

Glycerol gradient purification of the solubilized proteins, which has proved efficient in the separation of glycosyltransferases from *S. monoica* (Bulone *et al.*, 1990), was used to partially purify chitin synthase activity. As shown in Fig. 9, chitin synthase activity sedimented as a sharp peak ahead of the bulk of solubilized proteins, and the distribution of the proteins recognized by the anti-Chs2 270–460 antiserum followed the distribution of the chitin synthase activity. The enzyme activity was partially purified by product entrapment (Gay *et al.*, 1993) and probed with the anti-Chs2 270–460 antiserum. Among the proteins sedimented with chitin microfibrils, a single polypeptide of approximately 60 kDa was revealed by this technique (not shown). The protein had a smaller molecular mass than the proteins detected from the crude membranes and from the expected size deduced from the CHS2 sequence. This indicates that partial degradation of the enzyme(s) had occurred during the solubilization and purification procedures and that the 60 kDa protein was an active form of chitin synthase. This protein was not detected by the anti-Chs 2 8–137 (not shown); this may

Fig. 5. Comparison of the conserved amino acids in the Chs sequences. The diagram of the native Chs2 from *S. monoica* shows the different domains, the position of the catalytic amino acids as determined by Saxena *et al.* (1995) and the motifs determined as Chs signatures. The catalytic amino acids are indicated by an asterisk and the amino acids of the Chs signatures are indicated by a circle. S.m, *Saprolegnia monoica*; N.c, *Neurospora crassa*; S.c, *Saccharomyces cerevisiae*; C.a, *Candida albicans*; A.n, *Aspergillus nidulans*; R.o, *Rhizopus oligosporus*; U.m, *Ustilago maydis*.

Fig. 6. Comparison of the hydropathy profiles of chitin synthases. Deduced protein sequences were analysed according to Kyte & Doolittle (1982) and the profiles were aligned to the position of the conserved motif FEY. The localization of the catalytic A and B domains conserved among the glycosyltransferases (Saxena *et al.*, 1995) is indicated at the top. Inverted triangles indicate the position of the amino acid motifs determined as CHS signatures. C.a, *Candida albicans*; S.c, *Saccharomyces cerevisiae*; R.o, *Rhizopus oligosporus*; N.c, *Neurospora crassa*; U.m, *Ustilago maydis*; A.n, *Aspergillus nidulans*; S.m, *Saprolegnia monoica*.

reflect the proteolytic cleavage of the hydrophilic domain.

DISCUSSION

Using degenerate PCR primers, we amplified two conserved regions of chitin synthase genes from the cellulosic fungus *S. monoica*. Similar sequences were also amplified from two other Oomycetes, indicating that the chitin synthase system is widespread in this class

of organism. We have cloned and sequenced the *CHS2* gene from *S. monoica* and have determined its homology with other *CHS* genes. The conserved amino acid sequences derived from *CHS* genes have been useful in the phylogenetic analysis of chitinous fungi, confirming the current taxonomic groupings (Bowen *et al.*, 1992; Mehmman *et al.*, 1994). Compared to other complete predicted Chs polypeptides, Chs2 from *S. monoica* appears to be well separated (Fig. 10) confirming that Oomycetes are highly divergent from true, chitinous,

Fig. 7. Expression of chitin synthase genes of *Saprolegnia monoica*. (a) RT-PCR strategy. Primers used for RT-PCR (Table 1) were designed from the 5' and 3' regions bordering the two introns. An internal fragment was used as a probe to hybridize with the amplified products. (b) Total RNAs were isolated from freshly prepared protoplasts (lane 1), 24 h regenerated protoplasts (lane 2) and mycelium (lane 3). Ethidium bromide staining of the RT-PCR products separated on a 2% (w/v) agarose gel (A) and hybridization with *HpaII*-*PstI* fragment, used as probe (B). The size of the fragments are indicated on the left. The arrow indicates the predicted size of the genomic fragment delimited by the primers.

Fig. 8. Western blots of membrane-bound enzymes from *S. monoica*. Membrane-bound proteins were isolated from mycelial cell-free extracts by centrifugation at 48000 *g* for 30 min, separated by SDS-PAGE then transferred onto nitrocellulose and probed with the anti-Chs2 8-137 (a) and with the anti-Chs2 270-460 antiserum (b). Lanes 1 and 2 contained 150 µg and 300 µg protein, respectively.

Fig. 9. Glycerol gradient of digitonin-solubilized chitin synthase from *S. monoica*. Membrane-bound enzymes were solubilized with digitonin, layered onto a glycerol gradient and centrifuged at 120000 *g* for 4 h. Fractions were assayed for protein (□) and chitin synthase activity (■). Proteins from the gradient fractions were deposited onto nitrocellulose and probed with the anti-Chs2 270-460 serum.

