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Thctf1 transcription factor of *Trichoderma harzianum* is involved in 6-pentyl-2*H*-pyran-2-one production and antifungal activity

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ABSTRACT

We describe the cloning and characterization of the *Trichoderma harzianum Thctf1* gene, which shows high sequence identity with a transcription factor gene of *Fusarium solani* f. sp. *pisi*. In *T. harzianum*, disruption of the *Thctf1* gene by homologous recombination gave rise to transformants that in plate experiments did not show the yellow pigmentation observed in the wild-type strain. In several *Trichoderma* spp. a yellow pigmentation and a coconut aroma have been related to the production of 6-pentyl-2*H*-pyran-2-one (6PP) compounds. Prompted by this, we explored whether the loss of pigmentation in the *Thctf1* null mutants of *T. harzianum* might be related to the synthesis of 6PP. Chromatographic and spectroscopic analyses revealed that the disruptants did not produce two secondary metabolites, derived from 6PP and not previously described in the *Trichoderma* genus, that are present in wild-type culture filtrates. Since 6PP is a recognized antifungal compound, this ability was analyzed in both the disruptants and wild-type, observing that the *Thctf1* null mutants of *T. harzianum* had reduced antimicrobial capacity. Our results point to the significant role of THCTF1 in the production of secondary metabolites and in the antifungal activity of *T. harzianum*.

1. Introduction

Species of the common soil fungus *Trichoderma* are used in the biological control of a variety of plant pathogenic fungi (Monte, 2001; Benitez et al., 2004). The most frequently suggested *Trichoderma* mechanisms of biocontrol include mycoparasitism, competition for nutrients and antibiosis, as well as plant growth promotion (Harman, 2006) and plant defense signaling activation (Djonovic et al., 2007). It is clear that the abilities of *Trichoderma* to inhibit the growth of other fungi are probably due to the combined action of cell-wall degrading enzymes (CWDEs) together with the capacity of *Trichoderma* to produce different secondary metabolites (Lorito et al., 1994). The secondary metabolites with antibiotic activity produced by *Trichoderma* have been classified in different groups based on their biosynthetic origin or their chemical structure, and they include non-volatile (e.g. peptaibols) and volatile (e.g. simple aromatic metabolites, terpenes, the isocyano metabolites, some polyketides, butenolides and pyrones) compounds (Cardoza et al., 2005; Reino et al., 2008). Some metabolites have been associated with the antagonistic ability of *Trichoderma*, but none of them has been identified as a sole responsible agent of such biocontrol activity. One of the first volatile antifungal compounds isolated from *Trichoderma* species was 6-pentyl-alpha-pyrone (6PP). This compound is a non-toxic flavoring agent that was chemically synthesized for industrial purposes before its discovery as a natural product. The first identification of this pyrone was made in *Trichoderma viride* (Collins and Halim, 1972) and it has subsequently been isolated from several *Trichoderma* species (Dickinson, 1993; Keszler et al., 2000). 6PP is responsible for the "coconut aroma" associated with certain strains of *Trichoderma* (Bisby, 1939), and it is also well known that it can inhibit the growth of pathogens such as *Rhizoctonia solani* (Dennis and Webster, 1971). The biosynthesis of 6PP, and indeed of all compounds in this group, is a matter for conjecture. Some authors consider that the origin of these metabolites is linoleic acid (Serrano-Carreon et al., 1993), whereas others prefer to consider that these compounds are derived from a polyketide pathway (Sivasithamparam and Ghisalberti, 1998). Compounds derived from 6PP have been also isolated in *Trichoderma*. In this sense, the 6-pent-1-enyl-alpha-pyrone, which also presents the characteristic coconut odour, is able to inhibit the growth of the pathogens *R. solani* and *Botrytis cinerea* (Cooney and Lauren, 1999). Massoilactone and δ-decanolactone were patented by Hill et al. (1995) and, owing to their antifungal properties, are currently used as biocontrol agents of several plant diseases. Moreover, Evidente et al. (2003) have isolated another compound, viridepyronone, with antagonistic activity against *Sclerotium rolfsii*.

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This paper reports the isolation and characterization of the *Thctf1* gene in *T. harzianum*, and its functional analysis by a gene disruption strategy. The transformants did not produce two new 6PP derivatives and exerted a lower antimicrobial effect than the wild-type strain. These results relate the transcription factor THCTF1 of *T. harzianum* to the production of secondary metabolites and antifungal activity against plant pathogenic fungi.

2. Materials and methods

2.1. Bacterial strains

Escherichia coli DH5-alpha was used as a host for plasmid construction and propagation. This bacterial strain was grown in Luria–Bertani (LB) broth or on LB plates, which were supplemented with ampicillin (0.1 mg/ml), X-gal (40 μg/ml) and IPTG (10 μg/ml) when required.

2.2. Fungal strains

Trichoderma harzianum CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) (henceforth *T. harzianum* T34, or simply T34) was used as a source of DNA to clone the *Thctf1* gene and also as a host in the transformation experiments to disrupt the *Thctf1* gene. The *Trichoderma virens* T59 (NBT 59, NewBiotechnic S.A., Seville, Spain), *Trichoderma atroviride* T33 (IMI 352939, International Mycological Institute, CABI Bioscience, Egham, UK), *Trichoderma longibrachiatum* T27 (IMI 304059), *Trichoderma asperellum* T25 (IMI 296237) and *T. atroviride* T11 (IMI 352941) strains were used in Southern experiments. *Fusarium oxysporum* f. sp. *lycopersici* CECT 2866, *R. solani* CECT 2815 and *B. cinerea* B98, isolated from infected strawberries at our lab, were used as plant pathogenic fungi in antifungal assays. All fungal strains were maintained on PPG (Limon et al., 2004) or PDA (Sigma, St. Louis, MO) media.

2.3. EST database

Twenty-six cDNA libraries were constructed for the TrichoEST project using different mRNA populations from 10 strains belonging to eight *Trichoderma* species expressed under mycoparasitic and nutrient-stress conditions (Rey et al., 2004). Expressed sequence tags (ESTs) were generated by sequencing cDNA clones from the 5′ end, and an EST database was compiled with 13,814 unique ESTs (Vizcaino et al., 2006, 2007).

2.4. cDNA library

L03, a cDNA library constructed with RNA from *T. harzianum* T34, was used (Vizcaino et al., 2006). Biomass was obtained following a two-step liquid culture procedure. First, the fungus was grown in potato dextrose broth (PDB) (Difco Becton Dickinson, Sparks, MD) in Erlenmeyer flasks at 25 °C in an orbital incubator at 160 rpm for 2 days. The biomass was harvested, rinsed twice with sterile distilled water, and transferred to minimal medium (Penttilä et al., 1987) (MM: 15 g/l NaH_2PO_4, 5 g/l $(NH4)_2SO_4$, 600 mg/l $CaCl_2{\cdot}2H_2O$, 600 mg/l $MgSO_4{\cdot}7H_2O$, 5 mg/l $FeSO_4$, 2 mg/l $CoCl_2$, 1.6 mg/l $MnSO_4$, 1.4 mg/l $ZnSO_4$) under the following conditions in separate cultures: (i) 0.1% glucose, (ii) 1.5% chitin (Sigma), (iii) 100-fold increase in the concentration of metals or (iv) 1% of a 1:1 mixture of fungal cell walls from *Penicillium digitatum* and *B. cinerea*. The cultures were incubated for 8 or 12 h. RNA was extracted using the TRIZOL® reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. After total RNA extraction, equal amounts of RNA from the different growth conditions were mixed and used for mRNA purification, using Dynabeads (Dynal, Oslo, Norway). The cDNA library was constructed using the Uni-ZAP XR Vector System (Stratagene, La Jolla, CA) following the manufacturer's instructions.

2.5. Screening of a lambda genomic DNA library

A genomic library of *T. harzianum* T34 previously constructed in the bacteriophage vector lambda-GEM 11 (Lora et al., 1995) was used to isolate a genomic clone containing the *Thctf1* gene. Aliquots of the library were used to infect *E. coli* LE 392; phages were lifted onto Hybond-N^+ membranes (Amersham, Piscataway, NJ, USA) and hybridized with a specific probe for the 3′ end of the gene. The DNA from a positive phage was isolated and obtained to complete the sequence of the *Thctf1* gene. The *Thctf1* cDNA was PCR-amplified with the primers 2092-ATG (ATGTCTCCAGAAGCTGCGCC) and 2092-FTCUT3 (GCTCGCAGCGTAGTAACCAT) and phagemid DNA from the cDNA library L03 as template.

2.6. DNA and RNA manipulations

Standard molecular techniques were used throughout (Sambrook and Russell, 2001).

Mycelia for DNA extractions were obtained from PDB cultures incubated at 28 °C and 200 rpm for 2 days. Then, they were collected by filtration, washed with distilled water, frozen, and lyophilized. Fungal genomic DNA was isolated according to previously described protocols (Raeder and Broda, 1985).

For Southern analyses, 10 μg of genomic DNA was digested with restriction enzymes, electrophoresed on 0.7% agarose gels, and transferred to a Hybond-N^+ membrane. In these experiments, the probes were labeled using the DIG High Prime kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Hybridizations were carried out at 65 °C for 16 h. Membranes were washed under high-stringency conditions. Chemiluminescent detection was performed using the DIG nucleic acid detection kit (Roche).

