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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 (UDPGlcNAc) 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-García *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; Hyg^R, resistance to hygromycin; P_t, probability of the statistic absolute *t*.

The GenBank/EMBL/DDBJ accession numbers for the *chs1*, *chs2*, *chs3* and *chs*7 gene sequences reported in this paper are AY572421, AY572422, AY572423 and AY572424, respectively.

(Ruiz-Herrera & Martínez-Espinoza, 1999). Thus, chitosomes have been regarded as the vesicles responsible for the transport of CS from the endoplasmic reticulum (ER) to the cell surface. At present, CSs from filamentous fungi are classified into six categories, class I to class VI, based on conserved regions and the presence of specific myosin motor-like domains (Bowen *et al.*, 1992; Specht *et al.*, 1996; Ruiz-Herrera *et al.*, 2002; Roncero, 2002). The presence of multiple CS genes is common in a single fungal species (Munro & Gow, 2001; Roncero, 2002). In the budding yeast *Saccharomyces cerevisiae*, seven *chs* genes responsible for only three CS activities (CSI, CSII and CSIII) have been well characterized and the functions of the proteins encoded by the *S. cerevisiae* genes are the best understood among 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 (Fujiwara *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 (Af*chsA*, Af*chsB*, Af*chsC*, Af*chsD*, Af*chsF* and Af*chsG*) (Mellado *et al.*, 2003). Inactivation of Af*chsA* (class I), Af*chsB* (class II), Af*chsC* (class III) and Af*chsD* (class IV) does not lead to any obvious phenotypic defect (Mellado *et al.*, 1996a, b), whereas disruption of Af*chsE* (class V) and Af*chsG* (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 (Aufauvre-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 coniodophore 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* (Schoffelmeer *et al.*, 1999) and 20 % in *Aspergillus* spp. (Bull, 1970), compared to only 1–2 % in the yeast *S. cerevisiae* (Bulawa, 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, α-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 (Hyg^R) 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 *Bam*HI fragment was replaced by the Hyg^R cassette. In the case of *chs2*, *chs3* and *chs7*, the Hyg^R cassette was inserted into a *Bam*HI 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^R cassette were used for transformation of *F. oxysporum* 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 (García-Maceira *et al.*, 2000). Transformants were selected on hygromycin-containing plates, then purified by monoconidial 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 CHSI-1 5′-CTGAAGCTTACNATGTAYAAYGARGAY-3′ and CHSI-2 5′-GTTCTCGAGYTTRTAYTCRAARTTYTG-3′, designed based on highly conserved regions from different family I *chs* genes (Vidal-Cros & Boccara, 1998). For amplification of the *F. oxysporum chs7* gene the degenerate primers CHS7-1 5′-ATHAAYGGNTTYGTNG-GNTTYCAR-3′ and CHS7-2 5′-TCCCARAAYTTRTANACCATCA-TNAC-3′ were designed by comparison analysis between *chs7* from *S. cerevisiae* (Trilla *et al.*, 1999) and the corresponding orthologue gene identified at the *A. fumigatus* genome database (http://www.tigr.org/tdb/e2k1/afu1/release.shtml). *F. oxysporum* 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 using 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 *F. oxysporum* 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 *F. oxysporum* f. sp. *lycopersici* strain 4287, probed with fragments obtained by PCR amplification of *F. oxysporum* DNA using primers from conserved regions of different CSs 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 programs (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 *F. oxysporum* mycelium as described previously (Chomzczynski & Sacchi, 1987; Aljanabi & Martínez, 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 (García-Maceira *et al.*, 2000). First-strand cDNAs were generated from total RNA isolated from mycelia grown on SM or SM containing 1·2 M sorbitol as indicated in Fig. 4. PCR was performed using gene-specific primer pairs at the indicated amino acid positions and always flanking one intron. For amplification of *chs1* transcripts, CHSI-8 5′-TCGACTTCCTCCGATCTGAT-3′ (sense, from 561 to 566) and CHSI-22 5′-CCTTGTTCTTGAAGATGTCTG-3′ (antisense, from 690 to 696); for amplification of *chs2*, CHSII-12 5′-CCTCCTCCTTCAG-ATGGCAT-3′ (sense, from 154 to 160) and CHSII-23 5′-GTGGAG-ACTCGGGAACTT-3′ (antisense, from 923 to 928); for amplification of *chs3*, CHSIII-12 5′-GTGTCATGGGGAACAAAGGG-3′ (sense, from 829 to 835) and CHSIII-18 5′-CCTGTAACCCCAAAAGTAT-GT-3′ (antisense, from nucleotides 63 to 43 after the stop codon); for amplification of *chs7*, CHS7-9 5′-GCTGGGCGTTATGATGGT-3′ (sense, from 264 to 269) and CHS7-20 5′-GCGAGTAAGGCAGA-TCATAG-3′ (antisense, from 319 to 325) For each independent experiment the controls included both amplifications, the actin gene for normalization of the PCR conditions, and the *F. oxysporum* genomic DNA for comparison with the intron-containing amplified bands. The cDNA used as template was the same for each experiment, being the amount standardized and spectrophotometrically quantified for normalization of the amplification conditions.

