Ctf1, a transcriptional activator of cutinase and lipase genes in *Fusarium oxysporum* is dispensable for virulence

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SUMMARY

Cutinolytic enzymes are secreted by fungal pathogens attacking the aerial parts of the plant, to facilitate penetration of the outermost cuticular barrier of the host. The role of cutinases in soil-borne root pathogens has not been studied thus far. Here we report the characterization of the zinc finger transcription factor Ctf1 from the vascular wilt fungus *Fusarium oxysporum*, a functional orthologue of CTF1α that controls expression of cutinase genes and virulence in the pea stem pathogen *Fusarium solani* f. sp. *pisi*. Mutants carrying a $\Delta ctf1$ loss-of-function allele grown on inducing substrates failed to activate extracellular cutinolytic activity and expression of the *cut1* and *lip1* genes, encoding a putative cutinase and lipase, respectively, whereas strains harbouring a *ctf1^C* allele in which the *ctf1* coding region was fused to the strong constitutive *Aspergillus nidulans gpdA* promoter showed increased induction of cutinase activity and gene expression. These results suggest that *F. oxysporum* Ctf1 mediates expression of genes involved in fatty acid hydrolysis. However, expression of *lip1* during root infection was not dependent on Ctf1, and virulence of the *ctf1* mutants on tomato plants and fruits was indistinguishable from that of the wild-type. Thus, in contrast to the stem pathogen *F. solani*, Ctf1 is not essential for virulence in the root pathogen *F. oxysporum*.

INTRODUCTION

The plant cuticle forms a hydrophobic coating that covers nearly all above-ground parts of terrestrial plants and constitutes the interface between plant and environment. The main structural component of the plant cuticle is cutin, a polyester consisting of hydroxy and epoxy fatty acids of n-C_{16} and n-C_{18} types (Kolattukudy, 2002; Kolattukudy *et al.*, 1995). Fungal pathogens secrete cutinases and lipases that catalyse the hydrolysis of ester bonds from fatty acid polymers, thus facilitating fungal penetration through the cuticle (Hynes *et al.*, 2006; Kolattukudy, 1985; Voigt *et al.*, 2005). Both cutinases and lipases are esterase-like enzymes whose active sites share the catalytic triad serine–histidine–aspartic acid. Globally these enzymes are designated carboxylesterases, catalysing the hydrolysis and/or synthesis of long-chain acylglycerols (ten or more carbon units). The activity of lipases on triglycerides is usually enhanced at the lipid–water interface, an effect known as the interfacial activation phenomenon (Martínez *et al.*, 1992). Cutinases occupy an intermediate position between esterases and lipases, as they are active on soluble and on emulsified triglycerides (Kim *et al.*, 2003; Martínez *et al.*, 1992). Moreover, cutinases hydrolyse model substrates such as p-nitrophenyl palmitate (pNPP), p-nitrophenyl butyrate (pNPB) and triglycerides (Martínez *et al.*, 1992).

Regulation of fungal cutinases has been extensively studied in the pea stem pathogen *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*). Three cutinase genes have been reported in this species, *cut1*, *cut2* and *cut3*, which share a high degree of identity. While *cut2* and *cut3* are expressed constitutively at basal levels (Li *et al.*, 2002), expression of *cut1* is strongly induced by cutin monomers, and this transcriptional activation is mediated by the zinc finger transcription factor CTF1α (Li and Kolattukudy, 1997; Li *et al.*, 2002). In *Aspergillus nidulans*, two proteins with homology to CTF1α, *farA* and *farB*, regulate the expression of genes implicated in metabolism of short-chain and long-chain fatty acids (Hynes *et al.*, 2006). FarA and FarB bind *in vitro* to the same core DNA element CCGAGG that mediates binding of the cutinase transcription factor CTF1α (Li and Kolattukudy, 1997; Li *et al.*, 2002), suggesting that the transcriptional regulation of fatty acid metabolism may be widely conserved in fungi (Hynes *et al.*, 2006).

The role of cutinases in fungal pathogenicity to plants remains controversial. A number of gene knockout studies failed to show an essential role for cutinase in different pathogen species (Stahl and Schäfer, 1992; Sweigard *et al.*, 1992; Yao and Köller, 1995). By contrast, knockout of the cutinase gene *cut1* in *F. solani* f. sp. *pisi* resulted in decreased virulence on pea (Rogers *et al.*, 1994; Li *et al.*, 2002), and insertion of *cut1* into *Mycosphaerella*, a

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pathogen that normally requires wounds in the plant surface to cause infection, allowed the transgenic strains to penetrate an intact surface (Dickman *et al.*, 1989). Biochemical studies also support a role for cutinase in virulence. Immunolocalization with antibodies against Cut1 suggested that the cutinase was targeted to the growing tip of germinating spores of *F. solani* (Podila *et al.*, 1995). Application of cutinase antibodies to germinating spores inhibited infection of pea by the fungus (Maiti and Kolattukudy, 1979). Collectively, these studies support the view that cutinase is involved in virulence of fungal pathogens attacking the aerial parts of the plant.

Interestingly, disruption of the transcriptional regulator Ctf1α eliminated phytopathogenicity of *Fusarium solani* f. sp. *pisi*, but supplementation of the mutant with cutinase did not restore pathogenicity, suggesting that Ctf1α participates in the regulation of other genes essential for pathogenicity (Li *et al.*, 2002). The goal of the present work was to determine whether Ctf1 also regulates virulence factors in a pathogen which attacks the host plant through the roots, an organ that lacks a cuticle. To this aim, we have functionally characterized the *Fusarium oxysporum* transcription factor Ctf1, an orthologue of *F. solani* Ctf1α and of *A. nidulans* FarA. *F. oxysporum* is a soil-borne pathogen which causes economically important losses on a wide variety of crops and infects its host plants strictly through the roots (Di Pietro *et al.*, 2003). During penetration and root colonization, *F. oxysporum* secretes a battery of cell-wall-degrading enzymes such as polygalacturonases, pectate lyases and xylanases (Di Pietro and Roncero, 1998; García-Maceira *et al.*, 2001; Gómez-Gómez *et al.*, 2002; Huertas-González *et al.*, 1999; Ruiz-Roldán *et al.*, 1999). *F. oxysporum* therefore represents an excellent model to study the role of cutinases and lipases in fungal root pathogens. We find that *F. oxysporum* strains lacking a functional copy of the *ctf1* gene are impaired in induction of cutinase activity, as well as in expression of two genes encoding a predicted cutinase and lipase, respectively, during growth in culture. However, in contrast to *F. solani*, targeted disruption of the *ctf1* gene in *F. oxysporum* did not affect virulence on tomato plants, suggesting that transcriptional activation of cutinolytic genes plays differential roles in soil-borne and foliar plant pathogens.

RESULTS

### Cloning of the *ctf1* gene encoding a cutinase transcription factor of *F. oxysporum* f. sp. *lycopersici*

The *ctf1* gene, encoding a putative cutinase transcription factor of *F. oxysporum* f. sp. *lycopersici*, was cloned from a λEMBL3 genomic library and a lambda ZAP cDNA library of isolate 4287, using a probe generated by PCR with primers based on the coding sequence of the *ctf1α* gene from *F. solani* f. sp. *pisi* (Li and Kolattukudy, 1997) (Genbank accession number AAM22470).

