Role of chitin synthase genes in *Fusarium oxysporum*

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Three structural chitin synthase genes, *chs1*, *chs2* and *chs3*, were identified in the genome of *Fusarium oxysporum* f. sp. lycopersici, a soilborne pathogen causing vascular wilt disease in tomato plants. Based on amino acid identities with related fungal species, *chs1*, *chs2* and *chs3* encode structural chitin synthases (CSs) of class I, class II and class III, respectively. A gene (*chs7*) encoding a chaperone-like protein was identified by comparison of the deduced protein with Chs7p from *Saccharomyces cerevisiae*, an endoplasmic reticulum (ER) protein required for the export of ScChs3p (class IV) from the ER. So far no CS gene belonging to class IV has been isolated from *F. oxysporum*, although it probably contains more than one gene of this class, based on the genome data of the closely related species *Fusarium graminearum*. *F. oxysporum chs1−, chs2−* and *chs7−* deficient mutants were constructed through targeted gene disruption by homologous recombination. No compensatory mechanism seems to exist between the CS genes studied, since chitin content determination and expression analysis of the *chs* genes showed no differences between the disruption mutants and the wild-type strain. By fluorescence microscopy using Calcofluor white and DAPI staining, the wild-type strain and Δ*chs2* and Δ*chs7* mutants showed similar septation and even nuclear distribution, with each hyphal compartment containing only one nucleus, whereas the Δ*chs1* mutant showed compartments containing up to four nuclei. Pathogenicity assays on tomato plants indicated reduced virulence of Δ*chs2* and Δ*chs7* null mutants. Stress conditions affected normal development in Δ*chs2* but not in Δ*chs1* or Δ*chs7* disruptants, and the three *chs−* deficient mutants showed increased hyphal hydrophobicity compared to the wild-type strain when grown in sorbitol-containing medium. The chitin synthase mutants will be useful for elucidating cell wall biogenesis in *F. oxysporum* and the relationship between fungal cell wall integrity and pathogenicity.

INTRODUCTION

Chitin, an important structural cell wall component in many species of yeast and filamentous fungi but absent from plants and vertebrates, is a β(1,4)-linked polymer of N-acetylglucosamine which forms a fibrous polysaccharide. This taxonomic difference provides the rationale for considering chitin as a safe and largely selective target for developing antifungal control agents (Cohen, 1990). Chitin synthases (CSs) catalyse the transfer of N-acetylglucosamine from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) to a growing chain of β(1,4)-linked N-acetylglucosamine residues (chitin) (Ruiz-Herrera et al., 1992). The specific mechanism by which this polymer is synthesized in vivo by the different species appears to have selective characteristics. Fungal CSs are integral membrane-bound proteins that participate in the biosynthesis of the cell wall and are important for hyphal growth and differentiation (reviewed by Cabib et al., 1996; Roncero, 2002). Comparative analysis of the amino acid sequences deduced from fungal *chs* genes reveals the existence of a hydrophobic domain located towards the C-terminus, in agreement with the membrane location of these enzymes. Several authors have provided evidence for the existence of a type of specialized vesicles in the cytosol, named chitosomes, where most CS is accumulated (Bartnicki-Garcia et al., 1984). They synthesize chitin microfibrils through an asymmetric mechanism, accepting GlcNAc residues at the cytosolic face, and delivering chitin molecules at the inner face.

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Abbreviations: AUDPC, area under the disease progress curve; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CFW, Calcofluor white; CS, chitin synthase; DAPI, 4′,6-diamidino-2-phenylindole; ER, endoplasmic reticulum; HygR, resistance to hygromycin; P, probability of the statistic absolute t.

The GenBank/EMBL/DDBJ accession numbers for the *chs1*, *chs2*, *chs3* and *chs7* gene sequences reported in this paper are AY572421, AY572422, AY572423 and AY572424, respectively.
Enzymes have been elucidated in only a few cases (Fujiwara et al., 1999). The specific roles of these enzymes have been reported for representative filamentous fungal species (Bulawa, 1993; Roncero, 2002). Structural and functional analyses of chs genes and their products have been reported for representative filamentous fungal species, as implicated in the diverse morphologies of filamentous fungi. Nevertheless, the specific roles of these enzymes have been elucidated in only a few cases (Fujitwara et al., 2000; Lee et al., 2004; Yarden & Yanofsky, 1991). In the human-pathogenic fungus Aspergillus fumigatus, the chs gene family includes at least seven different genes, members of all six CS classes (AfchsA, AfchsB, AfchsC, AfchsD, AfchsF and AfchsG) (Mellado et al., 2003). Inactivation of AfchsA (class I), AfchsB (class II), AfchsC (class III) and AfchsD (class IV) does not lead to any obvious phenotypic defect (Mellado et al., 1996a, b), whereas disruption of AfchsE (class V) and AfchsG (class III) gives rise to altered phenotypes, suggesting that class III CS functions at the apical tips of the hyphae (Mellado et al., 1995) and class V CS is responsible for cell wall structural integrity (Auffauvre-Brown et al., 1997). In Aspergillus nidulans, five CS genes [chsA, chsB, chsC, chsE (identical to chsD) and csmA] have been reported so far. Based on phenotypes present in single or double disruption mutants, the function of each chs gene has been summarized as follows. chsB is a class III CS, required for normal hyphal growth and organization (Borgia et al., 1996), and csmA (class V) seems critical for the maintenance of hyphal wall integrity and the polarized synthesis of the cell wall (Horiuchi et al., 1999). The genes chsA, chsC and chsE appear to serve redundant functions during asexual morphogenesis such as conidia formation and conidiodore development (Motoyama et al., 1996; Fujiwara et al., 2000).