Determination of chitin content. Chitin content was measured by the method described by Din *et al.* (1996) with minor modifications (glusulase was used instead of β-glucuronidase). Mycelium was collected from germlings grown for 20 h minimal medium, washed twice with deionized water and lyophilized. Ten to twenty milligrams dry weight was suspended in 1 ml 6 % (w/v) KOH and incubated for 90 min at 80 °C. Glacial acetic acid (0·1 ml) was added to each sample and the insoluble material was collected by centrifugation at 13 000 ***g*** for 15 min, washed twice with water and suspended in 0·5 ml sodium phosphate buffer (pH 6·3). A 100 µl aliquot of a 5 mg ml^{-1} chitinase suspension (Sigma) was added, and the samples were incubated at 37 °C for 20 h. Following centrifugation at 13 000 ***g*** for 15 min, 450 µl of the supernatant was treated with 25 µl of a 10 000 units ml^{-1} solution of glusulase (New England Nuclear), for 2 h at 37 °C. Portions of 0·1 ml from each sample were removed and assayed for GlcNAc content (Reissig *et al.*, 1955).

Determination of conidiation. Conidial suspensions from wild-type and Δ*chs* mutant strains were inoculated on PDB medium at a concentration of 5×10^5 spores ml^{-1}, 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 counted in a haematocytometer under the microscope (Olympus, BH-2).

Morphological analyses by fluorescence microscopy. For microscopic analysis of the wild-type strain and Δ*chs1*, Δ*chs2*, Δ*chsV*, Δ*chs7* mutants, samples from shaken cultures were diluted with 3·7 % (v/v) formaldehyde, 50 mM phosphate buffer (pH 7), 0·2 % (v/v) Triton X-100, for fixation (Harris *et al.*, 1994), and stained with Calcofluor white (CFW) 10 µg ml^{-1} for 5 min, and/or 4′,6-diamidino-2-phenylindole (DAPI) 0·8 µg ml^{-1} (all from Sigma), and observed using a fluorescence microscope (Leica, DMR) with a 20× or 40× objective and a total magnification of 200 or 400, respectively, on screen.

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 *F. oxysporum* 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 *F. oxysporum* DNA inserts, designated *fchs1*, *fchs2* and *fchs3*, displayed highest homologies to the deduced polypeptides of the genes *chsI* 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 orthologue 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/tdb/e2k1/afu1/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′-CGCA-ACTCGTGAAAGGTA-3′ and λ-git 5′-AAGTCCAACCCA-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 AY572421, AY572422, AY572423 and AY572424.