For expression analyses, mycelia were obtained after growth in MM containing 2% glucose as carbon source at 28 °C and 200 rpm for 48 h. Then, the mycelia were harvested by filtration, washed with sterile water, and placed in fresh MM under the following conditions in separate cultures: 2% glucose, 0% glucose, nitrogen starvation, 1.5% chitin, 1% strawberry plants, 0.1% pectin (Sigma), 1% fungal cell walls from *B. cinerea*, or 1% olive oil (Sigma). Nitrogen starvation conditions corresponded to a 100-fold decrease in the concentration of ammonium sulfate in the medium (50 mg/l) containing 2% glucose as carbon source. After 4, 8 or 24 h of incubation, mycelia were collected by filtration, thoroughly washed with sterile water, lyophilized, and kept at −80 °C until RNA extraction. Fungal RNA was isolated as indicated previously using the TRIZOL® reagent.

For Northern analyses, 20 μg of total RNA was separated on a 1.2% formaldehyde–agarose gel and transferred to a Hybond-N^+ membrane. In these experiments, as well as for the screening of the T34 lambda genomic library, the probes were labeled radioactively using Ready-To-Go DNA labeling beads (^{32}P-dCTP) kit (Amersham). The hybridization conditions and membrane washes were as described above.

2.7. Probes used in hybridization experiments

In order to clone the *Thctf1* gene, a probe of 1517 bp containing the complete insert of the EST L03T34P022R02092 (henceforth, EST 2092) was amplified by PCR from the T34 genomic DNA using the oligonucleotides 2092-5′(+I) CACCATGATGCTGTTCCCTAA and 2092-FTCUT3.

For Northern experiments, a 1493 bp fragment was amplified by PCR using the T34 genomic DNA as template and the oligonucle-

otides 2092-COMPR2 TGGCGATATCCTTGGCTCATA and 2092-3′(+I) TCAAGTAGAGGTTTCGGGGTG.

For Southern experiments, fragments of 4737, 2748 and 2285 bp were amplified from the genomic DNA of the T34 strain, using the primer pairs 2092-FTCUT1 CCACTACTGGCTTCGTGTCTA and 2092-FTCUT3, 2092-intron1 CCGACCTACGAAGAACCAGC and 2092-FTCUT2 CCATTGACGCCTCTCCCTA, and 2092-FTCUT1 and 2092-intron2 ATTGAATCCGCATCATCGAG, respectively. Moreover, a 3008 bp fragment from the *amdS* gene was amplified using the primers *amdS*-fwd GACCAACCCGATAACCATTGA and *amdS*-rev TCACCACATTTCCCAGCAAC, and DNA from pGEM-T::*Thctf1*::*amdS* (Fig. 1) as template. Position (in bp) and orientation of these primers are shown in Fig. 1.

2.8. PCR procedures

Standard PCRs were accomplished using the *Taq* polymerase system (Biotools B&M Labs. S.A.), and the Expand Long Template PCR System (Roche) was used to amplify higher than 2-kb fragments, following the manufacturer's instructions.

2.9. Sequence analyses

Sequences were analyzed using the Lasergene package (DNASTAR Inc., Madison, WI). DNA-binding elements were found by looking for consensus sequences described elsewhere or by using the MatInspector program (http://www.genomatix.de/matinspector.html) with the TRANSFAC database restricted to fungi. The nucleotide sequence of *Thctf1*, including the promoter and terminator regions, was deposited in the GenBank database under Accession No. EU551672.

2.10. Plasmid constructions and Trichoderma transformation procedure

The plasmid pGEM-T::*Thctf1*::*amdS* (Fig. 1), carrying the disruption cassette for *Thctf1*, was constructed. For this, first the plasmid p3SR2 (Kelly and Hynes, 1985) was used as template to PCR-amplify the *amdS* gene of *Aspergillus nidulans,* which codes for acetamidase, using the primers *amdS-Hind*IIIA (CCAAGCTTTGCCGCGTCAAG) and *amdS-Hind*IIIB (GGAAGCTTTGTCTAGACTGG), adding the underlined HindIII restriction sites. Then, a *Thctf1* gene region of 4737 bp was PCR-amplified using the primers 2092-FTCUT1 and 2092-FTCUT3, and T34 genomic DNA as template, after which it was cloned into the pGEM-T vector, affording plasmid pGEM-T::*Thctf1* of 7737 bp. This plasmid was then digested with HindIII, treated with alkaline phosphatase (Roche), and finally ligated to the PCR-amplified *amdS* gene, previously digested with HindIII, to obtain the final vector pGEM-T::*Thctf1*::*amdS* of 10,999 bp (Fig. 1).

To obtain *Thctf1* disruptant strains, wild-type *T. harzianum* T34 was transformed with a linear fragment of 7999 bp obtained by PCR from the pGEM-T::*Thctf1*::*amdS* plasmid, using the primers 2092-FTCUT1 and 2092-FTCUT3. Protoplast preparation, transfor-

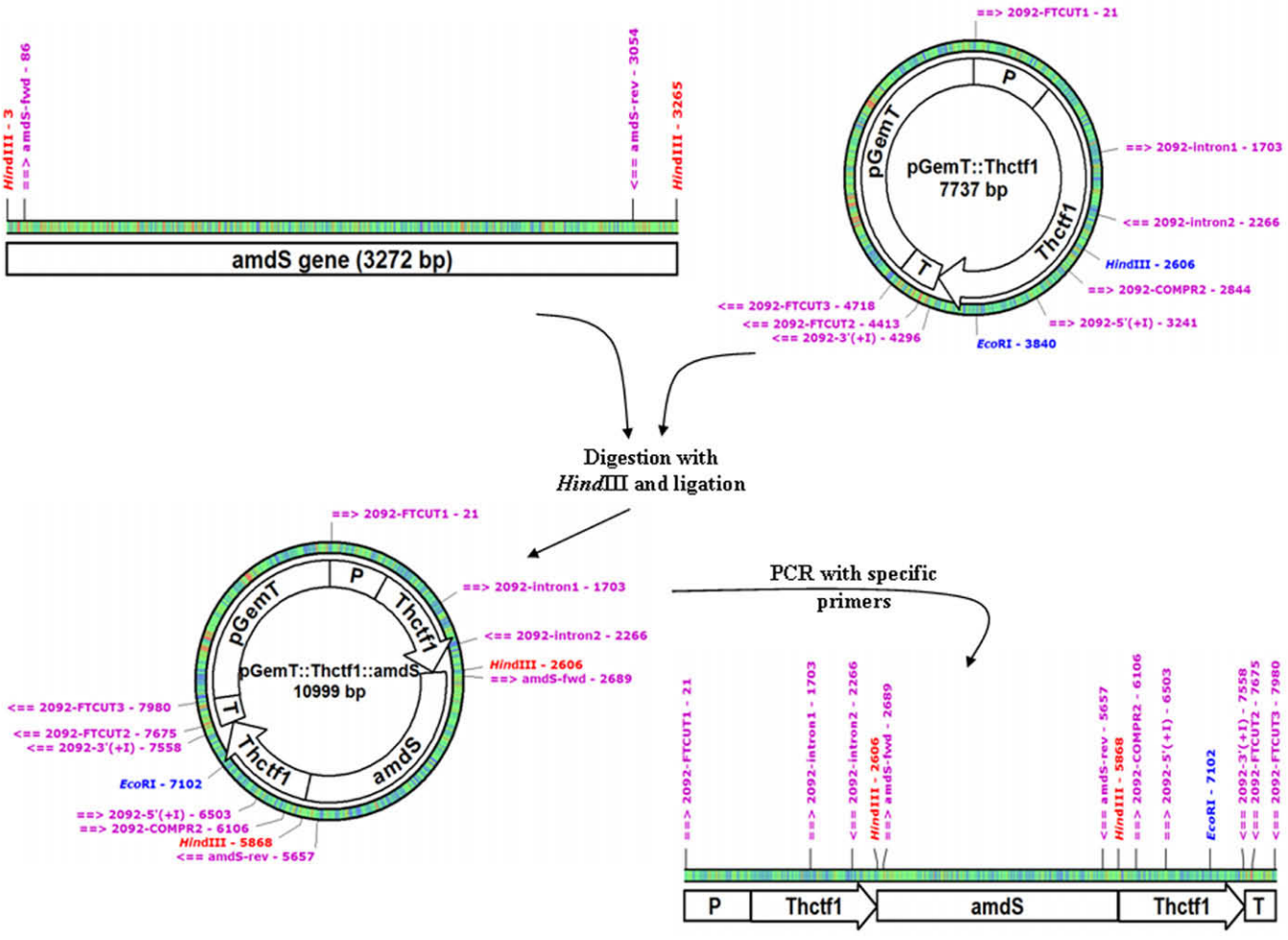

Fig. 1. Schematic representation of the strategy used for disruption of the *T. harzianum Thctf1* locus. The disruption cassette contained the *amdS* gene of *A. nidulans* flanked by 2606 bp of the 5′ region (including 903 bp of the promoter) and 2131 bp of the 3′ region (including 421 bp of the terminator) of the *Thctf1* gene. P and T are *Thctf1* gene promoter and terminator, respectively. HindIII and *Eco*RI restriction sites and the position and orientation of the used primers are marked. For further details, see Section 2.

mation, and transformant stabilization were carried out according to (Penttilä et al., 1987). Concentrations of $1 \times 10^7 - 2.5 \times 10^8$/ml of protoplasts and quantities of 2–5 μg of DNA were used.