Sequencing of hybridizing genomic and cDNA clones revealed the presence of an open reading frame (ORF) of 2712 nucleotides, interrupted by five introns of 59, 192, 92, 65 and 56 bp (supplementary Fig. S1). The *F. oxysporum ctf1* gene encodes a predicted protein of 904 amino acids with a molecular weight of 100.6 kDa, which shows 88.9 and 57.5% identity, respectively, to the cutinase and fatty acid transcription factors Ctf1α of *F. solani* (Li *et al.*, 2002) and FarA of *A. nidulans* (Hynes *et al.*, 2006). The Genbank accession number of *ctf1* is EF613328.

The predicted *F. oxysporum* Ctf1 protein contains a Cys_6Zn_2 binuclear cluster DNA-binding motif located close to the N-terminus (amino acid residues 52–85), a middle homology region (MHR) and three 'coiled coil' regions (predicted by the 'PEPCOIL' program) (supplementary Fig. S2A), all characteristic of the family of fungal regulatory proteins with Cys_6 zinc cluster DNA binding motifs (Schjerling and Holmberg, 1996; Todd and Andrianopoulos, 1997). The algorithm 'PROSITE' was used to predict 14 potential consensus phosphorylation sequences for casein kinase II, six potential sites for protein kinase C (PKC), three potential sites for tyrosine kinase, one potential site for cyclic AMP-dependent protein kinase A (PKA) and two potential sites for mitogen-activated protein kinase (MAPK) (supplementary Fig. S2B). Three putative nuclear localization signals with the residue patterns KRKK (position 86), KRHR (position 636) and PKRK (position 659) were detected, with a probability of 0.7 of Ctf1 being localized in the nucleus as predicted by the PSORT program (supplementary Fig. S2B). Southern analysis of genomic DNA treated with different restriction enzymes indicated that *ctf1* is present as a single copy in the *F. oxysporum* genome (data not shown). In agreement with this result, a BLAST search of the complete genome database of *F. oxysporum* (http://www.broad.mit.edu/annotation/genome/fusarium_group.1/MultiHome.html) detected a single gene corresponding to *ctf1*.

Construction of *ctf1* loss-of-function and overexpressing alleles

Loss-of-function mutants carrying a disrupted copy of the *ctf1* gene were generated using one-step gene replacement. A 2.4-kb *Bam*HI fragment within the *ctf1* coding region was replaced with the *hyg^R* cassette (Fig. 1A). This replacement results in disruption of the Cys_6Zn_2 DNA-binding motif, which is essential for proper function of CTF1α (Li and Kolattukudy, 1997). The knockout construct was introduced into protoplasts of the wild-type strain, and 40 hygromycin-resistant transformants were obtained and analysed by PCR with gene-specific primers ctf14 and ctf11 (see Table 1). Two transformants produced PCR banding patterns consistent with homologous replacement of the *ctf1* gene (data not shown). Southern blot analysis, using as a probe the *ctf1* genomic fragment which had been replaced by the hygromycin resistance cassette, confirmed that the hybridizing 8.3-kb *Bam*HI

(A)
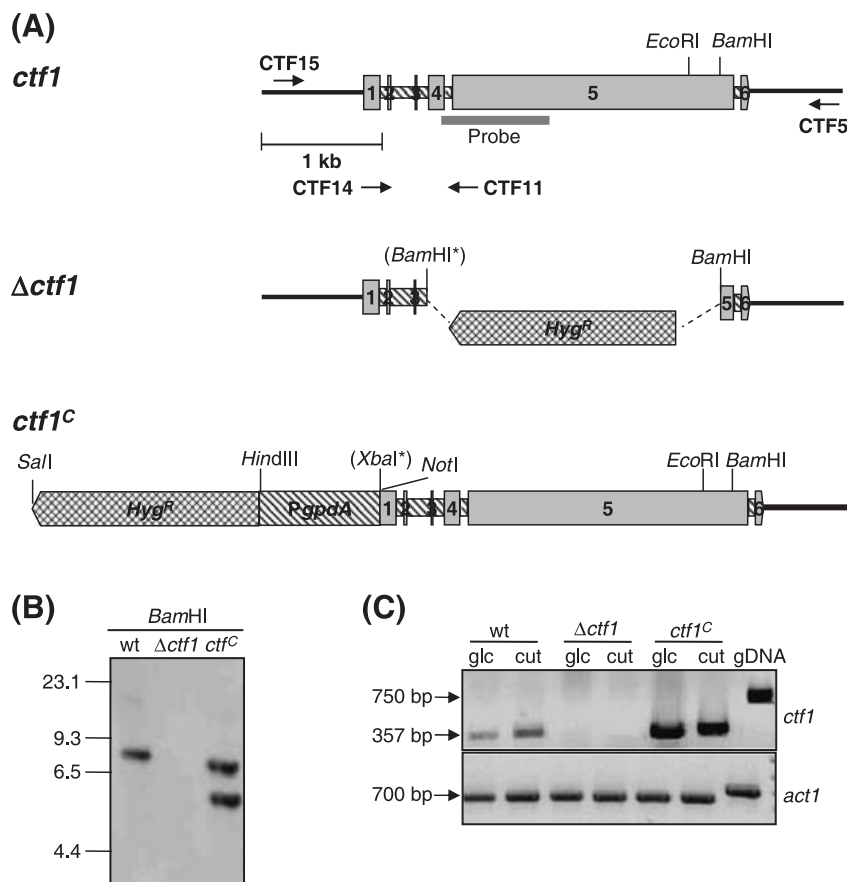
Fig. 1 Targeted disruption and overexpression of the *F. oxysporum ctf1* gene. (A) Physical maps of the *ctf1* locus, the gene replacement vector (Δ*ctf1* allele) and the *ctf1^C* allele (*ctf1* coding region fused to P*gpdA* from *A. nidulans*). The positions of the *ctf1* probe, the *Hyg^R* resistance cassette and the P*gpdA* promoter are indicated. The *Bam*HI and *Xba*I sites introduced by site-directed mutagenesis are marked with asterisks. (B) Southern hybridization analysis of wild-type strain 4287 and transformants Δ*ctf1* and *ctf1^C*. Genomic DNA treated with *Bam*HI was hybridized to the labelled *ctf1* probe indicated in (A). The Δ*ctf1* mutant shows homologous integration of the vector, whereas the constitutive *ctf1^C* mutant carries an ectopic insertion of the *ctf1^C* allele. Positions of DNA molecular size standards are shown. (C) Total RNAs from wild-type, Δ*ctf1* and *ctf1^C* strains grown in SM with 1% glucose or 0.2% apple cutin as carbon sources were used for RT-PCR analysis with *ctf1*-specific primers. *F. oxysporum* genomic DNA was used as a control template. The actin gene (*act1*) was used as a control for equal amounts of RNA.

fragment containing the wild-type *ctf1* allele was absent in transformant Δ*ctf1*, indicative of homologous integration-mediated gene replacement (Fig. 1B).

