

Involvement of *Trichoderma* Trichothecenes in the Biocontrol Activity and Induction of Plant Defense-Related Genes

M. G. Malmierca,^a R. E. Cardoza,^a N. J. Alexander,^b S. P. McCormick,^b R. Hermosa,^c E. Monte,^c and S. Gutiérrez^a

Area of Microbiology, University School of Agricultural Engineers, University of León, Campus de Ponferrada, Ponferrada, Spain^a; Bacterial Foodborne Pathogen and Mycology Unit, USDA/ARS, National Center for Agricultural Utilization Research, Peoria, Illinois, USA^b; and Spanish-Portuguese Centre of Agricultural Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, Salamanca, Spain^c

Trichoderma species produce trichothecenes, most notably trichodermin and harzianum A (HA), by a biosynthetic pathway in which several of the involved proteins have significant differences in functionality compared to their *Fusarium* orthologues. In addition, the genes encoding these proteins show a genomic organization differing from that of the *Fusarium tri* clusters. Here we describe the isolation of *Trichoderma arundinaceum* IBT 40837 transformants which have a disrupted or silenced *tri4* gene, a gene encoding a cytochrome P450 monooxygenase that oxygenates trichodiene to give rise to isotrichodiol, and the effect of *tri4* gene disruption and silencing on the expression of other *tri* genes. Our results indicate that the *tri4* gene disruption resulted in a reduced antifungal activity against *Botrytis cinerea* and *Rhizoctonia solani* and also in a reduced ability to induce the expression of tomato plant defense-related genes belonging to the salicylic acid (SA) and jasmonate (JA) pathways against *B. cinerea*, in comparison to the wild-type strain, indicating that HA plays an important function in the sensitization of *Trichoderma*-pre-treated plants against this fungal pathogen. Additionally, the effect of the interaction of *T. arundinaceum* with *B. cinerea* or *R. solani* and with tomato seedlings on the expressions of the *tri* genes was studied.

Trichothecenes are important mycotoxins known mainly for their phytotoxicity and for their toxic effects on animals and humans (16, 39). Trichothecenes are produced by the genera *Fusarium*, *Stachybotrys*, *Myrothecium*, and *Trichoderma* (14) as well as others (12). The trichothecene biosynthetic pathways in *Trichothecium* (33), *Fusarium* (5, 8, 23, 26, 29, 59), *Myrothecium* (53), and, recently, *Trichoderma* (11) have been studied. In *Fusarium* and *Trichoderma*, most of the genes involved in trichothecene biosynthesis are clustered, but the two genera display important genetic organizational differences. In *Fusarium*, the known genes for trichothecene biosynthesis are located in three different loci: one locus composed of a main cluster containing 12 genes, including *Tri5*, and the other two loci composed of 1 or 2 genes (43). In *Trichoderma*, only the *tri5* gene is located outside the main cluster (11). In both genera, *Tri5* encodes trichodiene synthase, a terpene cyclase that is the first committed enzyme of the trichothecene biosynthetic pathway, which converts farnesyl pyrophosphate (FPP) to trichodiene (24, 25). *Trichoderma* is the only trichothecene producer described so far in which the *tri5* gene is not located in the main *tri* cluster. The explanation for this gene location still remains unclear; however, a plausible explanation would be that the ancestral *tri* cluster included this gene and that during evolution, *tri5* was located outside the cluster in the *Trichoderma* trichothecene producers. Once trichodiene is produced in *Trichoderma*, it is then oxygenated by the *tri4*-encoded cytochrome P450 monooxygenase, utilizing three consecutive oxygenation steps at the C-2, C-12, and C-11 positions to produce isotrichodiol (11). The 3-step oxygenation by *Tri4* is also found for *Myrothecium* (37). Those trichothecenes that have an oxygenated C-3 position (such as T2-toxin, deoxynivalenol, and nivale-nol) have a *tri4*-encoded P450, which oxygenates four positions (C-2, C-12, C-11, and C-3) (36, 52). *Trichoderma Tri4* contains all the conserved structural features described for heme-thiolate proteins (2, 58), such as that seen for the *Tri11* protein, another monooxygenase (single step), which is involved in the C-4 hy-

droxylation of 12,13-epoxytrichothec-9-ene (EPT) to produce trichodermin in *Trichoderma* (11).