Fig. 10. Maximal parsimony dendrogram showing chitin synthase grouping. The tree was calculated by the program Phylip from amino acid sequences deduced from the chitin synthase genes shown. *R.o*, *Rhizopus oligosporus*; *C.a*, *Candida albicans*; *A.n*, *Aspergillus nidulans*; *S.c*, *Saccharomyces cerevisiae*; *N.c*, *Neurospora crassa* and *S.m*, *Saprolegnia monoica*.

fungi (Cavalier-Smith, 1986). The weak homology between these enzymes was confirmed by immunological probing. An antiserum directed against *S. monoica* Chs2 did not cross-react with proteins from *Sacch*.

cerevisiae, and antibodies against the yeast Chs2 enzyme did not recognize the proteins from *S. monoica* (unpublished data).

To date, chitin synthase gene families have been shown to be large in filamentous fungi. In *Neurospora crassa*, four classes of *CHS* genes have been identified (Din *et al.*, 1996). Mellado *et al.* (1995) found that *Aspergillus fumigatus* has at least six genes encoding various chitin synthases. In *S. monoica*, the presence of two *CHS* on two different chromosomes indicates that this enzyme activity is also encoded by a multigene family. We prepared and used polyclonal antibodies against two fragments of Chs2 to identify the polypeptide in cell free extracts. In addition to a polypeptide of 100 kDa, corresponding to the expected size of Chs2, several other bands of higher molecular mass reacted with the antibodies. These may represent other chitin synthases, confirming the existence of a multigene family. Sietsma *et al.* (1996) have shown that an antibody preparation against *Sacch. cerevisiae* Chs2 was capable of recognizing several polypeptides in *N. crassa*, which has several *CHS* genes. Immunodetection of several polypeptides may indicate homologous Chs; however, one cannot exclude that in both cases, other membrane proteins may react with the anti-Chs antibodies.

The topology of *S. monoica* Chs2 predicted from its sequence is that of a transmembrane protein containing two large cytoplasmic domains, one hydrophilic and one neutral. The putative *N*-glycosylation sites were located in these domains but as glycosyl residues from glycoproteins do not face the cytosol, this indicates that the protein is probably not *N*-glycosylated. In spite of the weak identity with Chs from chitinous fungi, Chs2 from *S. monoica* has striking similarities in size, charge and membrane topology with the enzymes of the other fungi. If the differences in the polypeptide sequences reflect the phylogenetic position of pseudofungi compared to the true fungi, genes of both types of organisms encode functionally equivalent enzymes which synthesize the same product, crystalline α -chitin. The presence of motifs conserved among the Chs sequences of these non-related organisms suggests their importance in the organization and/or the catalytic activity of the enzyme. The catalytic amino acids which characterize processive β -glycosyltransferases (Saxena *et al.*, 1995) and yeast Chs (Nagahashi *et al.*, 1995) were found at conserved positions in all the sequences. Two motifs were identified as specific signatures of Chs.

The antibodies directed against part of the neutral domain containing the catalytic amino acids detected a main protein of 100 kDa in crude membranes, and a polypeptide of 60 kDa entrapped in the chitin synthesized *in vitro* by detergent-solubilized enzymes. This protein may represent a truncated but still active form of the enzyme. We do not know if this form is the result of the proteolytic activation characterizing chitin synthase activity or the consequence of degradation by endogenous proteases. Recently, Uchida *et al.* (1996) have shown that trypsin treatment of the membranes from

Sacch. cerevisiae can generate truncated active polypeptides from the 100 kDa zymogenic Chs2, the size of these products ranging from 35 to 75 kDa. Therefore a small part, corresponding to the neutral domain of chitin synthases from cellulosic and chitinous fungi is sufficient for catalytic activity.

Two *CHS* genes were expressed in the mycelium and in regenerating protoplasts of *S. monoica*. Disruption strategies to inactivate *CHS* as performed in higher fungi are less feasible in *S. monoica* as the fungus is diploid. In order to determine the morphogenetic role of the gene products, we have used antisense RNA to inhibit Chs2 activity; preliminary data show that enzyme activity and hyphal morphogenesis of the transformants are not modified (M. Mort-Bontemps, unpublished data). These results agree with the fact that polyoxin D, a competitive inhibitor of Chs, does not alter hyphal morphology in *S. monoica* (Bulone *et al.*, 1992). Activity of the *CHS2* product seems to be dispensable during hyphal growth. However, it is still possible that additional genes are present in this organism and that other chitin synthase activities less sensitive to the inhibitor are involved in morphogenesis.

In conclusion, the results presented in this paper confirm clearly, and complement our previous data (Bulone *et al.*, 1992), that chitin can no longer be considered as a discriminative character between Oomycetes and higher fungi. They also indicate that chitin biosynthesis in *S. monoica* is mediated by multiple chitin synthases, encoded by a multigene family as in chitinous fungi. In spite of their divergent evolution, pseudofungi and fungi have evolved with conserved chitin synthase systems.

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