F. oxysporum strains carrying a constitutively overexpressed *ctf1^C* allele were produced by fusing the *ctf1* coding and terminator regions to the the *A. nidulans gpdA* promoter (P*gpdA*) (Fig. 1A). The entire construct including the *hyg^R* cassette was amplified by PCR and introduced into protoplasts of the wild-type strain. Thirty hygromycin-resistant transformants were selected, and the presence of the intact P*gpdA-ctf1* allele was confirmed by PCR with primers ctf4 and ctf16 (data not shown). Southern analysis of one of the transformants named *ctf1^C* revealed the presence of two hybridizing bands, one corresponding to the wild-type *ctf1* allele and an additional 6-kb band, consistent with ectopic insertion of the pP*gpdA-ctf1* construct into the genome (Fig. 1B).

RT-PCR analysis with gene-specific primers ctf14 and ctf11 flanking introns 1–4 (see Fig. 1A) confirmed the absence of a *ctf1* transcript in the knockout mutant and increased levels of transcript in the *ctf1^C* strain, independently of the carbon source (Fig. 1C). We concluded that the Δ*ctf1* mutant lacks a functional

copy of the *ctf1* gene, whereas transformant *ctf1^C* constitutively produces high amounts of *ctf1* transcript.

Mutations in *ctf1* affect fatty acid hydrolysis

Microscopic analysis of the fungal strains grown in liquid media containing different carbon sources, including wheat germ oil, did not reveal any apparent differences in growth and development between the wild-type, Δ*ctf1* and *ctf1^C* strains (Fig. 2A). By contrast, clear differences between wild-type and the *ctf1* mutants were observed on solid synthetic medium (SM) containing 1% olive oil with or without 0.1% sucrose. Vigorously growing colonies of the wild-type strain observed under UV light contained many fluorescent droplets surrounding the colony, indicating the presence of degradation products from fatty acid hydrolysis (Fig. 2B). The Δ*ctf1* mutant not only grew poorly, producing much less aerial mycelium than the wild-type, but also showed a complete absence of fluorescent droplets, indicative of a failure in hydrolysis of fatty acids. Conversely, the transformant *ctf1^C* exhibited significantly increased fatty acid hydrolysis compared with the wild-type strain. These results suggest that Ctf1 is

Table 1 Oligonucleotides used in this study.

Primer	Sequence
ctf1	GGAATGATAATCCGAAGGAGC
ctf2	TGGATCCATGTTCGGCTGCCAC
ctf5	TTGACTGAGGTATCTTGCCTA
ctf4	GCCATCATGTGGTCATCGG
ctf11	GGAATGATAATCCGAAGGAGC
ctf14	GTCGCAGTTGTCGGCACCA
ctf15	TTGTCATTCTCGGCAAAGTATT
ctf16	ATCCGTGGAACAGTCGCAAC
ctf17B	TATTCTGCA**GGATCC**ATGCGACGC
ctf18B	GCGTCGCAT**GGATCC**TGCAGAATA
ctf20X	**TCTAGA**ATGTCGTCAGGCAGCGGCACGC
ctf22	TTCCTCCATCATTGCTGCTTCT
cut9d	GT(N)TGGAT(H)CA(R)GG(N)GT(N)GG(N)GG
cut10d	T(Y)TG(N)A(R)(R)TT(Y)TT(N)GT(R)TA(N)CC(R)AA
cut14	GACTCCAGCAATCTTCTCCC
cut19	GGCAGCATCACCCGCAACG
lip11	GTTTTACATCCAACACGGCG
lip12	ATCTGGACATAAGAGTTCAGC
lip16	TTGGCGACGATACAGAAGCG
lip18	ACGAGACGACGATTTCCTTTC

Mutations introduced in the original sequences for creation of *Bam*HI restriction sites (B) are shown in bold. Degenerated primers are designated with a 'd', and use the UIPAC code: 'N' for A/C/T/G, 'R' for A/G; 'H' for A/T/C, and 'Y' for C/T.

required for expression of *F. oxysporum* genes involved in fatty acid hydrolysis.