2.11. Esterase activity assays

Cutinases are defined as extracellular esterases that break the ester bond of cutin, a heteropolymer of esters of hydroxylated fatty acids, derived from the cuticle of plants (Kolattukudy, 1985). Cutinase activity can be measured directly from the release of radioactivity by tritium-labeled cutin. However, this assay for cutinase activity is very tedious and the substrate is very expensive. Accordingly, in the present work esterase activity was quantified in the both T34 (wild-type) and ΔD1-38 (disruptant) cultures, using *p*-nitrophenylbutyrate (*p*-NPB) as substrate (Sweigard et al., 1992).

T34 and ΔD1-38 were grown in MM with 2% glucose, adding 1×10^5 spores/ml, and were incubated at 28 °C and 200 rpm for 48 h (preinduction). Inductions were performed in MM, without glucose, or in MM supplemented with 1.5% chitin or 1% olive oil. After 8 h of induction, the supernatant from each culture was recovered and precipitated with solid ammonium sulfate up to 80% saturation at 4 °C for 12 h. The samples were then centrifuged, resuspended again in distilled water, and dialyzed to remove the salts. *p*-NPB assays were carried out following a previously described protocol (Sweigard et al., 1992). The reaction mix was prepared by adding 50 μl from each sample to a 1-ml final volume of *p*-NPB solution. The reaction mix was incubated at 30 °C for 30 min and the hydrolysis rate of *p*-NPB was determined by measuring absorbance at 405 nm. Activity was quantified by performing a *p*-NPB standard curve. The assays were performed in triplicate in microtitre plates and three independent experiments were also carried out. *p*-NPB solution: 1 mmol/l *p*-NPB, 50 mmol/l taurodeoxycholic acid (TDOC), 10 mmol/l Tris–HCl, pH 8.0, 10 mmol/l NaCl. The protein concentration present in each sample was determined with the Bradford method (Bradford, 1976).

2.12. Phenotypic characterization of T. harzianum disruptants

To perform a phenotypic study of disruptants for the *Thctf1* gene, 15 μl containing 2×10^6 spores of *T. harzianum* strains were placed at the center of Petri dishes with MA (Malt Agar, Difco Becton Dickinson), PPG or PDA medium, and incubated at 25, 28 or 30 °C. Six plates were used for each condition. The growth, pigmentation and sporulation characteristics were examined daily over 10 days.

2.13. Isolation of metabolites

2.13.1. Fermentation

Seven 1-cm diameter agar plugs, obtained from 7-day old actively growing PDA cultures of T34 or ΔD1-38 strains were inoculated separately into seven Roux flasks, each containing 150 ml of the sterilized synthetic medium with the following composition: 0.5% glucose, 0.08% KH_2PO_4, 0.07% $KN0_3$, 0.02% $Ca(H_2PO_4)_2$, 0.05% $MgSO_4{\cdot}7H_2O$, 0.001% $MnSO_4{\cdot}4H_2O$, 0.0005% $CuSO_4{\cdot}5H_2O$ and 0.0001% $FeSO_4{\cdot}7H_2O$; pH 6.0. Surface cultures were incubated at 25 °C for 13 days.

2.13.2. Organic extraction and chemical methods

One liter of fermentation broth was filtered through 200 μm Nylon filters and saturated with NaCl, and the aqueous phase was extracted with ethyl acetate (EtOAc). The EtOAc extract was washed three times with H_2O and then dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure afforded a dense oil that was separated by means of flash chromatography on silica gel, with a mixture of ethyl acetate/hexane (20%, 30%, 50%, 80%, 100% in EtOAc) and methanol as solvents. The fractions were collected and purified by high-pressure liquid chromatography (HPLC). Extensive spectroscopic methods, specifically ^1H NMR and ^{13}C NMR, were then employed to analyze the presence of metabolites in each fraction.

^1H and ^{13}C NMR spectra were recorded on Varian Gemini 300 MHz and Varian Unity 400 MHz (^1H at 399.952 MHz and ^{13}C al 100.570 MHz) NMR spectrometers with $SiMe_4$ as the internal reference. Mass spectra were recorded on a GC-MS Thermoquest spectrometer (model: Voyager), and a VG Autospec-Q spectrometer. HPLC was performed on a Hitachi/Merck L-6270 apparatus equipped with a UV–vis detector (L 6200) and a differential refractometer detector (RI-71). TLC was performed on 0.2 mm-thick Merck Kiesegel 60 F_{254}, using different mixtures of hexane and ethyl acetate. Silica gel was used for column chromatography. HPLC was performed with a Hitachi/Merck L-6270 (Merck KGaA, Darmstadt, Germany) apparatus equipped with UV–vis detector (L4250) and a differential refractometer detector (RI-71). Purification by means of HPLC was accomplished with silica gel columns (LiChrospher Si-60, 10 μm, 1 cm wide, 25 cm long or LiChospher Si-60, 5 μm, 0.4 cm wide, 25 cm long). All chemical products were from Sigma and the solvents used were freshly distilled.

2.14. Antifungal assays

2.14.1. Growth assay on membranes

Five-mm-diameter PDA plugs of *T. harzianum* T34 or transformants were placed at the center of Petri dishes containing PDA or malt extract agar (MEA, Difco Becton Dickinson) media, on cellophane sheets or 10 kDa-cut-off cellulose membranes. After 2 days of incubation at 25 °C, the membranes were removed from the plates and a single 5-mm diameter mycelial plug of the pathogen, *F. oxysporum* f. sp. *lycopersici* or *R. solani* on PDA plates, and *B. cinerea* on MEA plates, was placed at the center of the plate. In parallel, the pathogens were grown on PDA or MEA (control plates). Each pathogen was tested in six plates. Growth diameters were calculated after 120, 96 and 72 h for *F. oxysporum* f. sp. *lycopersici*, *B. cinerea* and *R. solani*, respectively. The results are expressed as percentages of radial growth inhibition of each pathogen by *Trichoderma* strains.

2.14.2. Direct confrontation assay

In vitro confrontation assays between *Trichoderma* strains and the pathogens *F. oxysporum* f. sp. *lycopersici* and *R. solani* (on PDA plates), and *B. cinerea* (on MEA plates), were established as follows. Agar plugs cut from growing colonies of each fungus were placed 5.5 cm apart in parallel on PDA or MEA plates and incubated at 25 or 30 °C in the dark. The behavior of *T. harzianum* strains against each pathogen was examined visually until the *Trichoderma* strains had overgrown or surrounded the pathogen colony. The confrontation assays were performed in triplicate, and single cultures of *Trichoderma* strains and pathogens were used as control.

2.14.3. Spore germination assay

The antifungal activity of extracellular extracts from *T. harzianum* T34 and the transformants was evaluated in spore bioassays as follows: *T. harzianum* strains were inoculated in PDB medium at a final concentration of 10^5 spores/ml and were incubated at 28 °C on a rotary shaker at 200 rpm for 4 days. Supernatants were collected and 2 ml was fractionated by ultrafiltration through Centricon-10 (Amicon, Beverley, MA, USA). Then, both fractions were filter-sterilized using a 0.22 μm Ultrafree-MC filter (Millipore), and the proteins were quantified by the method of Bradford. Mixtures were prepared with 10 μl of the protein fraction (>10 kDa

or <10 kDa) at a concentration of 10 ng/μl, 90 μl of PDB medium and 2 μl of a conidial suspension of *F. oxysporum* f. sp. *lycopersici* (100 conidia) and were incubated in microtiter plates at 25 °C. The effect caused by *Trichoderma* extracts on the germination of *Fusarium* spores was analyzed with a stereomicroscope after 24, 30 and 50 h of incubation. In these assays, six replicates for each treatment were performed.

2.14.4. Mycelial biomass production assay

Liquid culture inhibition tests were performed as follows: 100 ml of PDB medium were inoculated with 1×10^5 conidia of *F. oxysporum* f. sp. *lycopersici* and a supernatant volume containing 100 μg of T34 or ΔD1-38 extracellular protein. Moreover, the <10 kDa fraction contained in the above supernatants was also tested. Cultures were incubated at 25 °C and 200 rpm for 64 h. Then, the pathogen mycelium was harvested by filtration with vacuum and its weight was measured. Single cultures of *F. oxysporum* f. sp. *lycopersici* were included as control. Three replicates for each treatment were carried out. Assay data obtained were analyzed using the unilateral Fisher's protected least-significance difference test (PLSD).

3. Results

3.1. The T34 Thctf1 gene

The 520-bp EST 2092 was selected for further characterization, based on its high degree of similarity with a cutinase transcription factor-encoding gene described in *F. solani* f. sp. *pisi*, using the BLASTX algorithm. The EST 2092 was obtained from the *T. harzianum* T34 cDNA library L03, which was constructed after growing the T34 strain under simulated mycoparasitic conditions.

The 1517-bp PCR fragment obtained with the primers 2092-5′(+I) and 2092-FTCUT3, and T34 genomic DNA as template was used as a probe to screen the T34 lambda-GEM 11 genomic library. One of the positive phages was selected, and its DNA was used to clone and sequence the 5′ end of the gene with a primer-walking strategy. As a result, 4861 bp were sequenced: 927 bp belonged to the promoter region and 521 bp to the transcriptional terminator. The ORF of the 2634 bp-long *Thctf1* gene encoded a protein of 877 amino acids, with a theoretical molecular mass of 97.03 kDa, and a deduced isoelectric point of 6.40 U, and it contained four introns of 111, 257, 334 and 77 bp. The primary structure of the deduced protein included the following amino acid percentages: 91 basic (K, R) (10.4%), 102 acid (D, E) (11.6%), 221 polar (N, C, Q, S, T, Y) (25.2%) and 292 hydrophobic (A, I, L, F, W, V) (40.3%).