Fusarium oxysporum, a vascular wilt pathogen with more than 100 specialized forms distributed worldwide, causes disease among a variety of important crop plants (Beckman, 1987). F. oxysporum has also been reported as an emerging opportunistic human pathogen in immunocompromised patients (Vartivarian et al., 1993). Between eight and twelve CS-encoding genes representative of all six categories described in filamentous fungi are present in the Fusarium graminearum genome, recently identified by in silico search of the database at Sequencing Project, Center for Genome Research (http://www.broad.mit.edu). Most of these genes have counterparts in the genomes of Neurospora crassa, A. nidulans and Magnaporthe grisea. Chitin can account for up to 10% of the cell wall of F. oxysporum (Schroffelmee et al., 1999) and 20% in Aspergillus spp. (Bulaw, 1970), compared to only 1–2% in the yeast S. cerevisiae (Bulaw, 1993). This difference may explain the high number of structural chs genes identified in filamentous fungal species such as A. fumigatus, A. nidulans and N. crassa, in contrast to only three genes encoding the catalytic subunits reported in S. cerevisiae (Roncero, 2002) and four genes in Candida albicans (Munro & Gow, 2001). In F. oxysporum f. sp. lycopersici a gene encoding a class V CS was isolated by random insertional mutagenesis and screening for pathogenicity mutants. The ChsV deduced protein carries a myosin domain in the N-terminal region and is required during host infection and for maintenance of cell wall integrity (Madrid et al., 2003). In this study, we report the isolation of four chs genes, chs1, chs2, chs3 and chs7 from F. oxysporum, and the construction of three targeted disruption mutants. We have characterized the deficient mutants for hyphal morphogenesis, nuclear distribution, and physiological and pathotypic behaviour.

**METHODS**

**Strains and culture conditions.** F. oxysporum f. sp. lycopersici wild-type strain 4287 (race 2) was obtained from J. Tello, Universidad de Almería, Spain. The mutant strain deficient in the class V CS gene chsV has been described elsewhere (Madrid et al., 2003). Microconidial suspensions were stored with glycerol at −80°C. The pathotype of the isolates was periodically confirmed by plant infection assays. For extraction of genomic DNA, mycelium was obtained from cultures grown in potato dextrose (glucose) broth (PDB, Difco) on a rotary shaker at 170 r.p.m. and 28°C as described previously (Di Pietro & Roncero, 1998). For phenotypic analysis of colony growth inhibition or hydrophobic characteristics, microconidia were collected from PDB, washed in sterile water, counted and transferred to synthetic medium (SM) plates (Di Pietro & Roncero, 1998) supplemented or not with the following metabolites at the concentrations indicated: sorbitol, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), caffeine, z-tomatine, Congo red and hydrogen peroxide (all from Sigma). SDS sensitivity was analysed on SM plates containing 0-025% (w/v) SDS. SM or PDA media were supplemented with hygromycin, for selection and/or maintenance of transformant phenotypes when required, at the appropriate concentrations.

**Construction of gene disruption vectors.** The gene replacement vectors pDChs1::hyg, pDChs2::hyg, pDChs3::hyg and pDChs7::hyg were constructed by following the general strategy of inserting the hygromycin-resistance (HygR) cassette from plasmid pH1B (Turgeon et al., 1987), interrupting the ORF of the corresponding chs gene. In the case of the chs1 gene an internal BamHI fragment was replaced by the HygR cassette. In the case of chs2, chs3 and chs7, the HygR cassette was inserted into a BamHI internal sequence newly created by site-directed mutagenesis with the use of two divergent and complementary specific primers containing this restriction site into the ORFs (Horton et al., 1989). Linear DNA fragments containing the different interrupted chs alleles were generated by amplification of the entire constructs using primer pairs flanking both ends of the disrupted genes as indicated in Fig. 3(a).

**Transformation-mediated gene replacement.** The final amplified constructs containing the F. oxysporum genomic DNAs with
the chs coding regions interrupted with the Hyg<sup>R</sup> cassette were used for transformation of <i>F. oxysporum</i> 4287 protoplasts to hygromycin resistance according to a protocol described previously (Di Pietro & Roncero, 1998). Briefly, microconidia were germinated for 14 h in SM before being submitted to protoplasting (Garcia-Maceira et al., 2000). Transformants were selected on hygromycin-containing plates, then purified by monoinatorial isolation by two consecutive rounds of single spore isolation before being stored as microconidial suspensions at −80 °C.