The *tri4* gene of *Trichoderma arundinaceum* spans 1,791 bp, with four introns of 70, 58, 56, and 53 bp, and encodes a protein of 517 amino acids, with a deduced molecular mass of 58 kDa and a pI of 7.9. A comparison of the *Tri4* protein with the nr database using the BLASTX algorithm resulted in high identities with *Tri4* proteins from *Trichothecium roseum* and *Fusarium* species (11). Lower identity values were obtained with P450 monooxygenases of a plethora of fungal species, such as *Myrothecium roridum*, *Aspergillus oryzae*, *Aspergillus clavatus*, and *Penicillium*, as representative examples.

Trichoderma species are also known for their biocontrol activity against phytopathogenic fungi (18, 31). This activity is carried out in several different ways, such as the production of extracellular hydrolytic enzymes (6) or the production of secondary metabolites with antifungal activity (48, 57). Proteins may also be involved in competition for nutrients conferring a nutritional advantage (17). Moreover, as revealed by research in the last decade, some *Trichoderma* strains can interact directly with roots, resulting in increased plant growth potential, resistance to diseases, and tolerance to abiotic stresses (21, 47).

Harzianum A (HA) has shown cytotoxic activity against some mammalian tumor cell lines (28). However, its phytotoxic ability has not been studied until now, although trichodermin, a trichothecene produced by *Trichoderma brevicompactum* strains

Received 10 February 2012 Accepted 26 April 2012

Published ahead of print 4 May 2012

Address correspondence to S. Gutiérrez, s.gutierrez@unileon.es.

Supplemental material for this article may be found at <http://aem.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AEM.00385-12

(14), has shown strong phytotoxic activity (50). Trichodermin has an acetyl group at C-4, while HA has an octa-2,4,6-trienedioic acid at that position. In both cases, it has been hypothesized that those lateral moieties might be added to trichodermol by an acyl-transferase encoded by the *tri3* gene (11).

T. arundinaceum might serve as a model to determine the beneficial effect of trichothecenes on *Trichoderma* biocontrol activity and also on the modulation of plant defense responses by this fungus.

In this article, we describe the effect of the disruption or silencing of *tri4* in *T. arundinaceum* on the gene expressions of the other *tri* genes involved in HA biosynthesis. In addition, we determined the abilities of the transformants (*tri4*-silenced or -disrupted) and the wild-type strain to produce HA, to act as a biocontrol agent, and to elicit plant defense responses in tomato plants.

MATERIALS AND METHODS

Strains, culture media, and culture conditions used in the present work.

Trichoderma arundinaceum IBT 40837 (Ta37) (IBT Culture Collection of Fungi at the Department of Biotechnology, Technical University of Denmark) was kindly provided by Ulf Thrane. For trichothecene analysis and RNA isolation, *Trichoderma arundinaceum* strains were grown by using a two-step procedure with CM (0.5% malt extract, 0.5% yeast extract, 0.5% glucose), followed by growth in PDB modified medium as described previously (11).

T. harzianum T34 (CECT 2413; Spanish Type Culture Collection, Valencia, Spain) was used as a control in the antifungal experiments.

The fungal phytopathogen *Botrytis cinerea* 98 was isolated from diseased strawberry plants, and *Rhizoctonia solani* CECT 2815, *Myrothecium roridum* ATCC 52485, and *Fusarium sporotrichioides* CECT 20166 were used as targets for the dual-confrontation assays. *B. cinerea* and *R. solani* were also included for growth assays on membranes.

All fungal strains were routinely maintained on PDA (2.4% PDB, 2% agar), except for *B. cinerea* and Ta37, which were maintained on MEA (2% glucose, 2% malt extract, 1% peptone, 2% agar [pH 5.6]) and PPG (2% [dry wt/vol] dehydrated potato flakes, 2% glucose, 2% agar), respectively.