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 -CATTCY- (Andrianopoulus & 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 -CCAAT- 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) -CCCCT- (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

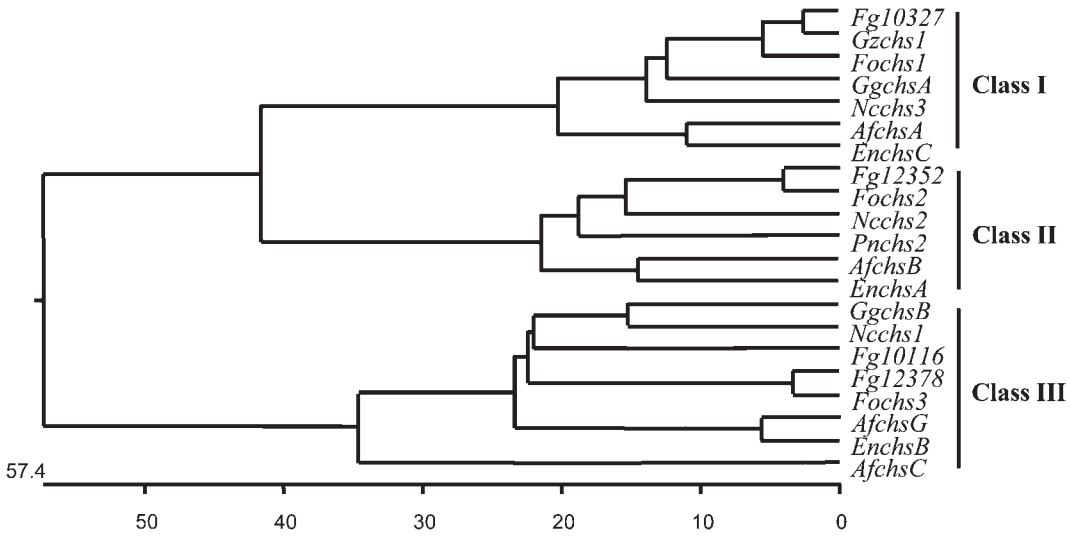

Fig. 1. Phylogenetic tree of relatedness between the predicted products of the *Fochs1*, *Fochs2* and *Fochs3* genes with fungal CSs belonging to class I, class II and class III. The deduced polypeptide sequences were aligned and subjected to CLUSTALW analysis. Species, genes and accession numbers are as follows: *F. oxysporum* f. sp. *lycopersici*, *chs1* (AY572421), *chs2* (AY572422) and *chs3* (AY572423); *F. graminearum*, Fg10327, Fg12352, Fg12378 and Fg10116, http://www.broad.mit.edu; *G. zeae chs1* (AJ312243); *G. graminicola chsA* (AY052545) and *chsB* (AY052546); *N. crassa chs1* (M73437), *chs2* (X77782) and *chs3* (AF127086); *Emericella nidulans*, *chsA* (D21268), *chsB* (D21269) and *chsC* (AB023911); *A. fumigatus*, *chsA* (AAB33397), *chsB* (AAB33398), *chsC* (X94245) and *chsG* (X94244); *Phaeosphaeria nodorum chs2* (AJ133695). The scale bar indicates relative divergence between sequences.