**Primers, PCR amplification and cloning of PCR products.** The primer pair initially used for amplification of CS domains was CHS-I-5′-CTGAAGGTCAATGAYAYGARGAY-3′ and CHSI-2 5′-GTTCTGAGYTTYAYTCAARATTYG-3′, designed based on highly conserved regions from different family 1 chs genes (Vidal-Cros & Bocca, 1998). For amplification of the <i>F. oxysporum</i> chs7 gene the degenerate primers CHS7-1 5′-CCTGAAYGCTACNAGTATATATATG-3′ and CHSI-2 5′-GTTCTGAGYTTYAYTCAARATTYG-3′, designed based on highly conserved regions from different family 1 chs genes (Vidal-Cros & Bocca, 1998). For amplification of the <i>F. oxysporum</i> chs7 gene the degenerate primers CHS7-1 5′-ATHAAAYGNTTYGTNG-GNTYCAR-3′ and CHS7-2 5′-TCCCARAAAYYRTTACACATCA-TNAC-3′ were designed by comparison analysis between chs7 from <i>S. cerevisiae</i> (Trilla et al., 1999) and the corresponding orthologue gene identified at the <i>A. fumigatus</i> genome database (http://www.tigr.org/tdb/e2k1/afu1/release.shtml). <i>F. oxysporum</i> genomic DNA was PCR-amplified with the following conditions: first cycle of 5 min at 94 °C, then 30 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C, followed by one cycle of 10 min at 72 °C. The PCR-amplified fragments obtained were analysed by gel electrophoresis and purified by the GeneClean Turbo (Q-BIO gene). The eluted fragments were cloned into pGEM-T vector (Promega) and their identities were verified by DNA sequencing before using them as probes for screening of <i>F. oxysporum</i> libraries.

**Nucleic acid manipulations and cloning of chs genes.** The chs1, chs2, chs3 and chs7 genes were isolated from a λ-EMBL3 genomic library of <i>F. oxysporum</i> f. sp. lycopersici strain 4287, probed with fragments obtained by PCR amplification of <i>F. oxysporum</i> DNA using primers from conserved regions of different Cses as described above. A cDNA clone from chs7 was isolated from screening of a λ-ZAP cDNA library (Roldán-Arjona et al., 1999). Screening of libraries, subcloning and other routine procedures were performed as described in standard protocols (Sambrook et al., 1989). Sequencing of both DNA strands was performed at the Servicio de Secuenciación de la Universidad de Córdoba, using the DyeDeoxy Terminator cycle sequencing kit (PE Biosystems) on an ABI Prism 377 genetic analyser. Analyses of sequencing data were carried out using the Lasergene program (DNASTar). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Total RNA and genomic DNA were extracted from <i>F. oxysporum</i> mycelium as described previously (Chomczynski & Sacchi, 1987; Aljanabi & Martinez, 1997). Southern and Northern hybridization analysis and probe labelling were performed as described in standard protocols using the non-isotopic digoxigenin labelling kit (Roche Diagnostics) according to the instructions of the manufacturer. RT-PCR amplification was carried out as described previously (Garcia-Maceira et al., 2000). First-strand cDNAs were generated from total RNA isolated from mycelia grown on SM or SM containing 1 mM hygromycin (Sigma). Portions of 0.1 ml from each sample were removed and assayed for GlcNAc content (Reissig et al., 1955).

**Determination of conidial content.** Conidial suspensions from wild-type and <i>AchV</i> mutant strains were inoculated on PDB medium at a concentration of 5 × 10<sup>4</sup> spores ml<sup>−1</sup>, and incubated on a rotary shaker at 28 °C. Samples were collected at regular intervals from 0 to 70 h, and the number of microconidia present in the cultures was haematocytometer under the microscope (Olympus, BH-2).

**Morphological analyses by fluorescence microscopy.** For microscopic analysis of the wild-type strain and Δchs1, Δchs2, ΔchsV, Δchs7, samples were cultured and incubated on a rotary shaker at 28 °C. Samples were collected at regular intervals from 0 to 70 h, and the number of microconidia present in the cultures was haematocytometer under the microscope (Olympus, BH-2).

**Pathogenicity assays on tomato plants.** Infection of tomato plants was performed as reported previously (Di Pietro & Roncero, 1998). Briefly, tomato seedlings of cv. ‘Vemar’ were inoculated with <i>F. oxysporum</i> f. sp. lycopersici strains by dipping the roots in a microconidial suspension, planting the seedlings in minipots with vermiculite and maintaining them in a growth chamber at 25 °C with 14 h light and 10 h dark. Plants immersed in sterile water were used as controls. For statistical analyses, the severity of disease symptoms was recorded from 1 week after the inoculation every 2 days until day 24 post-infection according to a scale ranging from 1 (healthy plant) to 5 (dead plant) (Di Pietro & Roncero, 1998). Fifteen plants were used for each treatment. The area under the disease progress curve (AUDPC) was calculated for each plant. The AUDPC means of the mutants were compared to that of the wild-type by Student’s t-test. All pathogenicity assays were performed at least twice with similar results. Plant seeds were kindly provided by Syngenta Seeds (El Ejido, Almería, Spain).
RESULTS