Solanum lycopersicum var. Marmande (Semillas Battle S.A., Barcelona, Spain) was used for fungus-plant interaction studies.

Xanthotoxin (Sigma, St. Louis, MO) was added, when indicated, to the culture medium in acetone for a 0.1 mM final concentration.

Construction of plasmids pΔtri4 and pURS4. Plasmid pURS4 was constructed by isolating a 554-bp fragment that corresponds to the second exon of the Ta37 *tri4* gene (see Fig. S1A in the supplemental material), using BamHI-SacI endonuclease digestion of plasmid pF42E4 containing the complete *tri4* open reading frame (ORF) (11). This fragment was treated with Klenow and ligated with plasmid pSIL, which contained a 150-bp intron, the *tss1* promoter, and the terminator region of the *cbh2* gene from *Trichoderma reesei* (49), which had been digested with BamHI, treated with Klenow enzyme, and dephosphorylated. The resulting plasmid, pSILtri4Sa, had one copy of the internal *tri4* fragment. This plasmid was digested with EcoRV, dephosphorylated, and ligated again with the same internal blunt-ended BamHI-SacI 554-bp fragment of the *tri4* gene, resulting in plasmid pSILtri4SaSb. This plasmid has two copies of *tri4* exon 2 in opposite orientations, separated by the above-mentioned 150-bp intron, which will form a hairpin RNA structure containing an intron (ihpRNA) in the transformed cell. Finally, a 3.2-kb SacI fragment from pSILtri4SaSb containing the whole silencing cassette was ligated with plasmid pUR5750 (15) (digested with SacI and dephosphorylated) to obtain the final construct, pURS4, of 17.7 kb (see Fig. S1B in the supplemental material).

A 777-bp fragment of the *tri4* gene (from bp 120 to 897) was amplified by using iProof high-fidelity DNA polymerase (Bio-Rad, Hercules, CA) and primer pair 2102/2103 (see Table S1 in the supplemental material)

(with an AscI restriction site at the end), using genomic DNA (GenBank accession number FN394491). The amplicon was band purified with the Ultraclean DNA purification kit (MoBio, Carlsbad, CA) and cloned into pCR4 Blunt TOPO (Invitrogen, Carlsbad, CA). The resulting plasmid was then cut with AscI and ligated with a chimeric hygromycin B gene (2.5 kb), which contained AscI restriction sites at both ends of the chimera (55), leading to the final 7.3-kb pΔtri4 vector (see Fig. S1B in the supplemental material).

Transformation of *Trichoderma arundinaceum*. Strain Ta37 was transformed according to the following two different procedures.

Plasmid pURS4 was transformed by using *Agrobacterium*-mediated transformation as described previously (9). The transformants were selected by hygromycin B resistance, and after two rounds of growth in selective medium, 10 transformants were chosen for PCR and Southern analysis.

Plasmid pΔtri4 was transformed by using a protoplast transformation protocol as described previously (38), with the only difference being that protoplasts were formed by incubating the mycelium with lytic enzymes (5 mg/ml lysing enzymes, 25 mg/ml driselase, and 0.05 mg/ml chitinase in a 0.7 M NaCl solution) (Sigma, St. Louis, MO) for 20 h. The transformants were selected by resistance to hygromycin B (100 μg/ml). The selected transformants were analyzed by PCR using primer pair Tri437-5/Tri437-3b (see Table S1 in the supplemental material), using the Terra PCR Direct polymerase mix (Clontech, Mountain View, CA), and by Southern hybridization to detect those transformants with the pΔtri4 vector inserted into the middle of the *tri4* gene.

Extraction and chemical analysis of trichothecenes and derivatives. Cultures were analyzed by high-performance liquid chromatography (HPLC) for HA as previously described (11).