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

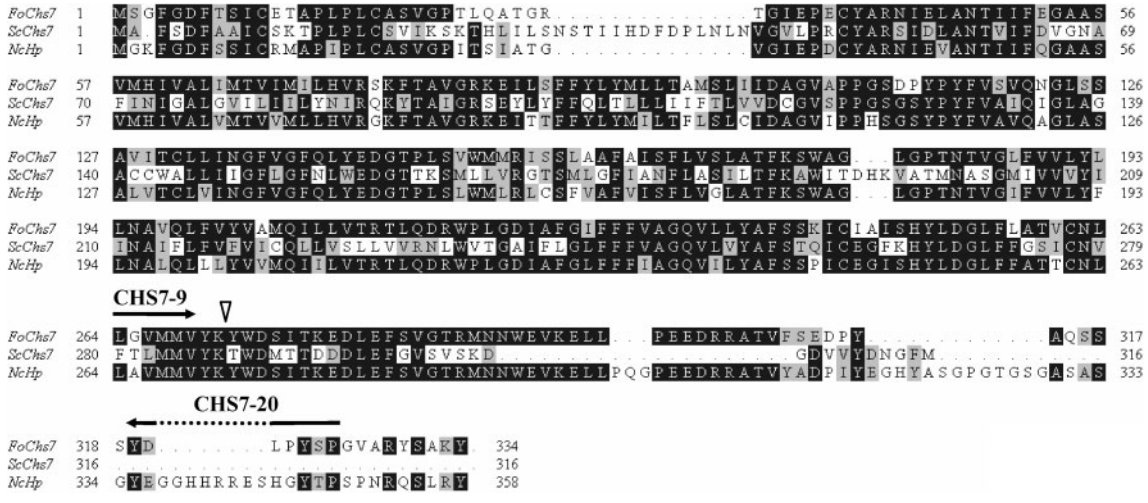

Fig. 2. Multiple alignment of the deduced amino acid sequences of *F. oxysporum chs7* (AY572424), *S. cerevisiae chs7* (SWISSPROT/AAB68984) and *N. crassa* NCU05720 (*chs7*-like hypothetical protein, from http://www.broad.mit.edu/annotation/fungi/neurospora). Location of primers used for RT-PCR are shown by arrows; the discontinuous part in CHS7-20 corresponds to the amino acids absent in the *Fochs7* gene product. The position of the intron is shown by an open triangle.