Isolation of *F. oxysporum* genes chs1, chs2, chs3 and chs7

PCR was used to amplify genomic DNA from the wild-type strain 4287 of *F. oxysporum* f. sp. lycopersici, with the degenerate primers CHSI-1 (a consensus between CHSI-1/CHSI-2) and CHSI-3 (Vidal-Cros & Boccara, 1998). The products were cloned, and the resulting recombinant plasmids initially characterized by restriction mapping revealed different restriction patterns, indicating the presence of diverse PCR products. DNA sequence analysis of several plasmid inserts allowed the identification of three different CS sequences. The translation products of these several plasmid inserts, designated fchs1, fchs2 and fchs3, displayed highest homologies to the deduced polypeptides of the genes chs1 from *Gibberella zeae* (GenBank accession number AJ312243), chs2 from *N. crassa* (X77782) (Din & Yarden, 1994) and chsB from *Glomerella graminicola* (AY052546), respectively. Isolation of the ortholog of *S. cerevisiae* CHS7 was accomplished by PCR amplification of *F. oxysporum* genomic DNA with a pair of degenerate primers deduced from the putative *A. fumigatus* orthologue gene by *in silico* identification in the available genome database (http://www.tigr.org/db/e2k1/afal1/release.shtml). The product was cloned and identified by sequencing and this *F. oxysporum* DNA insert was designated fchs7.

The *F. oxysporum* λ-EMBL3 genomic library available in our group was probed with the four PCR fragments (fchs1, fchs2, fchs3 and fchs7). The positive clones were subjected to PCR amplification using two λ primers, λ-int 5′-GCAGACTCGTGAAAGGTATA-3′ and λ-int 5′-AAGTCCACCCCA-GATAACGAT-3′. The *F. oxysporum* inserts present in the recombinant clones were amplified by PCR and sequenced using the strategy of DNA-walking with specific synthetic oligonucleotides (CHSI-4 to CHSI-19 for chs1, CHSII-4 to CHSII-21 for chs2, CHSIII-4 to CHSIII-19 for chs3, CHS7-3 to CHS7-15 for chs7). DNA sequences were determined and analysed by using the Lasergene Navigator and the BLAST algorithm (Altschul *et al*., 1990) at the NCBI. The gene sequences have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers AF572421, AF572422, AF572423 and AF572424.

Nucleotide sequences and predicted polypeptides of the chs1, chs2, chs3 and chs7 genes

The chs1 gene encodes a predicted polypeptide of 901 amino acids with a calculated mass of 102·3 kDa and a pI of 7·0, chs2 encodes a predicted polypeptide of 1041 amino acids with a calculated mass of 117·5 kDa and a pI of 7·5, and chs3 encodes a potential ORF of 978 amino acids with a calculated mass of 110·4 kDa and a pI of 7·0. The degree of identity at the amino acid level shared by the three polypeptides was 41% between FoChs1 and FoChs2, and 29·6% between FoChs2 and FoChs3. The N-terminal sequences of the three polypeptides diverge considerably, whereas the C-terminal sequences are more conserved as expected for the catalytic domains, with the motif QRRRW, essential for the catalytic activity (Cos *et al*., 1998), present in all three polypeptides. Comparison of the three predicted proteins with other fungal CSs gave the highest overall similarity with the following identified genes: FoChs1 with *G. zeae* Chs1 (86·8%), FoChs2 with *N. crassa* Chs2 (67·9%), and FoChs3 with *G. graminicola* ChsB (67·5%) (Din & Yarden, 1994; Yarden & Yanofsky, 1991). Based on sequence similarities at the amino acid level and conservation of catalytic motifs the *F. oxysporum* genes chs1, chs2 and chs3 presumably belong to class I, class II and class III CSs, respectively. Fig. 1 shows the phylogenetic tree of relatedness between the complete deduced amino acid sequences of *F. oxysporum* chs1, chs2 and chs3, with selected members of fungal family I CS-encoding genes, obtained by CLUSTALW analysis (PAM 250). According to the dendrogram, class I and class II fungal CSs share higher degrees of identity and thus have diverged before those of class III.

*F. oxysporum* chs7 encodes a predicted polypeptide of 334 amino acids with a calculated mass of 36·8 kDa and a pI of 4·9. FoChs7 has about 38·9% identity with Chs7p from *S. cerevisiae* (SWISSPROT/AAB68984), responsible for a chaperone involved specifically in ScChs3p export from the ER (Trilla *et al*., 1999), and 73·1% identity with a hypothetical protein deduced from the *N. crassa* genome database ‘assembly version 3’ (Galagan *et al*., 2003) (Fig. 2).

The position of introns present in the genes was located initially by comparison with other related CS polypeptides; when feasible this was confirmed by sequencing the corresponding cDNA isolated from a *F. oxysporum* cDNA library, as well as the transcriptional 5′ terminus and the poly(A) signal site. The promoter regions of the chs genes were analysed at the nucleotide level. The AbaA Response Element (ARE) binding sequence -CATTCC- (Andrianopoulos & Timberlake, 1994) was found in the promoter of the chs2 gene at positions −807, −467 and −462 relative to the ATG codon, and in gene chs7 at position −283. The sequence -CCAAAT- to which the HAP complex binds (Litzka *et al*., 1998) was present at position −632 in the chs1 promoter, at positions −996 and −921 in the chs2 promoter, at positions −588 in the chs3 promoter, and at position −690 in the chs7 promoter. The stress-response element binding site (STRE) -CCCCCT- (Estruch, 2000) was present at positions −363, −325, −308 in gene chs1, at positions −221, −186 in gene chs2, at positions −419 and −375 in gene chs3, and at positions −752, −377, −314, −263 and −175 in gene chs7.