3.2. Analysis of the THCTF1 protein

THCTF1 showed the highest degree of similarity (93% of identity) with a hypothetical cutinase transcription factor 1 beta protein of *Trichoderma reesei* (http://www.genome.jgi-psf.org/Trire2/Trire2.home.html-jgi/Trire2/103230/fgenesh5_pg.C_scaffold_1000863). This protein also showed high sequence similarity with a cutinase transcription factor 1 beta from *F. solani* (69.5%), showing 25.6% similarity with the cutinase transcription factor 1 alpha of this fungus.

As a result of the analysis of the structure of THCTF1 using the SMART data base (http://www.smart.embl.de), a $Cys_6Zn(II)_2$ binuclear cluster domain was located (smart 00066) at the N terminus from amino acid residues 63–108 of the protein. A fungal specific transcription factor domain (pfam 04082) was also found at position 352–446.

An *in silico* prediction of the secondary structure of the protein was performed using the PSIPRED program (Jones, 1999) and it was observed that in THCTF1 the alpha-helix regions were predominant. In addition, analysis of the protein sequence with the Scan Prosite application (http://www.expasy.org/tools/scanprosite) revealed the presence of putative glycosylation, phosphorylation, myristoylation and sulfation sites. The most probable cellular fate of THCTF1 was simulated using the PSORT II program (Nakai and Horton, 1999), which assigned the protein a nuclear location with 78.3% of probability.

3.3. Sequence analysis of the Thctf1 promoter

A 927-bp sequence of the *Thctf1* promoter region was obtained and investigated for the occurrence of protein-binding motifs that might provide some clue as to its regulation. The *Thctf1* promoter did not contain a TATA box adjusted to the 5′ TATA(T/A)AA 3′ consensus. However, similar sequences—TATATCT, TATAGTC and TATACTG—were found at positions −43, −427 and −810 with respect to the initiation triplet.

A theoretical analysis was also performed in order to search for binding sites for transcription factors whose consensus sequences have been described previously. This was done both manually and using the MatInspector application with the TRANSFAC database, restricted to fungi (http://www.genomatix.de).

Three 5′ HGATAR 3′ sequences, two in the sense strand (positions −601 and −743) and one in the antisense strand (position −878), were found. This sequence is involved in the binding of transcription factors (AreA in *A. nidulans* or Nit2 in *N. crassa*) related to nitrogen regulation (Ravagnani et al., 1997). There were also three 5′ SYGGRG 3′ sequences in the complementary strand (positions −528, −590 and -905). This sequence, which is specific for the binding of the Mig1/CreA/Cre1 protein (Mig1 in *S. cerevisiae*, CreA in A. *nidulans*, Cre1 in *T. reesei*), is responsible for catabolic repression (Kulmburg et al., 1993; Ilmen et al., 1996). Two 5′ AGGGG 3′ sequences were also found: one in the sense strand (−137) and the other in the antisense strand (−625). This is the binding sequence for the proteins Msn2p and Msn4p, which are involved in tolerance to several stress conditions in *S. cerevisiae* (Gorner et al., 1998).

3.4. Expression experiments in T34

Owing to the presence of consensus sequences in the *Thctf1* promoter for the carbon catabolite regulator Mig1/CreA/Cre1 and the global nitrogen regulator AreA/Nit2, we decided to investigate the regulation of *Thctf1* gene expression by carbon and/or nitrogen sources. It was also of interest to define whether the induction occurred in the presence of *B. cinerea* cell walls or chitin as carbon sources in order to simulate a mycoparasitic scenario and, additionally, in the presence of strawberry plants, pectin or olive oil. Samples of *T. harzianum* T34 mycelia grown at different times (4, 8 or 24 h) were collected for *Thctf1* expression studies; a hybridization signal was detected under all conditions tested (Fig. 2). In general, the highest *Thctf1* transcript intensity was observed after 24 h of induction, except in the presence of olive oil, where the highest transcript level was detected after 4 h of induction.

3.5. Thctf1 homologous genes in other Trichoderma species

A Southern blotting analysis was performed with a view to determine the copy number of the *Thctf1* gene in the *T. harzianum* T34 genome and the presence of homologs of this gene in other *Trichoderma* species. As a result, a single band was detected in the T34 genomic DNA digested with the endonuclease *Xho*I, an enzyme that does not cut inside the probe, and two bands were observed in the T34 genomic DNA digested with the endonucleases HindIII or *Eco*RI, two enzymes that cut once inside the probe. On the basis of these observations, it was concluded that the *Thctf1* gene was present in only one copy in the genome of T34 and that

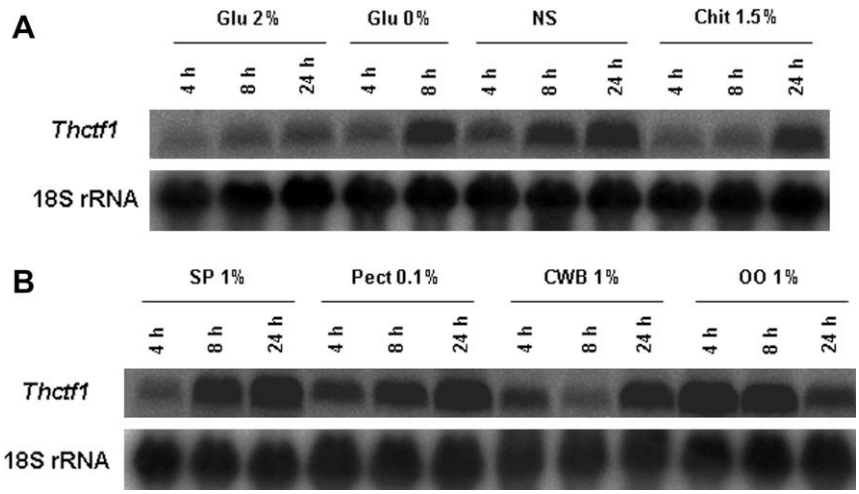

Fig. 2. Northern blot analysis of *Thctf1* gene expression. The experiments were carried out with total RNA extracted from mycelia of *T. harzianum* T34 grown in MM (Penttilä et al., 1987) containing: (A) 2% glucose (Glu 2%), 0% glucose (Glu 0%), nitrogen starvation (NS), 1.5% chitin (Chit 1.5%); and (B) 1% strawberry plants (SP 1%), 0.1% pectin (Pect 0.1%), 1% fungal cell walls from *B. cinerea* (CWB 1%), and 1% olive oil (OO 1%). Mycelia were cultivated for 4, 8 or 24 h. A 1493 bp fragment of the *Thctf1* coding region was used as probe and the 18S rRNA gene was used as a loading control.

a homologous gene was present in the other five strains investigated (Fig. 3).

3.6. Disruption of Thctf1 gene in T. harzianum

In order to gain further insight into the function of *Thctf1*, we constructed a *T. harzianum* strain lacking a functional copy of *Thctf1*. To this end, a disruption cassette consisting of the *A. nidulans amdS* gene flanked by 2606 bp of the *Thctf1* 5′ region (including 903 bp of the promoter) and 2131 bp of the *Thctf1* 3′ region (including 421 bp of the terminator) was constructed, and the resulting construct was transformed into *T. harzianum* T34. The best transformation frequencies were obtained with 2.5×10^8 protoplasts/ml and 5 μg of DNA.

Disruptants were selected in medium containing acetamide as the sole nitrogen source, and after several rounds of monosporic cultures 91 mitotically stable transformants (almost 100% of the conidia were able to grow in acetamide) were obtained and checked by PCR. A 4737-bp PCR product was predicted from the wild-type *Thctf1* locus when amplified with the primers employed, whereas a 7999-bp amplification product was expected in the transformants in which gene disruption had occurred. The 4737-bp fragment was present in 50 of the 91 transformants, indicating that these were not true disruptants (for example, ΔJ3-16). In eight transformant strains checked (designated Δ1-6-6, Δ2-11-1, Δ2-11-2, ΔJ2-11, ΔJ2-21, ΔD1-5, ΔD1-38 and ΔD1-43), this fragment was absent and hence they were selected for further Southern blotting analyses (Fig. 4).

Hybridization of *Eco*RI-digested genomic DNA using a 4737 bp fragment of the *Thctf1* gene as probe (Fig. 4A) resulted in the detection of 6.5 and 9.4 kb fragments in T34 and ΔJ3-16, whereas the eight putative disruptants showed restriction fragments of 6.5 and 12.6 kb. The *amdS* gene probe did not hybridize to wild-type T34 DNA, whereas hybridization signals were observed in all transformants (Fig. 4B), including ΔJ3-16, indicating that in this strain the *amdS* gene had been inserted in another region than the *Thctf1* locus.

Southern hybridization of *Xho*I-digested genomic DNA of wild-type T34, using a 2285 bp fragment of the *Thctf1* gene as probe, revealed the presence of a restriction fragment of 9.4 kb. Southern analysis also revealed that this probe hybridized to a single fragment of 12.6 kb in *Xho*I-digested DNA of the eight putative disruptants. However, ΔJ3-16 transformant showed identical hybridization profile with the 2285 bp probe than the wild-type, indicating that *Thctf1* gene was intact in this transformant (data not shown).