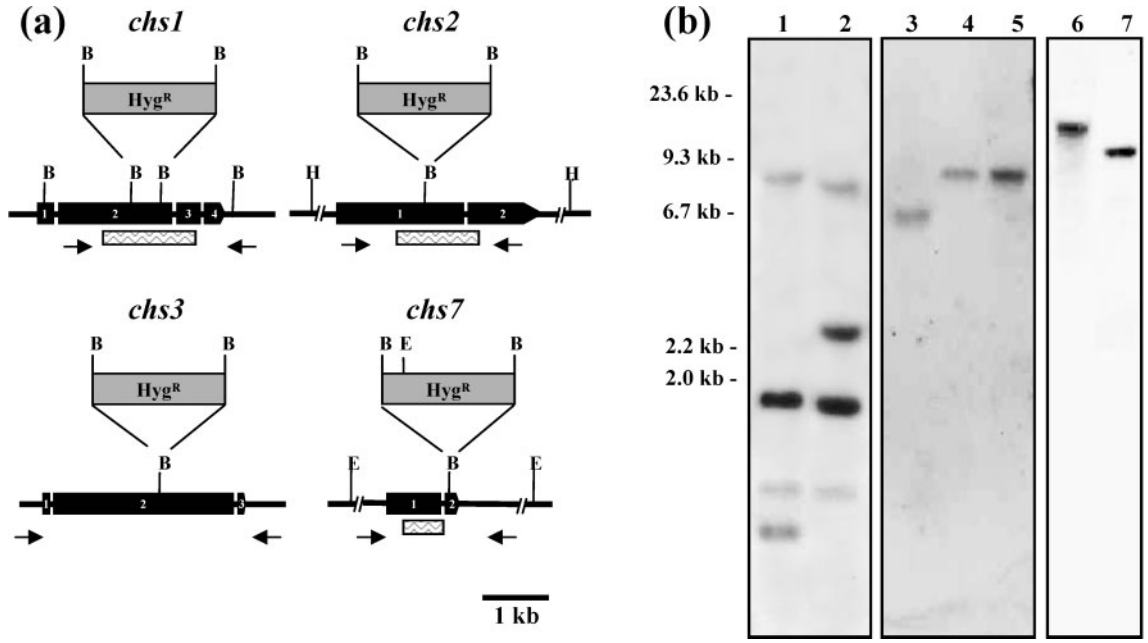

Fig. 3. Targeted replacement of *F. oxysporum* CS genes. (a) Physical maps of the genomic regions and strategy for construction of the different disruption vectors. The *chs1*, *chs2*, *chs3* and *chs7* coding regions are shown as black arrows with the orientation of the ORFs and the introns. Small arrows indicate the primers used for amplification of the gene replacement vectors designated pDChs1, pDChs2, pDChs3 and pDChs7. (b) Analysis of transformants Δ*chs1*, Δ*chs2.1*, Δ*chs2.6* and Δ*chs7* (lanes 2, 4, 5 and 7, respectively) and wild-type strain 4287 (lanes 1, 3, 6) by Southern blotting. Genomic DNAs were digested with *Bam*HI in lanes 1 and 2, with *Hin*dIII in lanes 3, 4 and 5, with *Eco*RI in lanes 6 and 7, then separated in a 0·7 % agarose gel, blotted onto a nylon membrane and hybridized. The corresponding gene probes are indicated as clear horizontal bars in (a). Size markers are shown on the left.

transformants were analysed by restriction enzyme digestion of genomic DNA and Southern hybridization. Transformants originated by gene replacement were obtained for *chs1*, *chs2* and *chs7* 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 Δ*chs1*), from 6·5 kb to 8·5 kb (in transformants Δ*chs2.1* and Δ*chs2.6*), from 10 kb to 8·5 kb (in transformant Δ*chs7*) (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 *chs* genes in wild-type and Δ*chs* 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, α-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 Δ*chs1*, Δ*chs2* and Δ*chs7* 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 *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 Δ*chs* mutants

Conidiation in the three Δ*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 Δ*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 *chs*-deficient mutants (Fig. 5) by fluorescence microscopy using CFW and DAPI staining. The wild-type strain, Δ*chs2* and Δ*chs7* mutants showed similar septation and even nuclear distribution, with each hyphal compartment containing only

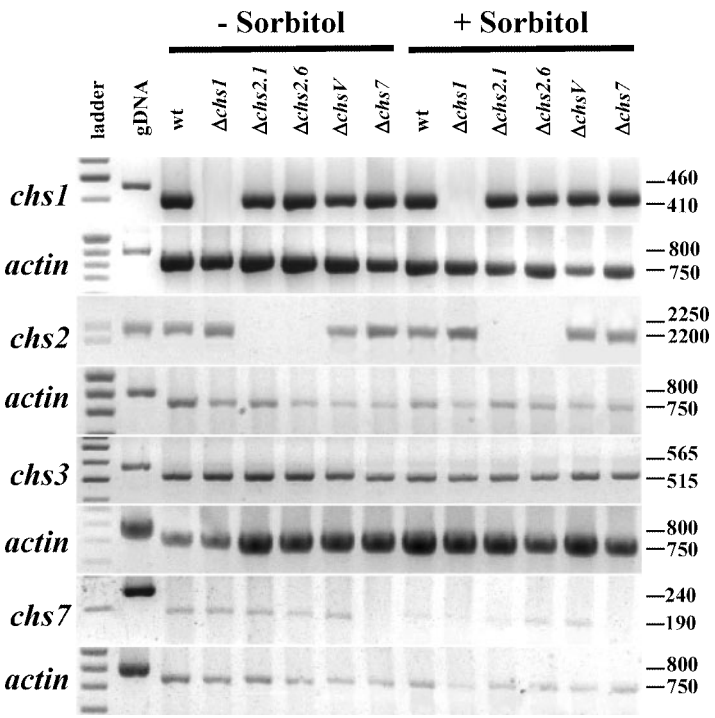

Fig. 4. Detection of *chs* gene transcripts. RT-PCR products were obtained from wild-type and Δ*chs1*, Δ*chs2*, Δ*chsV* and Δ*chs7* mutant strains, grown in SM or SM with 1·2 M sorbitol. PCR amplification using *F. oxysporum* genomic DNA as template was used as control. The DNA ladder is indicated.