**Targeted disruption of the chs1, chs2, chs3 and chs7 genes and molecular characterization of defective mutants**

Disruption vectors for chs genes were constructed by insertion of the hygromycin-resistance cassette gene into the
coding region of the corresponding genes (Fig. 3a). In the case of \textit{chs1} an internal \textit{BamHI} fragment was replaced by the \textit{Hyg}^R cassette (\textit{Fusarium}-disruption plasmid pDChs1). In the other three genes the \textit{Hyg}^R cassette was inserted into a \textit{BamHI} internal sequence created by site-directed mutagenesis with the use of two divergent complementary specific primers containing the \textit{BamHI} site (Horton et al., 1989). The resulting constructs were designated \textit{Fusarium}-disruption plasmids pDChs2, pDChs3 and pDChs7. Hygromycin resistance selection allowed the isolation of transformants harbouring the disrupted version of genes \textit{chs1}, \textit{chs2} and \textit{chs7}. A number of these hygromycin-resistant

**Fig. 1.** Phylogenetic tree of relatedness between the predicted products of the Fochs1, Fochs2 and Fochs3 genes with fungal CSs belonging to class I, class II and class III. The deduced polypeptide sequences were aligned and subjected to CLUSTALW analysis. Species, genes and accession numbers are as follows: \textit{F. oxysporum} f. sp. lycopersici, \textit{chs1} (AY572421), \textit{chs2} (AY572422) and \textit{chs3} (AY572423); \textit{F. graminearum}, Fg10327, Fg12352, Fg12376 and Fg10116, http://www.broad.mit.edu; \textit{G. zeae} \textit{chs1} (AJ312243); \textit{G. graminicola} \textit{chsA} (AY052545) and \textit{chsB} (AY052546); \textit{N. crassa} \textit{chs1} (M73437), \textit{chs2} (X77782) and \textit{chs3} (AF127086); \textit{Emicicella nidulans}, \textit{chsA} (D21268), \textit{chsB} (D21269) and \textit{chsC} (AB023911); \textit{A. fumigatus}, \textit{chsA} (AAB33397), \textit{chsB} (AAB33398), \textit{chsC} (X94245) and \textit{chsG} (X94244); \textit{Phaeosphaeria nodorum} \textit{chs2} (AJ133695). The scale bar indicates relative divergence between sequences.

**Fig. 2.** Multiple alignment of the deduced amino acid sequences of \textit{F. oxysporum} \textit{chs7} (AY572424), \textit{S. cerevisiae} \textit{chs7} (SWISSPROT/AAB68984) and \textit{N. crassa} NCU05720 (\textit{chs7-like} hypothetical protein, from http://www.broad.mit.edu/ annotation/fungi/neurospora). Location of primers used for RT-PCR are shown by arrows; the discontinuous part in \textit{CHS7-20} corresponds to the amino acids absent in the \textit{Fochs7} gene product. The position of the intron is shown by an open triangle.
Transformants were analysed by restriction enzyme digestion of genomic DNA and Southern hybridization. Transformants originated by gene replacement were obtained for \( \text{chs}1 \), \( \text{chs}2 \) and \( \text{chs}7 \) as shown by a shift of the hybridizing DNA fragments in wild-type strain 4287, from 1 kb to 3 kb, and the absence of some hybridizing bands (in transformant \( \text{Dchs}1 \)), from 6–5 kb to 8–5 kb (in transformants \( \text{Dchs}2.1 \) and \( \text{Dchs}2.6 \)), and from 10 kb to 8–5 kb (in transformant \( \text{Dchs}7 \)) (Fig. 3b). All 45 transformants obtained with the disruption vector pDChs3 showed ectopic integration of the transforming DNA, suggesting a non-viable phenotype for deletion of this CS class III gene (data not shown).

Attempts were made to determine the transcription levels of the different \( \text{chs} \) genes in wild-type and \( \text{Dchs} \) mutant strains by Northern analyses using total RNA obtained from mycelia grown on PDB or SM media with or without one of the following compounds: 1–2 M sorbitol, \( \alpha \)-tomatine, Congo red, hydrogen peroxide or caffeine. No detectable hybridization signal was obtained with any of the probes used. Therefore RT-PCR was used to determine the expression of these genes during hyphal growth, of \( \Delta \text{chs}1 \), \( \Delta \text{chs}2 \) and \( \Delta \text{chs}7 \) mutants, and the wild-type strain, in liquid media with or without osmotic stabilizer (Fig. 4). For PCR amplification of each gene transcript a pair of specific primers flanking an intron was used (as described in Methods). As shown in Fig. 4, no differences in the transcription levels of the \( \text{chs} \) genes between the different disruption mutants and the wild-type strain were observed with or without osmotic stabilizer in the growth medium.

**Conidiation, septum and nuclei distribution, physiological behaviour and colony hydrophobicity of \( \Delta \text{chs} \) mutants**

Conidiation in the three \( \Delta \text{chs} \) disruptants was examined microscopically and found to be indistinguishable from that in the wild-type strain (not shown). Initial inocula containing \( 1 \cdot 4 \times 10^5 \) micronidia ml\(^{-1} \) were germinated on PDB medium at 28°C and 170 r.p.m. Conidiation was determined by counting spores under the microscope at different time intervals. These \( \Delta \text{chs} \) mutants showed no significant difference in the number and the morphology of conidia produced in submerged cultures in comparison to the wild-type strain.