Cultures were also extracted and analyzed by gas chromatography low-resolution mass spectrometry (GCMS) for other trichothecene-related metabolites. GCMS measurements were made with a Hewlett Packard 6890 gas chromatograph fitted with an HP-5MS column (30-mm by 0.25-mm film thickness) and a 5973 mass detector. The carrier gas was helium with a 20:1 split ratio and a 20-ml/min split flow. The program used was as follows: the column was held at 120°C at injection, heated to 260°C at 25°C/min, and held for 4.4 min (total run time of 10 min). Individual components were isolated from concentrated extracts that were separated on a silica gel column eluted with hexane-ethyl acetate (3:1) and characterized by GCMS and nuclear magnetic resonance (NMR) spectroscopy.

Antifungal assays. (i) Direct confrontation assay. *In vitro* confrontation assays between *Trichoderma* strains (*T. arundinaceum* Ta37 and disruptant strain TaΔTri4) and the pathogens *R. solani*, *M. roridum*, *F. sporotrichioides* (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 cm apart in parallel on PDA or MEA plates and incubated at 28°C in the dark. The behaviors of *T. arundinaceum* strains against each pathogen were 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 controls. Photographs were taken after 10 days.

(ii) Growth assay on membranes. Five-millimeter-diameter PDA plugs of Ta37 or transformants (disruptant strain TaΔTri4 and silencing strain TaS4-9) were placed at the center of petri dishes containing PDA or MEA medium, on cellophane sheets, or on 10-kDa-cutoff dialysis membranes. After 2 days of incubation at 28°C, the membranes were removed from the plates, and a single 5-mm-diameter mycelial plug of the pathogens *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 three plates. Growth diameters were calculated after 72 and 96 h for *R. solani* and *B. cinerea*, respectively. The results are expressed as percentages of the inhibition of growth of each pathogen by *Trichoderma* strains.

(iii) **Antibiogram of an extracted Ta37 culture broth against *B. cinerea*.** Bioassay plates were made by inoculating 1×10^5 spores/ml of *B. cinerea* in warm (50°C) MEA (1% agar) medium. A 24-h-old culture of Ta37 was extracted with ethyl acetate and finally dissolved in acetonitrile, as previously described (11). We checked for the presence of any other compounds apart from HA and measured the HA concentration in the Ta37 sample by HPLC. Four different dilutions in acetonitrile (2.4, 1.2, 0.6, and 0.3 mg/ml HA) were tested against *B. cinerea*. Forty microliters of each dilution was added into holes with a 5.5-mm diameter and 3.5-mm depth that were formed in the bioassay plates. As a negative control, acetonitrile was placed into one hole. Plates were maintained at 4°C for 5 h to allow the solution to diffuse and then incubated for 1 week at 28°C to visualize the growth inhibition zone. The experiment was done in triplicate.

Tomato plant assays. *Solanum lycopersicum* var. Marmande tomato seeds were surface sterilized by washing for 10 min in 70% ethanol and 10 min in 50% sodium hypochlorite and thoroughly washed in sterile distilled water.

An *in vivo* assay was used to evaluate the abilities of *Trichoderma* strains to promote tomato plant growth. Tomato seeds were coated with an aqueous suspension containing 2×10^8 spores of *Trichoderma* per ml (1 ml of spore suspension/30 seeds) and then air dried in an open petri dish overnight under a laminar flow hood. Treated tomato seeds were sown in pots (32 pots per condition) containing commercial loamy field soil (Kekkilä 50/50; Projar S.A., Valencia, Spain), with the following composition: 90% organic matter (coconut shells and peat), 10% ash, 50% humidity, 1-4/1-6 decomposition degree (von Post), pH 5.5 to 6.6, 0.2% nitrogen, 0.1% total phosphorus, 0.5% calcium, 0.1% magnesium, 0.2% iron, 1.5 mg/kg of chlorides, and 80 g/liter of dry weight, previously autoclaved at 121°C for 1 h on two successive days. Pots with untreated tomato seeds were used as controls. The pots were incubated in a greenhouse at $22^\circ\text{C} \pm 4^\circ\text{C}$ and watered as needed. Measurements of stem height, stem diameter, and main root length were taken after 4 weeks.