Characterization of *F. oxysporum* genes involved in fatty acid hydrolysis

To study the role of Ctf1 in regulation of fatty acid hydrolysis of *F. oxysporum* further, the *cut1* and *lip1* genes were cloned encoding a putative cutinase and lipase, respectively. *cut1* was isolated from a λEMBL3 genomic library of *F. oxysporum* by screening with a probe obtained by PCR with degenerated primers derived from conserved regions of fungal cutinase genes. The nucleotide sequence revealed an ORF of 690 bp interrupted by one intron of 65 bp (supplementary Fig. S3A). The deduced amino acid sequence of the *cut1* gene product had 78.3, 76.5 and 76.1% identity, respectively, to *cut1* (P00590), *cut2* (AAL18696) and *cut3* (AAL18697) of *F. solani* f. sp. *pisi* (Li *et al.*, 2002; Soliday *et al.*, 1984) (supplementary Fig. S3B). The Cut1 protein contained the characteristic conserved catalytic triad Ser (120), Asp (175) and His (188) (Egmond and de Vlieg, 2000). A putative signal peptide was detected at the N terminus from amino acid residues 1–16. The mature Cut1 protein has a predicted molecular mass of 21.7 kDa and a pI of 8.1. The GenBank accession number of *F. oxysporum cut1* is EF613272.
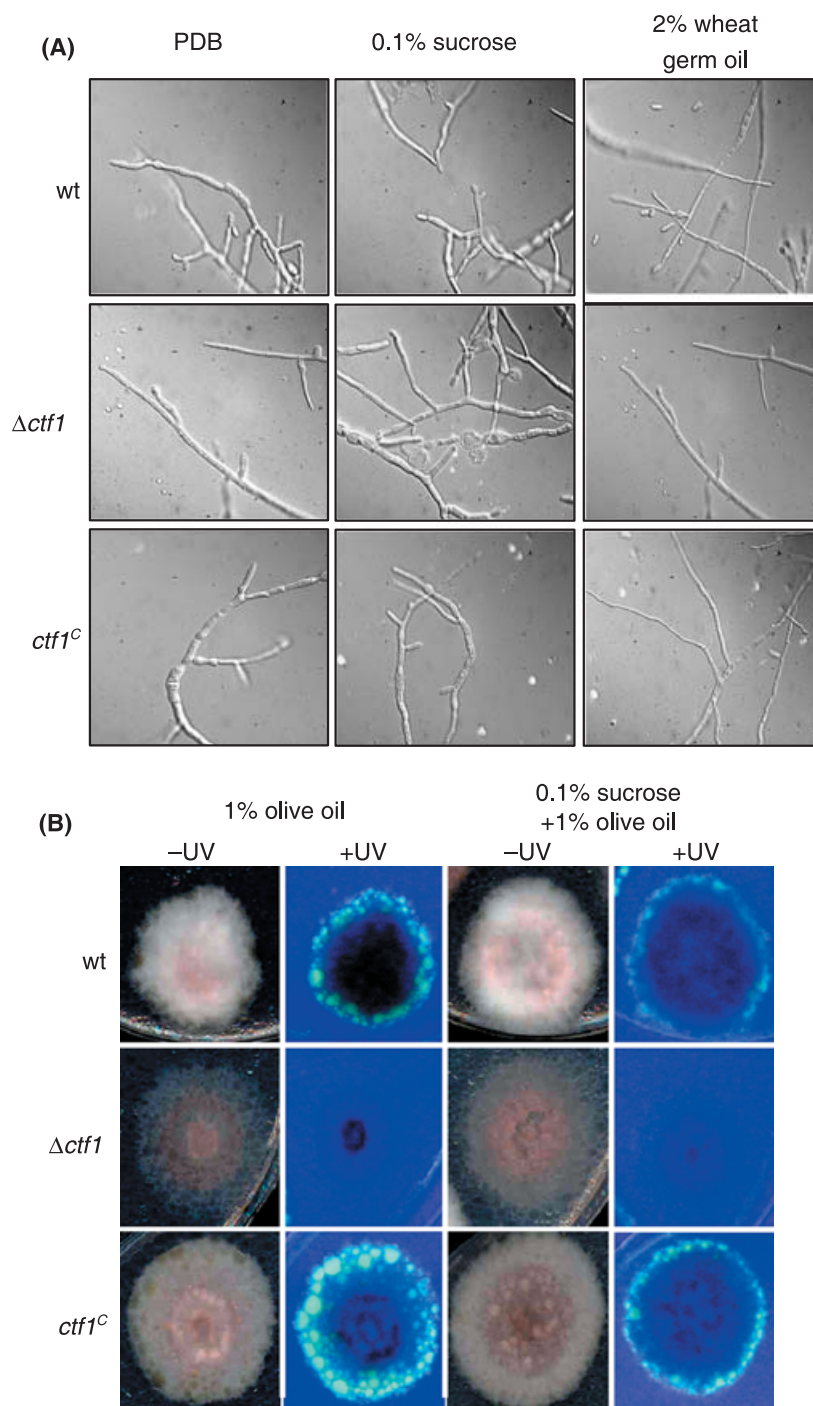
The *lip1* gene encoding a putative extracellular lipase of *F. oxysporum* was isolated by PCR amplification of *F. oxysporum* genomic DNA with primers lip16 and lip18, derived from the *F. verticilloides* genome database (http://www.broad.mit.edu/annotation/genome/fusarium-group/OrganismList.html#F%20-verticillioides). The nucleotide sequence of the *lip1* locus revealed an ORF of 1035 bp interrupted by two introns of 52 and 50 bp, respectively (supplementary Fig. S3C). The deduced amino acid sequence was 80% identical to the lipase Fgl1 from *F. graminearum* (AAQ23181) (Voigt *et al.*, 2005). The predicted Lip1 protein contains the characteristic catalytic triad, comprising a serine, a histidine and an aspartic acid, which is highly conserved in lipases (supplementary Fig. S3D) (Schrag *et al.*, 1991). A putative signal peptide is present at the N terminus, indicating that Lip1 is a secreted lipase. The predicted mature protein has a molecular mass of 34.8 kDa and a pI of 6.6. The Genbank accession number of the *lip1* gene is EF613329.

The promoter regions of *F. oxysporum cut1* and *lip1* each contained one copy of the CCGAGG DNA element (positions −711 and −495, respectively) mediating transcriptional activation by the transcription factors Ctfα and FarA (Hynes *et al.*, 2006).

Ctf1 regulates extracellular esterase and lipase activities and expression of *cut1* and *lip1* during growth in submerged culture

To test whether Ctf1 regulates extracellular esterase and lipase activities in *F. oxysporum*, supernatants of the wild-type strain and the *ctf1* mutants grown for various time periods in liquid SM with different carbon sources were analysed using colorimetric assays with pNPB or pNPP, respectively, as the substrate (Fig. 3). Esterase activity remained at basal levels in supernatants of all strains grown in SM without any carbon source (carbon starvation), but increased rapidly and to a similar extent in supernatants of the wild-type and the *ctf1*[C] strain grown on 0.2% apple cutin (Fig. 3A,B). By contrast, no induction of esterase activity on apple cutin was detected in supernatants of the Δ*ctf1* strains (Fig. 3B). Induction of extracellular esterase activity was both delayed and attenuated when glucose was supplemented as a carbon source in addition to apple cutin, probably due to preferential utilization of glucose by the fungus (Fig. 3C). Induction of lipolytic activity on 2% wheat germ oil was slightly increased in the *ctf1*[C] strain as compared with the wild-type, and was not detected in the Δ*ctf1* mutant (Fig. 3D). We conclude that Ctf1 mediates activation of extracellular esterase and lipase activities in *F. oxysporum* under inducing conditions, but not under carbon starvation or glucose-repressed conditions.

To test whether expression of the *cut1* gene depends on Ctf1, the level of *cut1* transcripts in mycelia grown under inducing conditions was determined by RT-PCR with gene-specific primers cut19 and cut14 which flank the intron (see supplementary

(A)

PDB   0.1% sucrose   2% wheat germ oil

wt

Δ*ctf1*

*ctf1^C^*

(B)

1% olive oil   0.1% sucrose +1% olive oil

−UV   +UV   −UV   +UV

wt

Δ*ctf1*

*ctf1^C^*

Fig. 2 Growth phenotype of *ctf1* mutants. (A) Microconidia of the indicated strains were germinated for 12 h in liquid PDB, transferred to SM with the indicated carbon sources, incubated for 24 h at 28 °C and 170 r.p.m. and observed under the microscope. (B) Strains were grown for 5 days on solid SM supplemented with the indicated carbon sources. Products of fatty acid hydrolysis are visible as fluorescence under UV light.