The positions of septa and the distribution of nuclei were examined in the wild-type and the \( \text{chs} \)-deficient mutants (Fig. 5) by fluorescence microscopy using CFW and DAPI staining. The wild-type strain, \( \Delta \text{chs}2 \) and \( \Delta \text{chs}7 \) mutants showed similar septation and even nuclear distribution, with each hyphal compartment containing only...
**Fig. 4.** Detection of *chs* gene transcripts. RT-PCR products were obtained from wild-type and Δ*chs1*, Δ*chs2*, Δ*chsV* and Δ*chs7* mutant strains, grown in SM or SM with 1·2 M sorbitol. PCR amplification using *F. oxysporum* genomic DNA as template was used as control. The DNA ladder is indicated.

**Fig. 5.** Fluorescence microscopy observation of germlings, grown for 14 h in PDB, from *F. oxysporum* wild-type (a) and different CS mutant strains, Δ*chsV* (b and b'), Δ*chs1* (c and c'), Δ*chs2* (d), Δ*chs7* (e). DAPI and CWF staining of nuclei, hyphae and septal walls (×200 magnification; ×400 in b' and c').
one nucleus (Fig. 5a, d, e), whereas in the Δchs1 mutant some compartments containing up to four nuclei could be seen (Fig. 5c, c'). The class V deficient mutant (ΔchsV) was also included in this analysis due to its abnormal morphology showing swollen, balloon-like structures along the hyphae, previously described (Madrid et al., 2003). These structures frequently contained up to eight nuclei (Fig. 5b, b'). These results indicate that nuclear sorting/distribution seems to be affected in Δchs mutants but not septum formation.

The growth of the Δchs1, Δchs2 and Δchs7 strains was determined in the presence of the detergent SDS, which affects membrane integrity. The Δchs2 mutant was at least 100 times more sensitive to SDS compared to the wild-type strain, whereas the Δchs1 and Δchs7 mutants were slightly more sensitive (Fig. 6a). No evidence for hyphal lysis was observed using the vital stain BCIP, as detected by the lack of a light blue zone surrounding the colonies of these three Δchs mutants (data not shown). No differences in colony growth rates of mutants were observed in the presence of other compounds assayed, including the chitin-binding dye CFW, plant defence compounds (α-tomatine, caffeine or hydrogen peroxide) or Congo red, which interferes with cell wall assembly.

To investigate whether inactivation of CS genes alters the hydrophobicity of the colony surface, drops of water were placed on the colony centre of the mutant strains Δchs1, Δchs2, Δchs7 and the wild-type, grown in synthetic medium with or without 1-2 M sorbitol, and observed after 48 h. All the Δchs-deficient mutants showed greater hyphal hydrophobicity than the wild-type strain when grown in sorbitol-containing medium (Fig. 6b). In this analysis the class V deficient mutant (ΔchsV) was also included because of its abnormal colony morphology (Madrid et al., 2003). This deficient mutant ΔchsV showed the same hydrophobicity phenotype as the wild-type strain (not shown) in both media.

**Chitin content of Δchs1, Δchs2 and Δchs7 mutant strains**

The total mycelial chitin content of the Δchs1, Δchs2 and Δchs7 mutants was measured by determining the amount of GlcNAc after digestion of the cell wall with chitinase and gluclusase. The chitin content of deficient mutants was found to be reduced only in Δchs1 and Δchs2, with a 10 % reduction in comparison to wild-type strain 4287 (Table 1). It has been previously described in A. fumigatus that only mutants defective in class III CS or homologues of this protein have a significant reduction in chitin content (Din et al., 1996), while mutants defective in the zymogen type of enzyme typically have a normal or a small reduction (10 %) in chitin content and CS activity (Mellado et al., 1996a).

**Pathotypic behaviour of Δchs1, Δchs2 and Δchs7 mutant strains**

To determine the effect of Δchs mutations on virulence of F. oxysporum, root infection assays with tomato plants were performed. Two-week-old plants were inoculated by immersing their roots in a microcomidal suspension of the wild-type strain, or the disruptants Δchs1, Δchs2 and Δchs7.
Table 1. Summary of Δchs phenotypes

Disease index refers to symptoms observed on tomato plants 21 days post-inoculation (dpi) with spore suspensions from the different strains. WT, wild-type; ND, not determined. Conidiation of all the mutants was indistinguishable from that of the WT.

<table>
<thead>
<tr>
<th>Chitin synthase class</th>
<th>Mutant</th>
<th>Chitin content/dry weight (% of WT)</th>
<th>Disease index 21 dpi</th>
<th>Colony surface hydrophobicity</th>
<th>Resistance to 0·025% SDS (10^3 spores)</th>
<th>Defects in morphology and cell biology</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Δchs1</td>
<td>90</td>
<td>5 ± 0</td>
<td>+</td>
<td>+</td>
<td>Normal shape but rarely cells with up to 4 nuclei</td>
</tr>
<tr>
<td>II</td>
<td>Δchs2</td>
<td>90</td>
<td>3·8 ± 0·33</td>
<td>+</td>
<td>ND</td>
<td>None</td>
</tr>
<tr>
<td>V</td>
<td>ΔchsV</td>
<td>90*</td>
<td>1 ± 0*</td>
<td>−</td>
<td>ND</td>
<td>Swollen cells with up to 8–10 nuclei</td>
</tr>
<tr>
<td>Chaperone-like</td>
<td>Δchs7</td>
<td>100</td>
<td>4·3 ± 0·30</td>
<td>+</td>
<td>+</td>
<td>None</td>
</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>100</td>
<td>5 ± 0</td>
<td>−</td>
<td>+ +</td>
<td>None</td>
</tr>
</tbody>
</table>

*Madrid et al. (2003).