Four-week-old plants obtained from tomato seeds coated with Ta37, disruptant strain Ta Δ Tri4, or water (control) were inoculated with $15 \mu\text{l}$ of *B. cinerea* conidial suspensions (1×10^6 spores/ml) in germination buffer (20 mM glucose and 20 mM KH_2PO_4) onto the leaf surface. Inoculated plants were incubated in a humid chamber under the conditions described above. Two inoculations were made per leaf on four leaves per plant for 16 plants per treatment and two replicates for each experiment. After 4 days, the necrotic lesions were measured. Leaves before and after pathogen inoculation were collected to extract RNA as described above. In parallel, uninoculated plants were used as controls.

Tomato-*Trichoderma* hydroponic cultures were carried out as previously described (45). First, sterile tomato seeds were placed inside Phytatray II boxes (Sigma, St. Louis, MO) (30 seeds per box) on a sterile gauze sheet over a sterile stainless steel screen, which held them 1 cm above 100 ml of Murashige and Skoog (MS) medium (Duchefa, Netherlands), and maintained at 25°C in a plant growth chamber with controlled light and humidity, as described above, for 2 weeks. Spores of *Trichoderma* (10^7 spores) were used to inoculate 250-ml flasks containing 100 ml of PDB medium. Each strain was cultured at 28°C and at 200 rpm in darkness for 48 h. Mycelia were then harvested by filtration, washed with sterile water, and used to inoculate Phytatray II boxes that contained 2-week-old tomato plants. Tomato-*Trichoderma* hydroponic cultures were maintained at 25°C and at 80 rpm for 20 h. Finally, *Trichoderma* mycelia attached to roots were recovered with a direct cold-water jet and used for gene expression analysis by quantitative PCR (qPCR).

Chitinase activity assays. Cultures of the different strains were grown in modified PDB medium at 28°C and at 250 rpm for 96 h. PDB was selected even when it was not the ideal medium to induce chitinases, since it was the best medium for the production of HA among all the examined media and conditions. Supernatant samples of 8 ml were taken every 24 h, and they were frozen until they were analyzed for chitinase activity.

The extracellular chitinase activity was quantified by using *p*-nitrophenyl- β -D-glucosaminide (*p*-nitrophenyl- β DG) as the substrate, which

was prepared at 12.5 mM in methanol–50 mM Tris-HCl (pH 6.0) (vol/vol).

The reaction mixture was prepared by the addition of 50 μl of supernatant, 1 mM substrate, and 50 mM Tris-HCl buffer (pH 6.0) in a 0.5-ml final volume. The reaction mix was incubated at 25°C for 1 h, and the reaction was stopped with 0.5 ml of methanol. The hydrolysis of *p*-NP β DG was determined by measuring the absorbance at a 410-nm wavelength. The activity was quantified by performing a *p*-NP standard curve. The total activity corresponded to nanomoles of *p*-NP formed in 1 min, and the specific activity corresponded to nanomoles formed in a minute per mg of dry weight. In all cases, the background caused by nonenzymatic substrate degradation was determined in a parallel assay with a mixture containing protein extract that had been treated for 5 min at 100°C and was subtracted from the values for nontreated enzyme extracts.

Nucleic acid extraction and manipulation. The procedures for fungal genomic DNA isolation and Southern hybridization were performed as described previously (11).

To isolate total RNA, *Trichoderma* strains were grown as indicated above. Mycelia were then recovered from modified PDB medium by filtration, washed with 0.9% NaCl, dried on absorbent filter paper, frozen with liquid nitrogen, and ground in a mortar. RNA was extracted by the phenol-SDS method (4) and treated with DNase and RNase protector (Fermentas, Vilnius, Lithuania).

The same procedure was also used to extract RNA from mycelia of the 5-mm interaction zone in confrontation assays and from tomato leaves.

The cDNA was synthesized by using 1 μg of total RNA and a reverse transcription system (Promega, Madison, WI).