Fig. S3A). Total RNA was obtained from mycelia of the wild-type, the Δ*ctf1* and the *ctf1^C^* strains grown for 20 h in SM supplemented with different carbon sources (1% glucose or 0.2% apple cutin) and subjected to RT-PCR. Higher levels of RT-PCR product corresponding to the *cut1* transcript were detected in mycelia of the wild-type grown on apple cutin than in those grown on glucose, suggesting that *cut1* transcription is triggered by inducing carbon sources (Fig. 4A). Activation of *cut1* by apple cutin was even more pronounced in the *ctf1^C^* strain than in the wild-type. By contrast, only a weak activation of *cut1* expression was observed in the Δ*ctf1* mutant. RT-PCR analysis of mycelia grown for 24 or 48 h on apple cutin confirmed the low level of *cut1* induction in the Δ*ctf1* mutant compared with the wild-type and *ctf1^C^* strain (Fig. 4B).
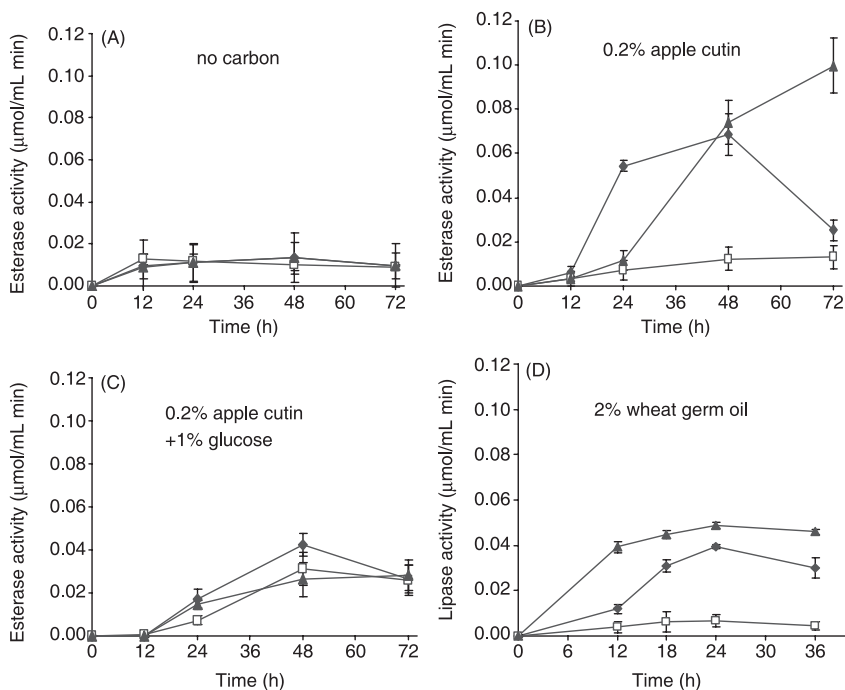
Fig. 3 Ctf1 regulates extracellular esterase and lipase activities in liquid cultures. Activities in supernatants were determined by a colorimetric assay using as substrate *p*-nitrophenyl butyrate (A–C) or *p*-nitrophenyl palmitate (D), respectively. Wild-type (filled diamonds), Δ*ctf1* (open squares) and *ctf1ᶜ* (filled triangles) strains were grown for 12 h in PDB and transferred for the indicated times to SM without carbon source (A), 0.2% apple cutin (B), 0.2% apple cutin plus 1% sucrose (C) or 2% wheat germ oil (D). Error bars indicate standard deviations from three independent experiments.

Northern hybridization analysis was used to study the effect of mutations in *ctf1* on expression of the *lip1* gene. Total RNA obtained from mycelia of the strains grown for 24 h on 2% wheat germ oil as the sole carbon source was hybridized to the *lip1* probe. Transcript levels of the *lip1* gene were slightly higher in the *ctf1ᶜ* strain than in the wild-type, whereas an extremely faint hybridization signal was detected in the Δ*ctf1* mutant (Fig. 4C). We conclude that efficient transcriptional activation of *cut1* and *lip1* on fatty acid substrates, but not on glucose, is mediated by the Ctf1 transcription factor.

Ctf1 is not a major regulator of *lip1* during root infection

Expression of *ctf1*, *cut1* and *lip1* during infection of tomato plants by *F. oxysporum* was determined in total RNA extracted from roots or stems of plants at several time points after inoculation with the different strains, using RT-PCR with gene-specific primers (Fig. 5). Both *ctf1* and *cut1* were expressed at very low levels. A faint signal corresponding to c*tf1* was detected in roots inoculated with the *ctf1ᶜ* strain at 7 and 10 days after inoculation. Likewise faint *cut1* transcript signals were observed in roots 10 days after inoculation with the three different strains and in stems infected with the wild-type or the *ctf1ᶜ* strain. By contrast, robust expression of *lip1* was detected in plants inoculated with all the strains, particularly in infected roots. No expression signal was detected in non-infected control roots.

Ctf1 is not required for virulence of *F. oxysporum* on tomato

The role of Ctf1 in virulence of *F. oxysporum* f. sp. *lycopersici* was determined both by plant and by fruit infection assays. Plant infection assays were performed by immersing the roots of 3-week-old tomato plants in microconidial suspensions of the different strains and planting them into minipots with vermiculite. Severity of wilt symptoms in plants inoculated with the wild-type strain increased steadily throughout the experiment. At the beginning, leaf tips turned yellow and curved, then the stalk weakened and decayed, and most of the plants were dead 20 days after inoculation. Plants inoculated with the Δ*ctf1* and *ctf1ᶜ* mutant strains showed a very similar symptom development as the wild-type during the entire period of the experiment (Fig. 6). The capacity to grow invasively on tomato fruit tissue was assayed by injecting microconidia of the strains directly into tomato fruits. The wild-type and the *ctf1* mutant strains efficiently colonized and macerated the fruit tissue, producing aerial mycelium on the surface (data not shown). We conclude that Ctf1 is not essential for virulence of *F. oxysporum*.
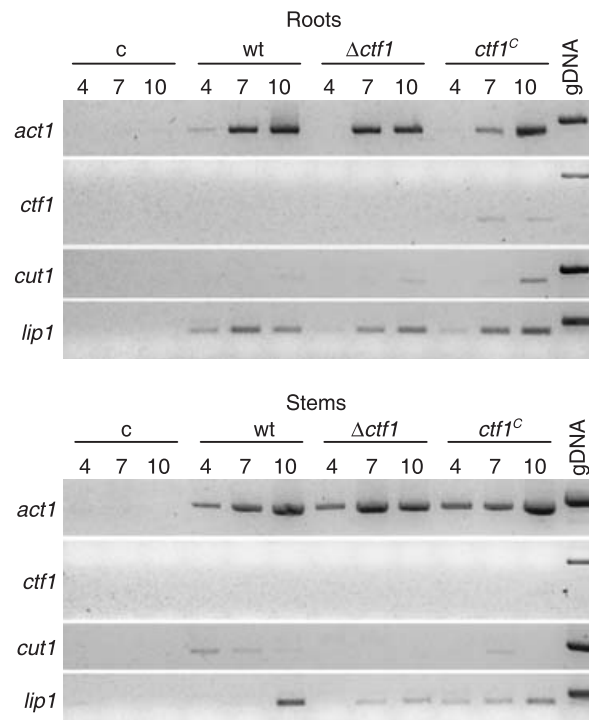
DISCUSSION