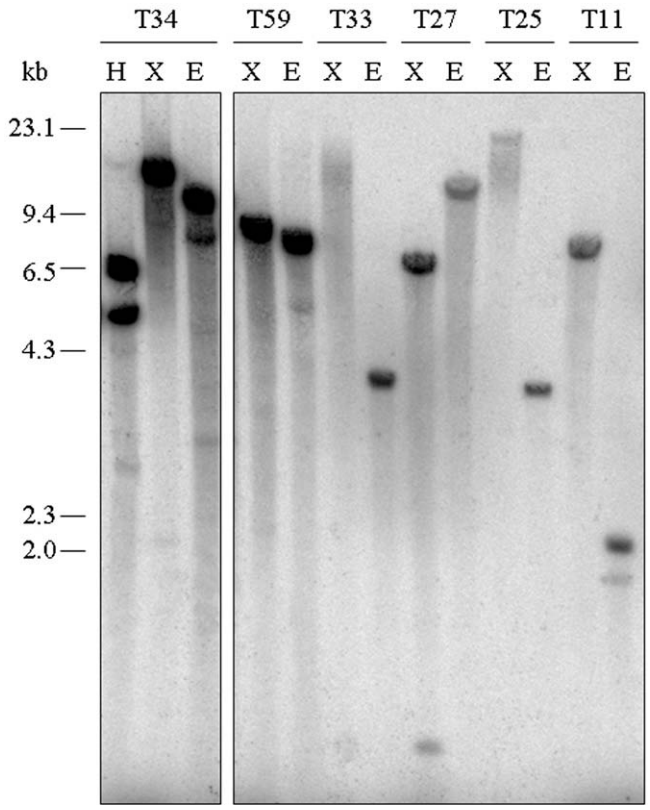

Fig. 3. Southern blot analysis of the *Thctf1* gene in different *Trichoderma* species. *Left*: Genomic DNA from *T. harzianum* T34 HindIII (H)-, *Xho*I (X)- or *Eco*RI (E)-digested. *Right*: Genomic DNA from *T. virens* T59, *T. atroviride* T33, *T. longibrachiatum* T27, *T. asperellum* T25 and *T. atroviride* T11 *Xho*I (X)- or *Eco*RI (E)-digested. Hybridization was carried out at 65 °C using a 2748-bp fragment of the *Thctf1* gene as probe. Molecular sizes (kb) are indicated.

To monitor *Thctf1* transcript levels in the wild-type and transformants, Northern blotting analyses were performed on total RNA isolated from *T. harzianum* T34 and the Δ2-11-2, ΔD1-5, ΔD1-38 and ΔJ3-16 transformant strains grown in a medium

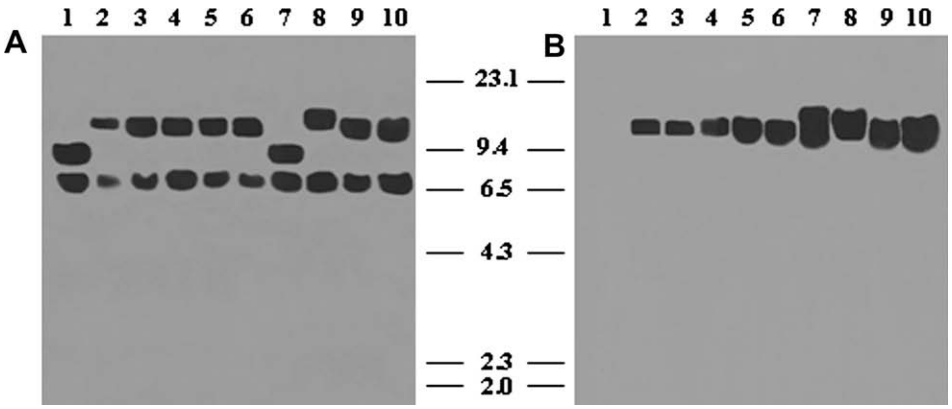

Fig. 4. Southern blot analysis of wild-type (*line 1*) and 9 transformants (*lines 2–10*, corresponding to Δ1-6-6, Δ2-11-1, Δ2-11-2, ΔJ2-11, ΔJ2-21, ΔJ3-16, ΔD1-5, ΔD1-38 and ΔD1-43 strains). Genomic DNAs were *Eco*RI-digested. (A) A 4737-bp fragment of *Thctf1* gene was used as probe. (B) A 3008-bp fragment of *amdS* gene was used as probe. The sizes of the marker bands are indicated at the right of the figure in kb.

supplemented with 1% olive oil (Fig. 5). No *Thctf1* mRNA was detected in transformants Δ2-11-2, ΔD1-5 or ΔD1-38, indicating that the *Thctf1* gene was not expressed in these transformants. The ΔD1-38 disruptant was chosen for further studies.

3.7. Phenotypic characterization of disruptants of T. harzianum

The wild-type, ΔD1-38 disruptant and ΔJ3-16 transformant strains were grown on MA, PPG and PDA plates at 25, 28 and 30 °C. With MA, a similar growth rate and degree of sporulation were observed in all the strains. With PPG, ΔD1-38 showed less sporulation and aerial mycelium production than T34 and ΔJ3-16 at 30 °C, but no differences were observed when growth was carried out at 25 °C. With PDA, ΔD1-38 exhibited a significantly reduced sporulation, growth rate and yellow pigment production with respect to the other strains after incubation at 25 or 30 °C. No phenotypical variation was observed between culture plates incubated at 28 and 30 °C.

3.8. Esterase activity assays

The T34 wild-type and ΔD1-38 disruptant strains were grown in MM without glucose (absence of a carbon source) or in MM supplemented with 1.5% chitin or 1% olive oil, conditions under which T34 had shown different esterase activity levels in previous studies (data not shown). In the presence of olive oil, the esterase activity of the ΔD1-38 strain was 1.7-fold higher than that of T34. However, for the rest of the culture conditions assayed, the differences between both strains were not significant. Similar results were obtained in two independent experiments.

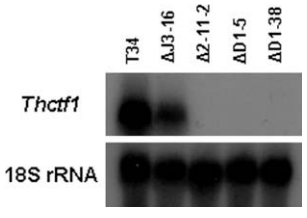

Fig. 5. Northern hybridization of total RNA from the wild-type strain T34 and the ΔJ3-16, Δ2-11-2, ΔD1-5 and ΔD1-38 transformants. Fungal mycelia were first grown on MM (Penttilä et al., 1987) containing 2% glucose as carbon source and were then transferred to MM containing 1% olive oil and harvested after 4 h. A 1493-bp fragment of the *Thctf1* coding region was used as probe, and the 18S rRNA gene was used as a loading control.

3.9. Secondary metabolite production

The T34 and disruptant ΔD1-38 strains were analyzed for their metabolite production. Fermentations were carried out separately for both strains in surface cultures and the broths were filtered and extracted with ethyl acetate and methanol, as indicated in Material and Methods. Evaporation of the solvents yielded 110 and 90 mg of a dense, brown oil from strains T34 and ΔD1-38, respectively. Analyses of both extracts revealed the presence of compounds in the T34 extract that were not present in the ΔD1-38 extract.

The brown oil extract (110 mg) obtained from the T34 strain was separated by means of column chromatography and final purification by HPLC. The polar fractions, eluted with EtOAc-hexane (80:20) and 100% in EtOAc, contained an intractable mixture (15.3 mg) of two compounds that could not be separated by HPLC. The mixture was acetylated by treatment with pyridine (2 ml) and acetic anhydride (3 ml). After 24 h, the reaction mixture was evaporated off at reduced pressure, yielding 16 mg of sample. The acetylated mixture was separated by means of semi-preparative HPLC, (LiChrospher Si-60, 10 μm, 1 cm wide, 25 cm long, EtOAc-hexane (1:1) as solvent, flow 3 ml/m). Two compounds 1 (1.5 mg) and **2** (2.0 mg) were isolated as acetyl derivatives and their structure was studied by ^{1}H NMR and ^{13}C NMR.

Both compounds **1** and **2** presented characteristic signals in the ^{1}H NMR spectrum of 6-pentyl-2*H*-pyran-2-one derivatives. The main differences observed in the ^{1}H NMR for compound **1** were a signal multiplet at δ 4.90, corresponding to a proton geminal to the acetoxy group; a signal doublet at δ 1.21, characteristic of a methyl group, and the characteristic singlet at 2.03 ppm of the acetate group. Its ^{13}C NMR spectrum contained carbonyl signals attributable to unsaturated δ-lactone and acetoxy carbons [δ 169 (s, Ac), 166.5 (s, C-6) 162.9 (s, C-2)], together with a signal at 70.2 characteristic of a carbon bearing an acetoxy group. Structure **1** (Fig. 6) was inferred from NMR spectral data and was confirmed by DEPT and COSY experiments.

The diacetoxy derivative **2** was obtained as an oil. The molecular formula –$C_{14}H_{18}O_6$– was confirmed by ^{13}C NMR spectral data. The IR revealed bands for the acetate groups and an unsaturated δ-lactone. The ^{1}H NMR study of compound **2** revealed that it retained the alpha-pyranone ring, together with two characteristic signals of protons geminal to the acetoxy groups at δ 5.28 (ddd, 1H) and 5.56 (d, 1H). Furthermore, the signals corresponding to the methyl of acetate groups, 2.03 (s) and 2.14 (s), together with signal methyl groups at 0.90 (t), corresponding to the methyl group at C-5′, allowed us to assign structure **2** to this compound (Fig. 6).