Labeling, hybridization, and immunological detection were carried out with a nonradioactive labeling and immunological detection kit with CDP-Star as the chemiluminescent substrate (Roche, Mannheim, Germany), as previously described (10).

qPCR experiments. In order to perform comparative studies of the *tri* gene or tomato plant defense gene expression levels, oligonucleotides were designed (see Table S1 in the supplemental material) based on the sequences of the studied genes (11, 54). In the case of *tri* gene expression, we ran GeNorm software (56) to determine the best reference genes among the α -actin, *gpd*, and β -tubulin genes. According to the GeNorm results, the α -actin and *gpd* genes were used as reference genes, and the β -tubulin gene was discarded. For tomato defense-related gene expression assays, we used α -actin as a reference gene. The qPCRs were carried out by using the Step One Plus system (Applied Biosystems). The reactions were performed with a total volume of 20 μl with the addition of the following components/reaction: 10 μl Express SYBR green qPCR Super-Mix Universal (Invitrogen, Carlsbad, CA), 0.4 μl forward primer (10 μM), 0.4 μl reverse primer (10 μM), 0.4 μl ROX reference dye (25 μM 5-carboxy-X-rhodamine in a solution containing 10 mM Tris-HCl [pH 8.6], 0.1 mM EDTA, and 0.01% Tween 20), cDNA, and H_2O to 20 μl . REST2009 software (40) was used to calculate the relative expression values and the significance of the differences between the gene expression levels. For each primer pair used in this work, we performed a standard curve with 320, 160, 80, 40, 20, and 10 ng cDNA for *tri* genes or 160, 80, 40, 20, 10, and 5 ng cDNA for the rest of the genes to determine the PCR amplification efficiency (E value). Each measurement was made in triplicate.

RESULTS

Disruption and silencing of the *T. arundinaceum tri4* gene. Plasmids p Δ tri4 and pURS4 (see Fig. S1 in the supplemental material) were transformed into *T. arundinaceum* IBT 40837 to obtain *tri4*-disrupted strain Ta Δ Tri4 and *tri4*-silenced strains TaS4-5 and TaS4-9, respectively. Ta Δ Tri4 should carry a disrupted, nonfunctional *tri4* gene, and TaS4-5 and TaS4-9 should have just the silencing cassette inserted.

For Ta Δ Tri4, 150 hygromycin B-resistant transformants were

TABLE 1 HA production in liquid medium by *Trichoderma* strains^a

Time (h)	Mean HA production (μg/ml) ± SD		
	Wild type	TaΔTri4	TaS4-9 ^b
24	18.60 ^a ± 0.35	0.00 ^d ± 0.00	18.22 ^a ± 1.61
48	18.28 ^a ± 0.50	0.00 ^d ± 0.00	12.98 ^{b,c} ± 0.10
72	14.76 ^b ± 0.67	0.00 ^d ± 0.00	12.85 ^{b,c} ± 0.50
96	10.38 ^c ± 0.78	0.00 ^d ± 0.00	10.38 ^c ± 1.28

^a For each column, values followed by different superscript letters are significantly different ($P < 0.001$).

^b The *tri4*-silenced transformants TaS4-9 and TaS4-5 produced similar levels of HA, with no significant differences between both strains.

analyzed by PCR using oligonucleotide pair Tri437-5/Tri437-3b (see Table S1 in the supplemental material), which amplified a 1.8-kb fragment in a nondisruptant transformant and a 9.1-kb fragment in a TaΔTri4 disruptant. Transformant 76 was the only one that displayed the expected 14.8-kb disruption band in a Southern analysis using BamHI-digested genomic DNA hybridized to a 777-bp *tri4* probe (amplified by PCR using primer pair 2102/2103) (see Fig. S2A in the supplemental material).