Plants were scored for vascular wilt symptoms at different time intervals (Di Pietro & Roncero, 1998). The development of the disease is shown in Fig. 7. Plants inoculated with the wild-type strain produced characteristic wilt symptoms starting 7 days after inoculation. Disease severity increased steadily throughout the experiment, and most of the plants were dead 20 days after inoculation, except for those inoculated with Δchs2 or Δchs7 mutants, which were delayed in the progression of disease. Sixteen days after inoculation most of these plants showed disease symptoms with a degree of 3 in a scale of 5 compared with those inoculated with the wild-type (Di Pietro, 1998). After the lag phase, the severity of wilting increased progressively, and most of the plants inoculated with wild-type and the rest of the Δchs mutants were dead after 24 days except for those infected with Δchs2 or Δchs7 mutants. The mean response of the mutant Δchs1 (AUDPC = 54·27) was not significantly (P = 0·3743) different from that of the wild-type (AUDPC = 56·40). The mutants Δchs2 and Δchs7 significantly (P = 0·0012, P = 0·0350, respectively) differed in virulence towards tomato plants (AUDPC = 37·10, AUDPC = 45·20, respectively) compared with the wild-type.

**DISCUSSION**

Previously a class V CS-encoding gene (chsV) had been isolated and shown to be required for pathogenicity during host infection by *F. oxysporum* f. sp. *lycopersici* (Madrid et al., 2003). The results presented here report the identification of three genes, chs1, chs2 and chs3, encoding structural CSs. The high degree of conservation in CS catalytic domains allowed the design of a unique pair of degenerate primers for PCR amplification of genomic DNA. Based on deduced amino acid sequences, these newly isolated *F. oxysporum* chs genes presumably encode class I, class II and class III enzymes, all belonging to family I (Bowen et al., 1992). The deduced amino acid sequences of the *F. oxysporum* chs genes share a considerable degree of identity, ranging from 41% to 30% and extending mainly between amino acids positions 190 to 600. Another gene, chs7, orthologous to the *S. cerevisiae* CHS7 which is required for functional ScChs3p activity (class IV) and responsible for its export from the ER (Roncero, 2002), has been isolated. Thus, the complexity of chitin synthesis in this pathogenic filamentous fungus, at the sequence and protein levels, appears to be similar to that in related filamentous fungi species. Interestingly, the most perturbed mould phenotype is seen when CSs of the mould-specific class III and V are disrupted, whereas minor or no phenotypes are seen when members of class I, II and IV are inactivated (Mellado et al., 2003). In accordance with this, targeted inactivation of genes chs1 and chs2 in *F. oxysporum* f. sp. *lycopersici*, from class I and class II respectively, caused no major effects in sporulation rates, chitin content,
morphology or hyphal growth, while the ΔchsV mutant (class V) displays morphological abnormalities and cell lysis, and is non-pathogenic and hypersensitive to plant antimicrobial defence compounds (Madrid et al., 2003); and all the attempts to disrupt the chs3 gene (class III) in *F. oxysporum* by gene-replacement-mediated transformation were unsuccessful, possibly due to a lethal phenotype of the null mutants. Nevertheless, in the human pathogen *A. fumigatus* two class III genes, chsC and chsG, have been characterized and replacement mutants independently targeted as well as double disruptants have been obtained, demonstrating the dispensable functions of the encoded proteins (Mellado et al., 1996a). In *A. nidulans*, disruptants in a class III gene (chsB), growing as minute colonies without conidia and producing hyphae with high degree of branching, have been isolated (Borgia et al., 1996). In spite of the numerous reports devoted to the molecular and cellular biology of chs genes and defective mutants derived therefrom, the localization and specific functions of the different CSs have not been elucidated completely in filamentous fungi. In *A. nidulans* the construction of single and double gene replacement mutants in CS genes, together with the use of vital reporter systems, such as β-galactosidase or green fluorescent protein, enabled the demonstration that chsA (class II) is expressed specifically during asexual differentiation, whereas chsB (class III) is ubiquitous throughout the fungal body and independent of the developmental status. chsC (class I) expression is temporally and spatially regulated, being moderate during sexual development and in the early phase of vegetative growth (Specht et al., 1996; Fujiwara et al., 2000; Lee et al., 2004).

No Δchs7 mutants have been described to date in filamentous fungi. *S. cerevisiae* Δchs7 mutants have reduced levels of CSIII activity (class IV) and chitin in their cell walls, defects comparable to those observed in the ScΔchs3 mutants (Trilla et al., 1999), and stronger than those detected in ScΔchs4 (Trilla et al., 1997), ScΔchs5 (Santos et al., 1997) or ScΔchs6 mutants (Bulawa, 1993), underscoring the relevance of this gene in the control of CSIII activity. The attempts to isolate a chs gene belonging to class IV in *F. oxysporum* failed; however, according to an in silico search in the *F. graminearum* database, the presence of one or more class IV chs genes can be expected in *F. oxysporum*. Nevertheless, the close relationship previously reported between Scchs7 and regulation of class IV CSs (Roncero, 2002) make it possible to refer to the Δchs7 *F. oxysporum* mutant as being defective in class IV activity.