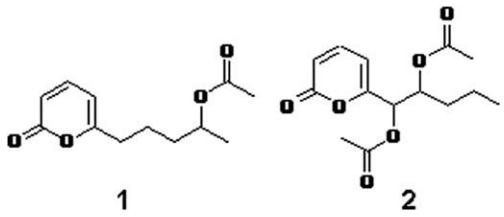

Fig. 6. Chemical structures of **1** and **2**; two compounds derived from 6-pentyl-2*H*-pyran-2-one.

The proposed structure was supported by homo- and heteronuclear-2D-correlation experiments.

3.10. Effect of the disruption of the Thctf1 gene on the antagonistic activity of Trichoderma

Four antifungal activity assays were performed: membrane growth, direct confrontation, spore germination and biomass production.

3.10.1. Growth assay on membranes

This assay was performed by growing the *T. harzianum* T34 or ΔD1-38 strains on cellophane and cellulose (cut-off 10 kDa) membranes to allow the presence of *Trichoderma* extracellular compounds in the medium. After removal of the membranes containing the mycelia, the effect of such hydrolytic enzymes plus metabolites (cellophane) or only metabolites (cellulose) on the growth of three different plant pathogenic fungi was determined. Table 1 summarizes the percentages of growth inhibition of *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *B. cinerea* by T34 or ΔD1-38. It may be observed that the inhibition values on cellophane were always generally higher than the values on cellulose, except in the case of *R. solani*, where an inhibition of approximately 77% was obtained with both membranes. Moreover, the inhibition of the growth of *F. oxysporum* and *R. solani* caused by the T34 and ΔD1-38 strains was higher than the inhibition of *B. cinerea* growth on both cellophane and cellulose.

Similarly, the inhibition values calculated for the growth of the three pathogens on cellophane and cellulose assays were higher with T34 than with ΔD1-38. Thus, on cellophane the percentage of inhibition of T34 against *F. oxysporum* f. sp. *lycopersici* was 33.8% higher than that measured with ΔD1-38, and on cellulose the inhibition values of T34 against *F. oxysporum* f. sp. *lycopersici* and *B. cinerea* were respectively 12.3% and 11.2% higher than those measured with ΔD1-38.

3.10.2. Direct confrontation assay

Plate confrontation experiments between T34 or ΔD1-38 and the pathogens *F. oxysporum* f. sp. *lycopersici*, *R. solani* and *B. cinerea* were carried out at 25 and 30 °C, and plates were observed after 8 days of incubation (data not shown). In all cases, after this incubation time, the three pathogens completely covered the surface of 90 mm diameter PDA or MEA plates used as control. In the dual cultures *Trichoderma* strains overgrew the colonies of *R. solani* and *B. cinerea*, and surrounded the colonies of *F. oxysporum*. At 30 °C, ΔD1-38 showed a slower and more irregular growth pattern than T34, such that while T34 overgrew or surrounded the pathogens, ΔD1-38 did not even approach them. At 25 °C, these differences were less remarkable. Both *Trichoderma* strains were able to inhibit the growth of the three pathogens tested reducing the colony diameter of *R. solani*, *B. cinerea* and *F. oxysporum* to no more than 25 mm, but no significant differences could be observed between the inhibition effect of T34 and ΔD1-38 in this assay. However, the above differences in pigmentation and sporulation between T34 and ΔD1-38 were again observed in the plate confrontation assays carried out at 25 and 30 °C.

Table 1
Percentages of growth inhibition of *F. oxysporum*, *R. solani* and *B. cinerea* by T34 and ΔD1-38 grown on cellophane or cellulose (cut-off 10 kDa) membranes.

% Growth inhibition	T34		ΔD1-38	
	Cellophane	Cellulose	Cellophane	Cellulose
F. oxysporum (120 h)	69.0 ± 1.7	46.1 ± 2.3	35.1 ± 1.4	33.8 ± 2.1
R. solani (96 h)	77.6 ± 2.9	77.1 ± 2.4	76.1 ± 2.8	72.5 ± 1.9
B. cinerea (72 h)	31.9 ± 1.6	18.0 ± 3.1	25.5 ± 5.6	6.8 ± 4.3

3.10.3. Spore germination assay

Supernatants of T34 and ΔD1-38 4 days PDB cultures were separated in two fractions (<10 kDa or >10 kDa sizes) as it was indicated in Section 2. The highest inhibition of *F. oxysporum* spore germination was always observed in the presence of *Trichoderma* fractions containing molecules with MW <10 kDa. T34 always showed a higher degree of inhibition than ΔD1-38, not only the <10 kDa fraction (Fig. 7A), but also the >10 kDa extract.

3.10.4. Mycelial biomass production assay

One hundred μg of 4 days supernatants and <10 kDa fractions contained in 100 μg supernatants of T34 and ΔD1-38 were tested against *F. oxysporum* f. sp. *lycopersici* in PDB cultures. After 64 h, T34 supernatant reduced the mycelium weight 23% more than did the ΔD1-38 supernatant, indicating a lower antifungal activity of the disruptant. As it could be expected, the <10 kDa fraction, containing low weight molecules like 6PP, showed more antifungal activity in T34 than in ΔD1-38 (Fig. 7B). No significative differences were observed between the *F. oxysporum* f. sp. *lycopersici* mycelium weight in the presence of <10 kDa fraction of ΔD1-38 and the control. Mycelial biomass production was significative lower in the presence of <10 kDa fraction of T34, 36% and 34% lower than the ΔD1-38 <10 kDa fraction and control, respectively.

4. Discussion

In recent decades, *Trichoderma* strains have been successfully used in the biocontrol of plant pathogenic fungi in the natural environment (Monte, 2001). The EU-funded *Trichoderma* functional genomic project "TrichoEST" (http://www.trichoderma.org) was initiated in 2001 to identify genes and gene products from 12 strains of biotechnological value, corresponding to eight *Trichoderma* species (Rey et al., 2004). As result of this project, an extensive EST collection was obtained after growing *Trichoderma* under mycoparasitic, plant-interaction and nutrient-stress conditions. More than 34400 clones were sequenced, of which 8710 belonged to *T. harzianum* T34 and corresponded to 3478 unique sequences (Vizcaino et al., 2006). A high number of ESTs were similar to genes with hydrolytic activities on fungal cell-wall, and only a few were related to plant cell-wall polymer degradation. One of these ESTs (EST 2092) showed a high degree of similarity with the transcription factor CTF1 beta, a cutinase activator previously described in *F. solani* f. sp. *pisi* (Li et al., 2002). Cutinases, which are extracellular esterases excreted by plant pathogenic fungi, catalyze the hydrolysis of cutin, the structural polymer of the cuticle of higher plants. This enzyme is involved in the penetration of fungal infection structures into plant cells and thus plays a crucial role in pathogenesis (Shaykh et al., 1977; Maiti and Kolattukudy, 1979; Köller and Kolattukudy, 1982). Since *Trichoderma* is a genus employed as a biocontrol agent and is defined as an opportunistic

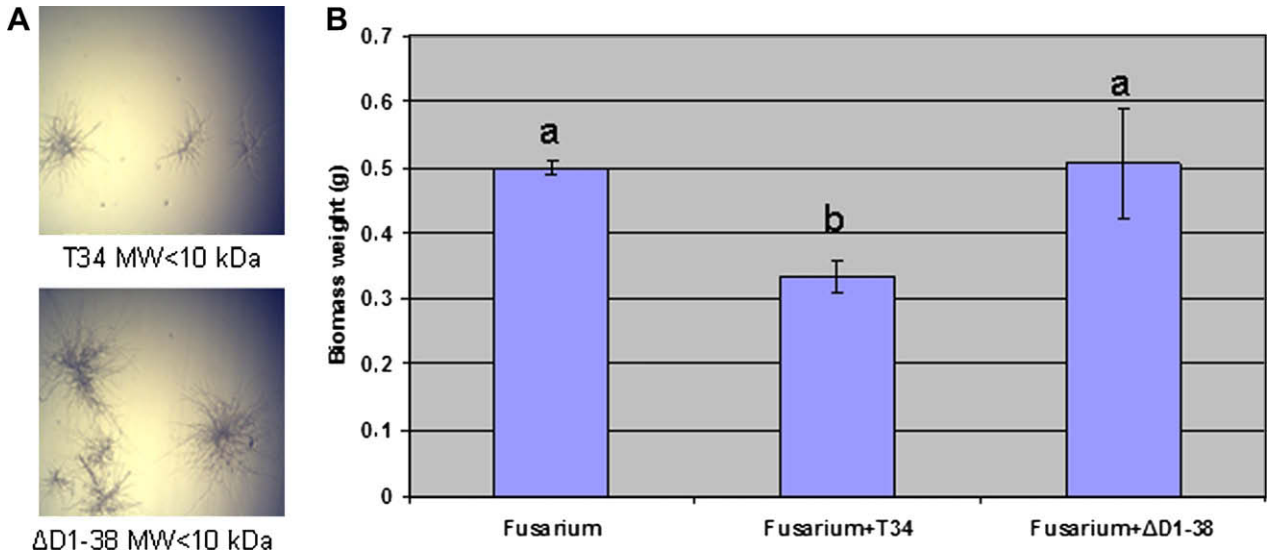

Fig. 7. (A) *In vitro* inhibition of the hyphal growth of *F. oxysporum* f. sp. *lycopersici* by <10 kDa fractions of T34 or ΔD1-38 strains. (B) Biomass accumulation in *F. oxysporum* f. sp. *lycopersici* after growth in PDB medium at 25 °C for 64 h in presence of <10 kDa fractions of T34 and ΔD1-38 strains. Values are means of three replicates with the corresponding standard deviation. Bars with different letters differ significantly (Fisher's test).

and avirulent plant symbiont (Harman et al., 2004), the study of EST 2092 was considered, even though no homologous cutinase *Fusarium* genes were found in the *Trichoderma* EST database and the sequenced genomes of *T. reesei* and *T. virens*.