Infection by fungal pathogens causes severe losses in crop plants worldwide. For fungal pathogens attacking the aerial parts of the plant, the first contact between pathogen and host occurs on the
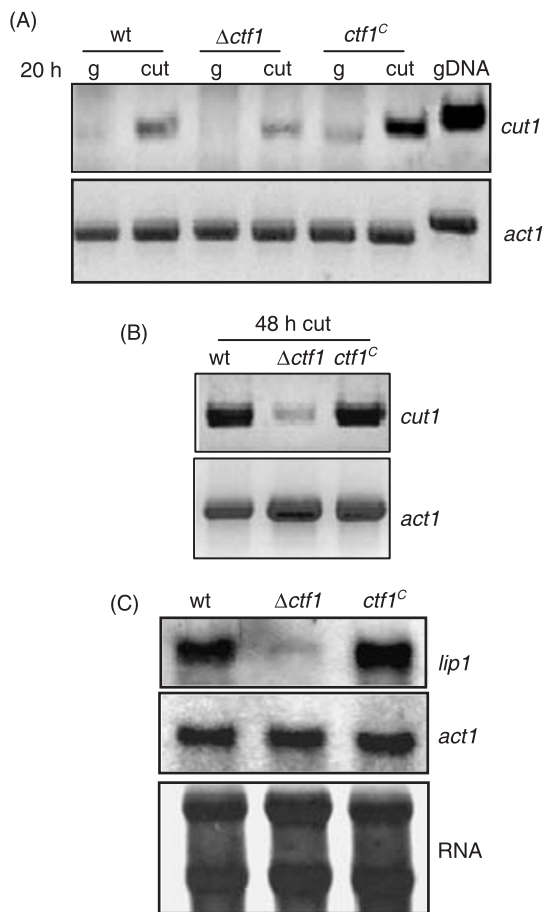
Fig. 4 Ctf1 regulates expression of *cut1* and *lip1* in axenic culture. (A) Transcript levels of the *cut1* gene determined by RT-PCR. The indicated strains were grown for 12 h in PDB and transferred for 20 h to SM with 1% glucose or 0.2% apple cutin as carbon source. (B) Expression of *cut1* determined by RT-PCR in the indicated strains grown for 48 h in SM with 0.2% apple cutin. (C) Northern analysis for *lip1* transcript accumulation. Strains were grown for 12 h in PDB and transferred for 24 h to SM with 2% wheat germ oil as carbon source. Upper panel: the filter hybridized to the *lip1* probe. Middle panel: the filter hybridized to the *act1* probe. Lower panel: total RNA stained on the filter with methylene blue.

Fig. 5 Ctf1 is not required for expression of *lip1* during infection of tomato plants. RT-PCR products showing the expression pattern of *ctf1*, *cut1* and *lip1* during infection of tomato plants by the wild-type strain, the Δ*ctf1* mutant and the *ctf1^C* strain. cDNA generated from total RNA isolated at the indicated time points (days after inoculation) from roots and stems of infected plants were used as templates for PCR with gene-specific primers (see Experimental procedures). Uninoculated plants were used as control. gDNA refers to PCR with genomic DNA as template.

plant surface, which is protected by a cuticle layer (Agrios, 1997). By contrast, soil-borne pathogens enter their host plants through the roots, which lack a cuticle layer. While a role of lipolytic and cutinolytic enzymes during infection of aerial plant pathogens has been established (Li *et al.*, 2002; Rogers *et al.*, 1994; Voigt *et al.*, 2005), the function of these enzymes has not been investigated in soil-borne pathogens. To test the role of cutin-degrading enzymes in virulence of the soil-borne vascular wilt pathogen *F. oxysporum*, we cloned and mutated the gene encoding the transcription factor Ctf1, the deduced amino acid sequence of which shows a high degree of identity with *F. solani* f. sp. *pisi* CTFα, which regulates the inducible cutinase gene *cut1* (Li *et al.*,

2002), and with *A. nidulans* FarA, which controls expression of genes involved in fatty acid metabolism (Hynes *et al.*, 2006). The predicted *F. oxysporum* Ctf1 protein contains three putative nuclear localization signals, and its amino terminus shows the $Zn_2(II)Cys_6$ DNA binding domain, characteristic of regulatory proteins with diverse function. This domain follows the canonical pattern $CX_2CX_6CX_8CX_2CX_6C$, with both repeats (CX_2CX_6C) separated by a variable number of residues (eight in CTFα and CTF1). This group of transcription factors binds to promoter regions at two conserved palindromic triplets $GCC(N)_xCCG$, where N has a variable length (e.g. 11 in *Saccharomyces cerevisiae* GAL4) (Liang *et al.*, 1996). In *F. solani* f. sp. *pisi*, the DNA motifs recognized by CTFα have the opposite orientation and are separated only by two nucleotides ($GCCN_2GGC$; Li *et al.*, 2002). FarA and FarB, two transcription factors regulating fatty acid metabolism in *A. nidulans*, bind to the conserved motif CCGAGG in the promoter regions of the target genes (Hynes *et al.*, 2006). The promoter of the *F. oxysporum ctf1* gene contains a copy of the CCGAGG motif at position −374, suggesting that Ctf1 may regulate its own expression. This hypothesis is supported by the observation that
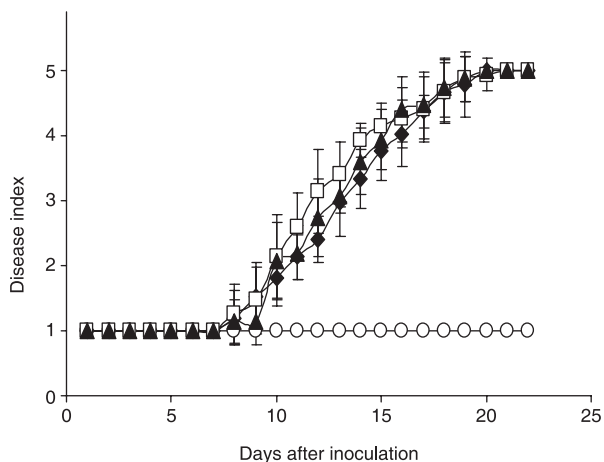
Fig. 6 Ctf1 is not required for virulence of *F. oxysporum*. Incidence of *Fusarium* wilt caused on tomato plants (cultivar Vemar). Severity of disease symptoms was recorded at different times after inoculation, using an index ranging from 1 (healthy plant) to 5 (dead plant). Symbols refer to plants inoculated with the wild-type strain 4287 (filled diamonds), loss-of-function mutant Δ*ctf1* (open squares), constitutive mutant *ctf1^C* (filled triangles) and the water control (open circles). Error bars represent standard deviations from 20 plants for each treatment. The experiment was repeated twice with similar results.

ctf1 transcript levels were higher in mycelia grown on cutin as opposed to those grown on glucose (Fig. 1C).

Mutants of *F. oxysporum* carrying either a loss-of-function or a constitutively expressed allele of *ctf1* did not show differences in growth and development in submerged culture even when grown on fatty acid-containing carbon sources such as olive oil or wheat germ oil. However, clear differences were detected on solid media with olive oil as carbon source. The phenotype of the Δ*ctf1* mutants under these conditions (poor growth, reduced aerial hyphae, absence of UV-fluorescent droplets) was consistent with the notion that these mutants were impaired in hydrolysis of fatty acids. This suggests that Ctf1 controls expression of the major lipolytic enzymes produced by *F. oxysporum* under these conditions. As CTFα and FarA regulate genes involved in fatty acid metabolism and therefore affect the production of encoded enzyme activities (Hynes *et al.*, 2006; Li *et al.*, 2002), we tested the role of *F. oxysporum* Ctf1 in production of extracellular esterase and lipase activities on apple cutin extract and wheat germ oil, respectively. Esterase activity was clearly induced by apple cutin in the wild-type strain, and this induction was abolished by deletion of *ctf1*, such that activity remained at basal levels similar to those produced under carbon starvation conditions (Fig. 3). Similar results were obtained for lipase activities. Thus, Ctf1 is required for efficient induction of extracellular lipolytic enzymes in *F. oxysporum*. By contrast, the differences in enzyme activities between the wild-type and the constitutive *ctf1^C* strain were much smaller, and esterase activity in the *ctf1^C* strain was still

subject to induction by fatty acid substrates. These results are consistent with a model in which activation of Ctf1 occurs, at least in part, at the post-translational level. Similar results have been reported recently for the zinc finger transcription factors XlnR controlling expression of xylanase genes in *F. oxysporum* (Calero-Nieto *et al.*, 2007) and Zfr1 regulating fumonisin production in *F. verticillioides* (Flaherty and Woloshuk, 2004).