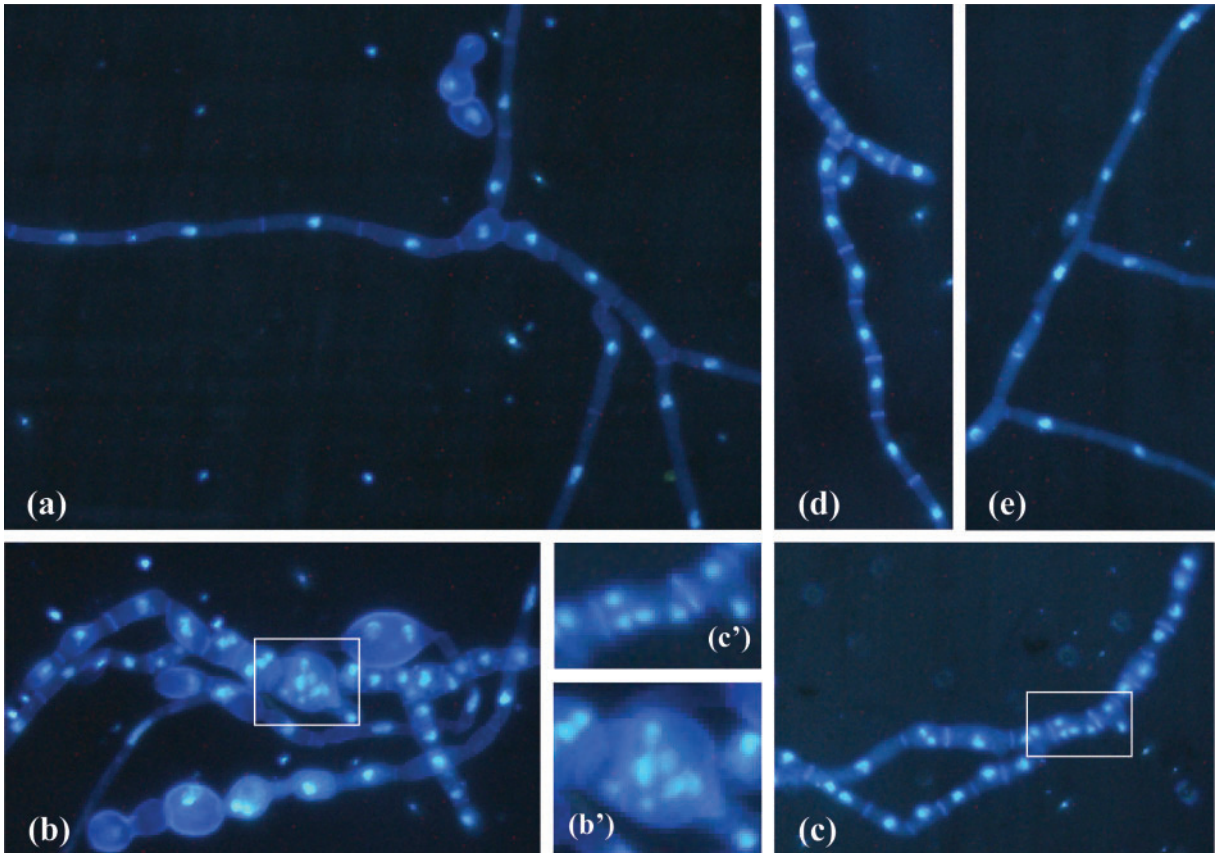

Fig. 5. Fluorescence microscopy observation of germlings, grown for 14 h in PDB, from *F. oxysporum* wild-type (a) and different CS mutant strains, Δ*chsV* (b and b′), Δ*chs1* (c and c′), Δ*chs2* (d), Δ*chs7* (e). DAPI and CWF staining of nuclei, hyphae and septal walls (× 200 magnification; × 400 in b′ and c′).