Here we report the cloning and characterization of the *Thctf1* gene of *T. harzianum* T34 following a genomic approach. THCTF1 shows all the characteristics of a transcription factor. The presence of putative nuclear localization signals suggested that THCTF1 might be a nuclear protein. The N-terminal $Cys_6Zn(II)_2$ binuclear cluster motif found in this protein is also present in other regulatory proteins from filamentous fungi (Andrianopoulos and Hynes, 1990; Woloshuk et al., 1994; Suarez et al., 1995) and yeasts (Kim and Michels, 1988; Andre, 1990; Marczak and Brandriss, 1991; Yuan et al., 1991). However, THCTF1 does not share homology with any other regions of those factors.

It has been reported that the expression of the transcription factor CTF1 from *F. solani* f. sp. *pisi* might be regulated by catabolic repression or, alternatively, that CTF1 may be expressed constitutively but binds its recognition sequence only after an activation regulated by the catabolite level (Kämper et al., 1994). In the present work, we detected *Thctf1* expression in all the culture conditions assayed, including when the mycelia were cultured in a medium with 2% glucose, although three CreA/Cre1 consensus-binding sites (Cubero and Scazzocchio, 1994), related to catabolic repression, were found in the *Thctf1* promoter. Significant transcripts were detected when chitin or *B. cinerea* cell walls were present in the media, although no sequences were found in the *Thctf1* promoter that matched MYC motifs (Cortes et al., 1998), which have been suggested to be involved in the mycoparasitic response. It is curious that the expression level increased with time under the different assay conditions, except when the medium contained olive oil, which is considered to be a good substrate for esterases, where the highest transcript level was detected after 4 h of induction. In light of all these results, it seems likely that the expression of *Thctf1* would be regulated by at least two different systems.

Southern analyses revealed that the *Thctf1* gene was present as a single copy in *T. harzianum* T34, and a homologous gene was also present in other *Trichoderma* species. To investigate the possible function of *Thctf1* from *T. harzianum* T34, transformants without the functional copy of this gene were obtained. This was an important part of our study because the frequency of homologous recombination in *Trichoderma* is very low (Mach and Zeilinger, 1998) and has only been achieved once in *T. harzianum* (Rosado et al., 2007).

The ΔD1-38 disruptant showed a 1.7-fold increase in esterase activity with respect to the wild-type when olive oil was present in the medium. However, no differences were observed in activity levels when another two different growth conditions, such as the presence of chitin or the absence of a carbon source in the culture medium, were assayed. In view of the overall results, the role of THCTF1 in the regulation of esterase genes is not clear. Considering only the esterase activity on olive oil, which was increased in ΔD1-38, it could be speculated that *Thctf1* could affect the expression of esterase genes in the presence of an ester-rich substrate.

The growth, sporulation, and pigmentation of the wild-type and ΔD1-38 strains were assayed under several growing conditions and the observed differences were found to depend on the medium and on the incubation temperature. In PDA, the ΔD1-38 disruptant, as well as another five disruptants tested (data not shown), did not show the yellow pigmentation produced by pyrone-like metabolites observed in the wild-type and the ectopic transformant ΔJ3-16, used as a control. This phenotypic difference suggested that THCTF1 could be involved in the biosynthesis of some of these secondary metabolites. Chromatographic studies demonstrated that the disruptant and the wild-type produced fatty acids, phthalates and mixtures of polyhydroxylated compounds, whose structures have not yet been described, although some products were observed exclusively in extracts from T34. Spectroscopic analyses of these compounds confirmed that the wild-type produced two metabolites, derived from 6PP and not previously described in *Trichoderma* that were not produced by ΔD1-38. Because of the small amounts of compounds **1** and **2** obtained, the stereochemistry and absolute configuration could not be determined. Work is currently in progress to synthesize these compounds and determine their absolute configuration.

Four different inhibition tests were performed: growth assay on cellophane and cellulose membranes, direct confrontation assay, spore germination assay and mycelial biomass production assay. ΔD1-38 exhibited poorer antagonistic properties than the wild-type against three plant pathogenic fungi in three of the four inhibition tests. In dual cultures, the inhibition behavior, measured as

colony diameter reduction of the three pathogens was similar in both *Trichoderma* strains and the reduced ability of ΔD1-38 to overgrow or surround the pathogen colonies could be due to the slower and irregular growth of this strain with respect to T34. In the other cases, ΔD1-38 showed minor antifungal activity. Considering that the differences between the wild-type and disruptant strains, as regards the inhibition of *F. oxysporum* f. sp. *lycopersici* spore germination and the mycelium biomass reduction, were more patent when supernatants containing molecules of less than 10 kDa were used, it may be concluded that the THCTF1 transcription factor must be related to the production of secondary metabolites from *T. harzianum* T34, affecting its biocontrol properties.

Volatile pyranone derivatives can contribute to increasing plant resistance to insects and pathogens (Eckermann et al., 1998), and it is well known that 6PP metabolites are plant growth regulators (Cutler et al., 1986). *Trichoderma* biocontrol strains can indeed produce 6PP compounds, but the fact that these metabolites are degraded to less bioactive products suggests that 6PP has a useful but only transitory effect and may not persist in the environment (Hanson, 2005); accordingly, a signaling role for these molecules cannot be ruled out. *T. atroviride* mutants with reduced 6PP production have been reported to exert a strong antagonistic effect against *R. solani* (Mukherjee et al., 2007) and this suggests that 6PP might have functions other than solely those of the antibiotic type. It has also been suggested that 6PP could act as plant growth enhancers at low doses but that they would have an inhibitory effect at higher concentrations, and/or that they would be involved in the production of auxin inducers (Vinale et al., 2008). In *T. atroviride*, a relationship between 6PP and sesquiterpene regulation in heterotrimeric G protein-disrupted mutants (Reithner et al., 2005) and 6PP down-regulation by an MAPK (Reithner et al., 2007) has been described, but no 6PP biosynthesis-related genes have yet been implicated in plant growth promotion by *Trichoderma* or indeed any other microorganism. The THCTF1 transcription factor could help to understand this underexplored ability of *Trichoderma* in plant-interaction systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2008.10.008.

References

Andre, B., 1990. The *UGA3* gene regulating the GABA catabolic pathway in *Saccharomyces cerevisiae* codes for a putative zinc-finger protein acting on RNA amount. Mol. Gen. Genet. 220, 269–276.

Andrianopoulos, A., Hynes, M.J., 1990. Sequence and functional analysis of the positively acting regulatory gene *amdR* from *Aspergillus nidulans*. Mol. Cell. Biol. 10, 3194–3203.

Benitez, T., Rincon, A.M., Limon, M.C., Codon, A.C., 2004. Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol. 7, 249–260.

Bisby, G.R., 1939. *Trichoderma viride* Pers ex Fr And notes on *Hypocrea*. Trans. Br. Mycol. Soc. 23, 149–168.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Cardoza, R.E., Hermosa, M.R., Vizcaino, J.A., Sanz, L., Monte, E., Gutierrez, S., 2005. Secondary metabolites produced by *Trichoderma* and their importance in the biocontrol process. In: Mellado, E., Barredo, J.L. (Eds.), Microorganisms for Industrial Enzymes and Biocontrol. Research Signpost, Kerala, pp. 1–22.

Collins, R.P., Halim, A.F., 1972. Characterization of the major aroma constituent of the fungus *Trichoderma viride* (Pers.). J. Agric. Food Chem. 20, 437–438.

Cooney, J.M., Lauren, D.R., 1999. Biotransformation of the *Trichoderma* metabolite 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) by selected fungal isolates. J. Nat. Prod. 62, 681–683.

Cortes, C., Gutierrez, A., Olmedo, V., Inbar, J., Chet, I., Herrera-Estrella, A., 1998. The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. Mol. Gen. Genet. 260, 218–225.

Cubero, B., Scazzocchio, C., 1994. Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of *Aspergillus nidulans*. EMBO J. 13, 407–415.

Cutler, H.G., Cox, R.H., Crumley, F.G., Cole, P.D., 1986. 6-Pentyl-alphapyrone from *Trichoderma harzianum*: its plant growth inhibitory and antimicrobial properties. Agric. Biol. Chem. 50, 2943–2945.

Dennis, C., Webster, J., 1971. Antagonistic properties of species-groups of *Trichoderma* II. Production of volatile antibiotics. Trans. Br. Mycol. Soc. 57, 41–48.

Dickinson, J.M., 1993. Microbial pyran-2-ones and dihydropyran-2-ones. Nat. Prod. Rep. 10, 71–98.

Djonovic, S., Vargas, W.A., Kolomiets, M.V., Horndeski, M., Wiest, A., Kenerley, C.M., 2007. A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. Plant Physiol. 145, 875–889.

Eckermann, S., Gudrun Schröder, G., Schmidt, J., Strack, D., Edrada, R.A., Helariutta, Y., Paula Elomaa, P., Kotilainen, M., Kilpeläinen, I., Proksch, P., Teeri, T.H., Schröder, J., 1998. New pathway to polyketides in plants. Nature 396, 387–390.