Different approaches were used to isolate *F. oxysporum* cutinase genes, including PCR amplification of genomic DNA or RT-PCR with mRNA obtained from the wild-type strain and *ctf* mutants under different growth conditions, using primers derived from conserved regions of *F. solani* cutinase genes. All these approaches yielded the same amplified fragment, indicating the existence, in *F. oxysporum*, of a single gene encoding this structural class of cutinases, as opposed to three (*cut1*, *2* and *3*) in *F. solani* (Li *et al.*, 2002). The different number of cutinase genes in the two *Fusarium* species may point to divergent evolutionary mechanisms during adaptation of the infection process in root and aerial plant pathogens. In fungal leaf and stem pathogens, multiple genes encoding cuticle-degrading enzymes may have evolved from a common ancestor by duplication and may have adapted to the specific requirements encountered during host penetration. The product of the *F. oxysporum lip1* gene has 80% identity to *F. graminearum* Lip1, an extracellular lipase required for virulence on wheat (Voigt *et al.*, 2005). The promoters of both *cut1* and *lip1* genes contained the element CCGAGG, which mediates binding of the fatty acid hydrolysis-regulating transcription factor FarA from *A. nidulans* (Hynes *et al.*, 2006). The key role of Ctf1 in regulation of *cut1* and *lip1* was substantiated by RT-PCR and Northern analysis showing that expression of the two genes was nearly abolished in the Δ*ctf1* mutant. Thus, the role of this transcription factor in activation of genes involved in fatty acid hydrolysis appears to be broadly conserved in ascomycetes.

The roles of cutinases and lipases in the infection process of fungal plant pathogens has been highly controversial and may vary substantially between different enzymes and species. Targeted disruption of *ctfα* in *F. solani* f. sp. *pisi* resulted in loss of virulence on pea, but the decreased level of secreted cutinase was not sufficient to fully explain the loss of virulence, as addition of exogenous cutinase did not restore pathogenicity (Li *et al.*, 2002). In the present work we failed to detect significant differences in virulence between wild-type and Δ*ctf1* mutants, in both tomato plant and fruit infection assays. Thus, Ctf1, a major regulator of cutinase and lipase activity during growth in axenic culture, is not required for pathogenicity of *F. oxysporum*. This result can be interpreted in two ways. First, neither cutinase nor lipase may act as virulence factors in *F. oxysporum*. At first, this interpretation appears convincing because *F. oxysporum* infects roots, an organ which lacks a cuticle. However, the results from RT-PCR show that the *lip1* gene is highly expressed during infection of roots, both in the wild-type and the Δ*ctf1* strain. Thus, under these conditions

lip1 expression appears to be largely independent of the presence of Ctf1. Consequently, Ctf1 appears to regulate *lip1* under the *in vitro* conditions studied, whereas during root infection this function could be fulfilled by another transcriptional regulator. Therefore, a similar role of *lip1* in virulence of *F. oxysporum* as reported for its orthologue *fgl1* in *F. graminearum* (Voigt *et al.*, 2005) cannot yet be ruled out.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

F. oxysporum f. sp. *lycopersici* 4287 (race 2) was obtained from J. Tello, Universidad de Almería, Spain. The pathotype of the strain was periodically confirmed by plant infection assays. Fungal strains were stored at −80 °C with glycerol as a microconidial suspension (Di Pietro and Roncero, 1998). For extraction of DNA and microconidia production, cultures were grown in potato dextrose broth (PDB, Difco, Detroit, MI) at 28 °C with shaking at 170 r.p.m. as described previously (Di Pietro and Roncero, 1998).

For phenotypic analysis of colony growth, a 5-μL drop containing 1×10^6 freshly obtained microconidia was transferred on 1.5% (w/v) agar plates of synthetic medium (SM) (Di Pietro and Roncero, 1998), containing 1% (v/v) olive oil or 1% olive oil and 0.1% (w/v) sucrose as carbon source and 0.1% NH_4NO_3 as the nitrogen source, and incubated at 28 °C for 5 days as described previously (Di Pietro and Roncero, 1998). For microscopic analysis, strains were observed in a Leica DMR microscope using the Nomarsky or phase contrast techniques, or in a Leica MZ FLIII stereomicroscope.

For analysis of gene expression or extracellular cutinase and lipase production, freshly obtained microconidia from wild-type and mutants strains were inoculated in PDB medium at a final concentration of 5×10^6 mL^{-1} and germinated for 12 h at 28 °C and 170 r.p.m. Germlings were washed twice in sterile water and transferred to liquid SM (as above without agar) containing either 0.2% (w/v) apple cutin prepared as described (Walton and Kolattukudy, 1972), 1% (w/v) glucose or 2% (v/v) wheat germ oil (Sigma) as the carbon source.

Nucleic acid manipulations and cloning of the *ctf1*, *cut1* and *lip1* genes

Total RNA and genomic DNA were extracted following previously reported protocols (Chomczynski and Sacchi, 1987; Raeder and Broda, 1985). Southern and Northern blot analyses and probe labelling were carried out as described (Di Pietro and Roncero, 1998) using the non-isotopic digoxigenin labelling kit (Roche Diagnostics SL). The expression analysed by RT-PCR was performed using Superscript II reverse transcriptase (Gibco-BRL, UK). The primers used to amplify the gene transcripts were ctf11 and ctf14

for *ctf1*, cut14 and cut19 for *cut1*, and lip11 and lip18 for *lip1* (Table 1). Other routine DNA procedures were performed according to standard protocols (Sambrook and Russell, 2001).