Promoter analyses of the *F. oxysporum* chs DNA sequences studied identified potential recognition site motifs for different transcription activators such as AbA, one of the key regulatory transcription factors involved in asexual development in *Aspergillus* spp. Several putative ARE elements are found in chs2 and chs7 genes, suggesting a developmentally regulated mechanism similar to that described for the *A. nidulans* chsC (Park et al., 2003). The stress response sequence STRE was present at different positions in the promoters of the four genes analysed. The chs genes might have a special role in cell proliferation and/or in maintaining the structural integrity of the cell wall and may therefore be activated in response to stress signals (Wang et al., 2002), explaining the higher sensitivity of the Δchs1, Δchs2 and Δchs7 mutants to alterations of the membrane caused by detergents, as well as their stronger hyphal hydrophobicity when grown on medium of high osmotic pressure. These different hydrophobic properties shown by colonies of Δchs1, Δchs2 and Δchs7 mutants on sorbitol plates, in comparison to wild-type and ΔchsV mutant, are reminiscent of phenotypes of mutants affected in hydrophobin assembly or production. These small secreted proteins are fundamental to the development of fungi; to date, more than 20 hydrophobin-encoding genes have been identified and proven to be ubiquitous in filamentous fungi (Wessels, 1997). The existence of hydrophobic proteins in the outer layer of conidial walls and their involvement in the construction of the conidial outer wall has been demonstrated in the human pathogen *A. fumigatus*, as have their interactions with the cell wall components altering the surface properties of the fungus (Paris et al., 2003).

The mutants Δchs2 (class II) and Δchs7 (related to class IV) show a significant reduction of virulence, and ΔchsV (class V) has been previously reported as non-pathogenic (Madrid et al., 2003). These results indicate the critical importance of the cell wall in the pathogenicity of *F. oxysporum*. The reduction in virulence of these chs mutants might be indicative of higher sensitivity to plant defence compounds caused by permeability differences in the cell wall. Accordingly, colonial growth of these mutants was more sensitive to SDS than that of the wild-type, suggesting an altered composition and structure of the cell wall that may be affecting hyphal permeability. Nevertheless, comparable levels of resistance to plant defence compounds, like α-tomatine, caffeine and hydrogen peroxide, as well as to compounds interfering with cell wall assembly, such as CFW or Congo red, were observed in Δchs2 and Δchs7 in comparison with wild-type (data not shown), while ΔchsV showed a higher sensitivity (Madrid et al., 2003). The association between the cell wall and pathogenicity has been widely reported, and also specifically with CSs. For instance, the reduction of virulence in *Ustilago maydis* by disruption of the genes Umchs6 (class V) and Umchs5 (class IV), and reduced virulence in a *Botrytis cinerea* Δchs1 mutant (class I) have been reported (Garcera-Teruel et al., 2004; Xoconostle-Cázares et al., 1997; Soulie et al., 2003).

The cell wall defects of the ΔchsV and Δchs1 mutant strains could lead to cell cycle alterations that produce the occasional multinuclear phenotype. In *A. nidulans* it has been demonstrated that the NUDC protein, involved in nuclear migration, has an important role in cell wall biogenesis. Defective nudC mutants show aberrant wall deposition such as overproduction of both chitin and glucan, giving grossly abnormal cell walls uniformly
distributed over the cell membrane and the formation of spherical rather than polar cells, suggesting a possible relationship between fungal cell wall biosynthesis and nuclear migration (Chiu et al., 1997). Deletion of the class V CS gene in *F. oxysporum* causes cell swelling and lysis (Madrid et al., 2003), producing aberrant spherical cell structures with up to eight nuclei and suggesting that altered nuclear distribution through mycelium is a consequence of this cell wall deficiency. The requirement of cell wall integrity for dynein anchoring and correct nuclear positioning has been indicated previously in related fungal systems (Chiu et al., 1997). The presence of a myosin motor-like domain in *A. nidulans* CsmA and *F. oxysporum* ChsV proteins suggests that the localization of chitin synthesis may be guided by association with cytoskeletal structures (Fujinawa et al., 1997; Madrid et al., 2003). Whereas in general the morphological phenotype of the *F. oxysporum* CS 1 null mutants (Δchs1) was indistinguishable from wild-type, some abnormal cells with more than one nucleus were observed, supporting the occurrence of similar pleiotropic effects between cell wall structure and nuclear partitioning.

The *F. graminearum* genome (Fusarium graminearum Sequencing Project, Center for Genome Research, http://www.broad.mit.edu), has five representatives of the three mould-specific CS classes (class III, class V and class VI). The identification and characterization of the other components that participate in *Fusarium* cell wall biogenesis will be crucial for the understanding of the process at the molecular level, as well as for the elucidation of the functional relationships with pathogenicity. The results presented here together with other previously reported (Madrid et al., 2003) support the view that CSs may play a role in fungal pathogenesis, and therefore represent potential targets for antifungal intervention (Odds et al., 2003).

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**REFERENCES**