Evidente, A., Cabras, A., Maddau, L., Serra, S., Andolfi, A., Motta, A., 2003. Viridepyronone, a new antifungal 6-substituted 2*H*-pyran-2-one produced by *Trichoderma viride*. J. Agric. Food Chem. 51, 6957–6960.

Gorner, W., Durchschlag, E., Martinez-Pastor, M.T., Estruch, F., Ammerer, G., Hamilton, B., Ruis, H., Schuller, C., 1998. Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev. 12, 586–597.

Hanson, J.R., 2005. The chemistry of the bio-control agent, *Trichoderma harzianum*. Sci. Prog. 88, 237–248.

Harman, G.E., 2006. Overview of mechanism and uses of *Trichoderma* spp. Phytopathology 96, 190–194.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M., 2004. *Trichoderma* species: opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. 2, 43–56.

Hill, R.A., Cutler, H.G., Parker, S.R., 1995. *Trichoderma* and metabolites as control agents for microbial plant diseases (PCT Int. Appl. WO). Chem. Abstr. 123, 220823.

Ilmen, M., Thrane, C., Penttilä, M., 1996. The glucose repressor gene *cre1* of *Trichoderma*: isolation and expression of a full-length and a truncated mutant form. Mol. Gen. Genet. 251, 451–460.

Jones, D.T., 1999. Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. 292, 195–202.

Kämper, J.T., Kämper, U., Rogers, L.M., Kolattukudy, P.E., 1994. Identification of regulatory elements in the cutinase promoter from *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*). J. Biol. Chem. 269, 9195–9204.

Kelly, J.M., Hynes, M.J., 1985. Transformation of *Aspergillus niger* by the *amdS* gene of *Aspergillus nidulans*. EMBO J. 4, 475–479.

Keszler, A., Forgacs, E., Kotai, L., Vizcaino, J.A., Monte, E., Garcia-Acha, I., 2000. Separation and identification of volatile components in the fermentation broth of *Trichoderma atroviride* by solid-phase extraction and gas chromatography–mass spectrometry. J. Chromatogr. Sci. 38, 421–424.

Kim, J., Michels, C.A., 1988. The *MAL63* gene of *Saccharomyces* encodes a cysteine-zinc finger protein. Curr. Genet. 14, 319–323.

Kolattukudy, P.E., 1985. Enzymatic penetration of the plant cuticle by fungal pathogens. Annu. Rev. Phytopathol. 23, 223–250.

Köller, W., Kolattukudy, P.E., 1982. Mechanism of action of cutinase: chemical modifications of the catalytic triad characteristic for serine hydrolases. Biochemistry 21, 3083–3090.

Kulmburg, P., Mathieu, M., Dowzer, C., Kelly, J., Felenbok, B., 1993. Specific binding sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. Mol. Microbiol. 7, 847–857.

Li, D., Sirakova, T., Rogers, L., Ettinger, W.F., Kolattukudy, P.E., 2002. Regulation of constitutively expressed and induced cutinase genes by different zinc finger transcription factors in *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*). J. Biol. Chem. 277, 7905–7912.

Limon, M.C., Chacon, M.R., Mejias, R., Delgado-Jarana, J., Rincon, A.M., Codon, A.C., Benitez, T., 2004. Increased antifungal and chitinase specific activities of *Trichoderma harzianum* CECT 2413 by addition of a cellulose binding domain. Appl. Microbiol. Biotechnol. 64, 675–685.

Lora, J.M., de la Cruz, J., Llobell, A., Benitez, T., Pintor-Toro, J.A., 1995. Molecular characterization and heterologous expression of an endo-beta-1, 6-glucanase gene from the mycoparasitic fungus *Trichoderma harzianum*. Mol. Gen. Genet. 247, 639–645.

Lorito, M., Peterbauer, C., Hayes, C.K., Harman, G.E., 1994. Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. Microbiology 140, 623–629.

Mach, R.L., Zeilinger, S., 1998. Genetic transformation of *Trichoderma* and *Gliocladium*. In: Kubicek, C.P., Harman, G.E. (Eds.), *Trichoderma* and *Gliocladium*, vol. 1. Taylor and Francis, London, pp. 225–242.

Maiti, I.B., Kolattukudy, P.E., 1979. Prevention of fungal infection of plants by specific inhibition of cutinase. Science 205, 507–508.

Marczak, J.E., Brandriss, M.C., 1991. Analysis of constitutive and noninducible mutations of the PUT3 transcriptional activator. Mol. Cell. Biol. 11, 2609–2619.

Monte, E., 2001. Understanding *Trichoderma*: between biotechnology and microbial ecology. Int. Microbiol. 4, 1–4.

Mukherjee, M., Mukherjee, P.K., Kale, S.P., 2007. CAMP signalling is involved in growth, germination, mycoparasitism and secondary metabolism in *Trichoderma virens*. Microbiology 153, 1734–1742.

Nakai, K., Horton, P., 1999. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem. Sci. 24, 34–36.

Penttilä, M., Nevalainen, H., Ratto, M., Salminen, E., Knowles, J., 1987. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61, 155–164.

Raeder, U., Broda, P., 1985. Rapid preparation of DNA from filamentous fungi. Lett. Appl. Microbiol. 1, 17–20.

Ravagnani, A., Gorfinkiel, L., Langdon, T., Diallinas, G., Adjadj, E., Demais, S., Gorton, D., Arst Jr., H.N., Scazzocchio, C., 1997. Subtle hydrophobic interactions between the seventh residue of the zinc finger loop and the first base of an HGATAR sequence determine promoter-specific recognition by the *Aspergillus nidulans* GATA factor AreA. EMBO J. 16, 3974–3986.

Reino, J.L., Guerrero, R.F., Hernández-Galan, R., Collado, I.G., 2008. Secondary metabolites from species of the biocontrol agent *Trichoderma*. Phytochem. Rev. 7, 89–123.

Reithner, B., Brunner, K., Schuhmacher, R., Peissl, I., Seidl, V., Krska, R., Zeilinger, S., 2005. The G protein alpha subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites. Fungal Genet. Biol. 42, 749–760.

Reithner, B., Schuhmacher, R., Stoppacher, N., Pucher, M., Brunner, K., Zeilinger, S., 2007. Signaling via the *Trichoderma atroviride* mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection. Fungal Genet. Biol. 44, 1123–1133.

Rey, M., Llobell, A., Monte, E., Scala, F., Lorito, M., 2004. Genomics of *Trichoderma*. In: Khachatourians, G.G. (Ed.), Fungal Genomics. Elsevier Science, Amsterdam.

Rosado, I.V., Rey, M., Codon, A.C., Govantes, J., Moreno-Mateos, M.A., Benitez, T., 2007. QID74 Cell wall protein of *Trichoderma harzianum* is involved in cell protection and adherence to hydrophobic surfaces. Fungal Genet. Biol. 44, 950–964.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, New York, USA.

Serrano-Carreon, L., Hathout, Y., Bensoussan, M., Belin, J.M., 1993. Metabolism of linoleic acid or mevalonate and 6-pentyl-alpha-pyrone biosynthesis by *Trichoderma* species. Appl. Environ. Microbiol. 59, 2945–2950.

Shaykh, M., Soliday, C., Kolattukudy, P.E., 1977. Proof for the production of cutinase by *Fusarium solani* f. *Pisi* during penetration into its host, *Pisum sativum*. Plant Physiol. 60, 170–172.

Sivasithamparam, K., Ghisalberti, E.L., 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*. In: Kubicek, C.P., Harman, G.E. (Eds.), *Trichoderma* and *Gliocladium*, vol. 1. Taylor and Francis, London, pp. 139–191.

Suarez, T., de Queiroz, M.V., Oestreicher, N., Scazzocchio, C., 1995. The sequence and binding specificity of UaY, the specific regulator of the purine utilization pathway in *Aspergillus nidulans*, suggest an evolutionary relationship with the PPR1 protein of *Saccharomyces cerevisiae*. EMBO J. 14, 1453–1467.

Sweigard, J.A., Chumley, F.G., Valent, B., 1992. Disruption of a *Magnaporthe grisea* cutinase gene. Mol. Gen. Genet. 232, 183–190.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Woo, S.L., Lorito, M., 2008. *Trichoderma*–plant–pathogen interactions. Soil Biol. Biochem. 40, 1–10.

Vizcaino, J.A., Gonzalez, F.J., Suarez, M.B., Redondo, J., Heinrich, J., Delgado-Jarana, J., Hermosa, R., Gutiérrez, S., Monte, E., Llobell, A., Rey, M., 2006. Generation, annotation and analysis of ESTs from Trichoderma harzianum CECT 2413. BMC Genomics 7, 193.

Vizcaino, J.A., Redondo, J., Suarez, M.B., Cardoza, R.E., Hermosa, R., Gonzalez, F.J., Rey, M., Monte, E., 2007. Generation, annotation, and analysis of ESTs from four different *Trichoderma* strains grown under conditions related to biocontrol. Appl. Microbiol. Biotechnol. 75, 853–862.

Woloshuk, C.P., Foutz, K.R., Brewer, J.F., Bhatnagar, D., Cleveland, T.E., Payne, G.A., 1994. Molecular characterization of *aflR*, a regulatory locus for aflatoxin biosynthesis. Appl. Environ. Microbiol. 60, 2408–2414.

Yuan, G.F., Fu, Y.H., Marzluf, G.A., 1991. *Nit-4*, a pathway-specific regulatory gene of *Neurospora crassa*, encodes a protein with a putative binuclear zinc DNA-binding domain. Mol. Cell Biol. 11, 5735–5745.