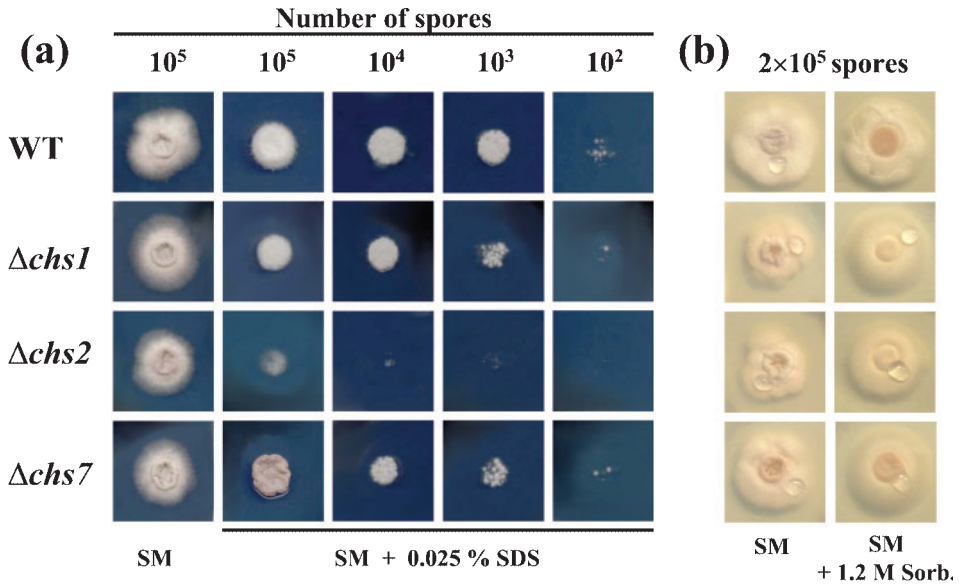

Fig. 6. (a) Colony growth of *F. oxysporum* wild-type 4287 and mutant strains Δ*chs1*, Δ*chs2* and Δ*chs7*, after 3 days at 28 °C on SM plates containing 0·025 % SDS. Tenfold serial dilutions starting with 10^5 spores (columns 1 and 2) were spotted in drops onto the SM plates. First column, control plates without SDS. (b) Hydrophobicity of colony surfaces of the wild-type strain and different CS-deficient mutants grown on synthetic medium (1 % glucose) with or without 1·2 M sorbitol.

one nucleus (Fig. 5a, d, e), whereas in the Δ*chs1* mutant some compartments containing up to four nuclei could be seen (Fig. 5c, c′). The class V deficient mutant (Δ*chsV*) 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 Δ*chs* mutants but not septum formation.

The growth of the Δ*chs1*, Δ*chs2* and Δ*chs7* strains was determined in the presence of the detergent SDS, which affects membrane integrity. The Δ*chs2* mutant was at least 100 times more sensitive to SDS compared to the wild-type strain, whereas the Δ*chs1* and Δ*chs7* 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 Δ*chs* 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 Δ*chs1*, Δ*chs2*, Δ*chs7* and the wild-type, grown in synthetic medium with or without 1·2 M sorbitol, and observed after 48 h. All the *chs*-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 (Δ*chsV*) was also included because of its abnormal colony morphology (Madrid *et al.*, 2003). This deficient mutant Δ*chs*V showed the same hydrophobicity phenotype as the wild-type strain (not shown) in both media.

Chitin content of Δ*chs1*, Δ*chs2* and Δ*chs7* mutant strains

The total mycelial chitin content of the Δ*chs*1, Δ*chs2* and Δ*chs7* mutants was measured by determining the amount of GlcNAc after digestion of the cell wall with chitinase and glusulase. The chitin content of deficient mutants was found to be reduced only in Δ*chs1* and Δ*chs2*, 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 Δ*chs1*, Δ*chs2* and Δ*chs7* mutant strains

To determine the effect of *chs* 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 microconidial suspension of the wild-type strain, or the disruptants Δ*chs1*, Δ*chs2* and Δ*chs7*.

Table 1. Summary of Δ*chs* 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.