Cloning of the *ctf1* gene was achieved by PCR amplification of *F. oxysporum* 4287 genomic DNA using the primers ctf1 and ctf2 (Table 1) derived from the *ctf1α* gene of *F. solani* (Genbank accession number P52958). For isolation of *cut1* gene the degenerated primers cut9d and cut10d were used (Table 1), which were derived from conserved sequence domains of fungal cutinases. Cloning of the *lip1* gene was done using the primers lip11 and lip12 derived from a putative lipase gene in the *F. verticillioides* genome sequence database (http://www.broad.mit.edu/annotation/genome/fusarium-group/OrganismList.html#F%20verticillioides) with homology to the *fgl1* gene from *F. graminearum* (AY292529.1). PCRs were routinely performed with the Long Template PCR system (Roche Diagnostics, SL). The amplified DNA fragments were cloned into the pGEM-T vector (Promega, Madison, WI) and used to screen a λEMBL3 genomic library of *F. oxysporum* f. sp. *lycopersici* isolate 4287. Library screening, subcloning and other routine procedures were performed according to standard protocols (Sambrook and Russell, 2001). The cDNA clone of *ctf1* gene was obtained from a lambda ZAP cDNA library of *F. oxysporum* (4287) (Roldán-Arjona *et al.*, 1999). Sequencing of both DNA strands of the obtained clones was performed at the Servicio de Secuenciación Automática de DNA, SCAI (University of Córdoba, Spain) using the Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI Prism 377 Genetic Analyser apparatus (Applied Biosystems). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul *et al.*, 1990) at the National Center for Biotechnology Information (Bethesda, MD).

Construction of plasmids vectors and fungal transformation

Gene replacement vector pD*ctf1* was constructed as follows: a *Bam*HI site was introduced at position 515–521 of the *ctf1* coding region using the overlapping method (Ho *et al.*, 1989) with the primers ctf17B and ctf18B (Table 1), and the final amplification product was cloned into vector pGEM-T. This construct was treated with *Bam*HI and the excised internal 2.4-kb *Bam*HI fragment was replaced with the hygromycin B resistance gene (*hph*) under the control of a *Cochliobolus heterostrophus* promoter (Turgeon *et al.*, 1987), resulting in disruption of the *ctf1* coding region (supplementary Fig. S1A). The linear fragment containing the interrupted *ctf1* allele was PCR-amplified with primers ctf5 and ctf15, and used to transform protoplasts of *F. oxysporum* wild-type strain following the protocol of Di Pietro and Roncero (1998).

The constitutively expressed *ctf^C* allele was obtained by introducing an *Xba*I site immediately upstream of the ATG start

codon of *ctf1* using primers ctf20X and ctf22, and cloning the resulting fragment containing the *ctf1* coding and 3′ flanking regions into pGEM-T. The vector pPgpdA-*ctf1 was con*structed by excising the *ctf1* allele using *Xba*I and *Not*I and cloning into pGEM-T containing the *gpdA* promoter (Punt *et al.*, 1987). The PgpdA-*ctf1* DNA fragment was excised with *Hind*III and *Not*I, and cloned into a vector containing the *Hyg^R* cassette (Punt and van den Hondel, 1992) (Fig. 1A). The final construct containing the *ctf1^C* allele under the control of the *gpdA* promoter plus the hyg cassette was amplified using the universal forward and reverse primers. The amplification product was purified and used to transform protoplasts of the 4287 wild-type strain to hygromycin resistance as described previously (Di Pietro and Roncero, 1998).

Determination of cutinase and lipase activity

For the activity assays, culture supernatants of the wild-type and mutant strains collected at different times of growth under the conditions described above were used as the enzyme source. Each assay was carried out in triplicate in 96-well microtitre plates. Esterase assays were performed using as substrate *p*-nitrophenyl butyrate (pNPB; Sigma) (Davies *et al.*, 2000; Kolattukudy *et al.*, 1981). A 20-μL volume of crude supernatant was mixed with 200 μL of reaction buffer containing 50 mM sodium phosphate buffer, pH 7, 0.4% (v/v) Triton X-100 and 1.76% (v/v) PNB in acetonitrile, and incubated at 37 °C for 30 min.

Lipase activity was determined using as substrate *p*-nitrophenyl palmitate (pNPP; Sigma) (Winkler and Stuckman, 1979). A 10-μL volume of supernatant (concentrated 12.5× with acetone) was added to 240 μL of reaction buffer, containing 2 mM pNPP in isopropanol, 1% gum arabic (Sigma), 0.1% Triton X-100 (Merck), and 50 mM bis-Tris-propane (Sigma), at pH 8 and incubated at 37 °C for 30 min.

Production of *p*-nitrophenol in both assays was measured at 405 nm using a multiwell spectrophotometer (SpectraFluor Plus). One unit of enzymatic activity was defined as 1 nmol/min *p*-nitrophenol released from the substrate. Calibration curves were made using standard *p*-nitrophenol solutions.

Virulence assays

Tomato plant inoculation assays were performed as described previously (Di Pietro and Roncero, 1998). Briefly, 2-week-old tomato seedlings (cultivar Vemar) were inoculated with *F. oxysporum* strains by immersing the roots in a microconidial suspension, planted in vermiculite and maintained in a growth chamber. At different times after inoculation, severity of disease symptoms was recorded using an index ranging from 1 (healthy plant) to 5 (dead plant). Twenty plants were used for each treatment. Assays for invasive growth on tomato fruits (cultivar Daniela) were

carried out as described (Di Pietro *et al.*, 2001). Virulence experiments were performed three times with similar results.

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SUPPLEMENTARY MATERIAL

Fig. S1 Amino acid sequence alignment of the predicted *F. oxysporum ctf1* from *F. oxysporum* (Fo, EF613328) with Ctf1α from *F. solani* (Fs, AAM22470) and FarA from *A. nidulans* (An, ABD51992). Identical amino acids are highlighted on a shaded background. Dashes indicate gaps in the alignment.

Fig. S2 Characteristics of the deduced *F. oxysporum ctf1* gene product. (A) Putative functional motifs are: $Cys_6Zn(II)_2$ DNA binding domain (the six conserved cysteine residues are indicated by asterisks); Middle Homology Region (MHR); and three putative

'coiled coils'. (B) Predicted sites in Ctf1 for N-glycosylation, phosphorylation by protein kinases and nuclear localization signals.

Fig. S3 Identification of *cut1* and *lip1* encoding a putative cutinase and lipase of *F. oxysporum*. (A) Physical map of the *cut1* locus. Primers used for RT-PCR analysis are indicated. (B) Phylogenetic tree of the predicted products of cutinase genes from the genome databases of filamentous fungi: Cut1 from *F. oxysporum* (Fo, EF613272), Cut1 (M29759), Cut2 (AF417004) and Cut3 (AF417005) from *F. solani* (Fs), Cut1 (XM_383066) and Cut2 (XM_383633) from *F. graminearum* (Fg), Cut1 (XM_741414), Cut2 (XM_746327) and Cut3 (XM_750180) from *A. fumigatus* (Af), and Cut1 from *M. grisea* (XM_365241, Mg). (C) Physical map of the *lip1* locus. The probe used for northern analysis of *lip1* expression is indicated. (D) Phylogenetic tree of the predicted *F. oxysporum lip1* gene product with lipases from filamentous fungi: Lip1 from *F. oxysporum* (Fo, EF613329), Fgl1 from *F. graminearum* (Fg, AY292529), Lip1 from *F. heterosporum* (Fh, S77816), NhL1 from *F. solani* (Fs, AJ271094), Lip1 from *M. grisea* (Mg, XM_370519), LipA from *A. niger* (Ani, DQ680030), Lip1 from *A. nidulans* (An, XM_676223) and Lip1 from *N. crassa* (Nc, XM_956273).

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