Chitin synthase		Chitin content/dry weight (% of WT)	Disease index 21 dpi	Colony surface hydrophobicity	Resistance to 0·025 % SDS (10^3 spores)	Defects in morphology and cell biology
Class	Mutant					
I	Δ*chs1*	90	5±0	+	+	Normal shape but rarely cells with up to 4 nuclei
II	Δ*chs2*	90	3·8±0·33	+	−	None
V	Δ*chsV*	90*	1±0*	−	ND	Swollen cells with up to 8–10 nuclei
Chaperone-like	Δ*chs7*	100	4·3±0·30	+	+	None
WT	WT	100	5±0	−	++	None

*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 Δ*chs2* or Δ*chs7* 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 strain (Di Pietro & Roncero, 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 Δ*chs* mutants were dead after 24 days except for those infected with Δ*chs2* or Δ*chs7* mutants. The mean response of the mutant Δ*chs1* (AUDPC=54·27) was not significantly (P_t=0·3743) different from that of the wild-type (AUDPC=56·40). The mutants Δ*chs2* and Δ*chs7* significantly (P_t=0·0012, P_t=0·0350, respectively) differed in virulence towards tomato plants (AUDPC=37·10, AUDPC=45·20, respectively) compared with the wild-type.

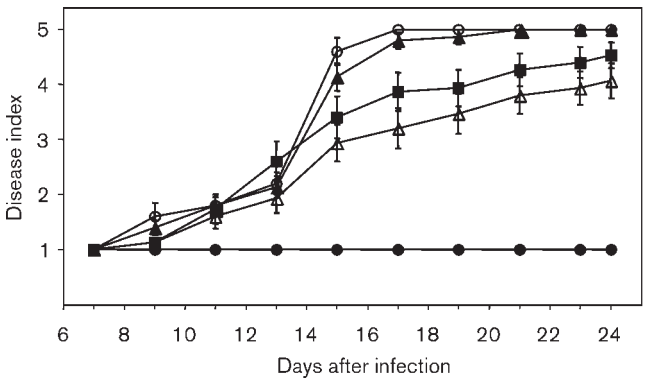

Fig. 7. Virulence of *F. oxysporum* f. sp. *lycopersici chs*-deficient mutants on tomato plants (cv. 'Vemar'). Severity of disease symptoms was recorded at different times after inoculation, using an index ranging from 1 (healthy plant) to 5 (dead plant). Symbols refer to plants inoculated with the wild-type strain 4287 (○), Δ*chs1* (▲), Δ*chs2* (△), Δ*chs7* (■) and the non-inoculated control (●). Error bars indicate the standard error from 15 plants for each treatment.

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 Δ*chsV* 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 Δ*chs7* mutants have been described to date in filamentous fungi. *S. cerevisiae* Δ*chs7* mutants have reduced levels of CSIII activity (class IV) and chitin in their cell walls, defects comparable to those observed in the ScΔ*chs3* mutants (Trilla *et al.*, 1999), and stronger than those detected in ScΔ*chs4* (Trilla *et al.*, 1997), ScΔ*chs5* (Santos *et al.*, 1997) or ScΔ*chs6* 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 Sc*chs7* and regulation of class IV CSs (Roncero, 2002) make it possible to refer to the Δ*chs7 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 AbaA, 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 Δ*chs1*, Δ*chs2* and Δ*chs7* 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 Δ*chs1*, Δ*chs2* and Δ*chs7* mutants on sorbitol plates, in comparison to wild-type and Δ*chsV* 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 Δ*chs2* (class II) and Δ*chs7* (related to class IV) show a significant reduction of virulence, and Δ*chsV* (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 Δ*chs2* and Δ*chs7* in comparison with wild-type (data not shown), while Δ*chsV* 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 Um*chs6* (class V) and Um*chs5* (class IV), and reduced virulence in a *Botrytis cinerea* Δ*chs1* mutant (class I) have been reported (Garcerá-Teruel *et al.*, 2004; Xoconostle-Cázares *et al.*, 1997; Soulie *et al.*, 2003).

The cell wall defects of the Δ*chsV* and Δ*chs1* 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 (Fujiwara *et al.*, 1997; Madrid *et al.*, 2003). Whereas in general the morphological phenotype of the *F. oxysporum* CS 1 null mutants (Δ*chs1*) 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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