For TaS4-5 and TaS4-9, 48 hygromycin B-resistant transformants were isolated by *Agrobacterium*-mediated transformation. Ten of these transformants were analyzed by Southern transfer, with 4 transformants (transformants 1, 5, 6, and 9) showing the expected bands of 3.1 and 1.4 kb when they were hybridized with a 554-bp probe corresponding to an internal fragment of the *tri4* gene. They did not show, as expected, any hybridization signal when they were hybridized with a 7.9-kb BglII probe corresponding to the region of plasmid pURS4 that was not transferred to the fungus (no T-DNA region) (see Fig. S2B in the supplemental material), indicating that only the “T-DNA” region of the transforming plasmid had been integrated into the fungal genome. Finally, transformants 5 and 9, which exhibited the correct Southern patterns, were selected for further studies.

Trichothecene production in TaΔTri4 and TaS4-9 transformants. The disrupted *tri4* mutant, TaΔTri4 transformant 76, does not produce harzianum A (Table 1), as determined by HPLC analysis of supernatants from cultures grown in PDB, although trace amounts of this compound were detected under some culture conditions. The two *tri4*-silenced transformants produced slightly smaller amounts of HA than did the wild-type strain at 48 and 72 h of growth in liquid production medium (Table 1).

The GCMS method used for trichothecene analysis did not detect HA but did detect trichodermol, the immediate HA precursor, in cultures of wild-type *T. arundinaceum* and both the TaS4-5 and TaS4-9 transformants (Fig. 1D). Extracts of TaΔTri4 did not contain trichodermol, isotrichodiol, or trichodiene but contained other low-molecular-weight components. Based on its mass spectral fragmentation and NMR spectroscopy, the main component was identified as 12,13-epoxytrichoene-2-ol (see Table S2 in the supplemental material). Two minor components were tentatively identified as 12,13-epoxytrichoene-2,8-diol (see Table S3 in the supplemental material) and 12,13-epoxytrichoene-2,16-diol (see Table S4 in the supplemental material). 12,13-Epoxytrichoene-2-ol was also found in extracts of TaS4-5, TaS4-9, and wild-type Ta37 cultures that had been treated with the P450 inhibitor xanthotoxin (Fig. 1B and C).

Effect of *tri4* silencing or disruption on expression of the *tri* genes. The relative expression levels of the *tri* genes in the *tri4*-

disrupted and -silenced transformants were analyzed and compared with that in the wild-type strain.

The disruption negatively affected the expressions of the *tri4* and *tri5* genes ($P = 0.00$) (Fig. 2) in comparison with the wild-type strain, while the levels of *tri6* expression were similar for both strains. Note that the level of *tri4* expression in the disruptant was above zero, which is most likely due to the fact that the primers used to detect *tri4* expression were located at the 5' end of the gene, before the insertion of the disrupting vector. The *tri4* promoter and 5' end of the gene should be intact, and transcription should be initiated. On the other hand, in the TaS4-9 transformant, the *tri4*, *tri5*, *tri6*, and *tri11* genes were repressed significantly ($P = 0.00$) in comparison with the wild-type strain. *tri4* had very low levels of expression, as the expression ratio was 0.015 ($P = 0.00$) compared with the wild-type strain. This was expected since it is a *tri4*-silenced transformant, indicating that the silencing process had worked properly.

It was noted that for both types of transformants, a reduction in the *tri5* gene transcription level was found, with expression ratios of 0.191 ($P = 0.00$) and 0.180 ($P = 0.00$) for TaΔTri4 and TaS4-9, respectively (Fig. 2).

***tri4* gene disruption or silencing resulted in an increase in the chitinase activity in comparison with that of the control strain.** Chitinase behavior, which is involved in the *Trichoderma* mycoparasitic activity, increased about 2.5-fold in the TaΔTri4 and TaS4-9 transformants in comparison with the strain transformed with plasmid pUR5750 without the insert (Table 2). It is important to note that the level of chitinase activity was higher in both *tri4* transformants than in the control strain, even though these transformants had very different levels of HA production.

Silencing and disruption of the *tri4* gene negatively affect the antagonistic activity of *Trichoderma*. Two *in vitro* antifungal assays were used: growth on membranes and direct confrontation.

(i) Growth on membranes. *Trichoderma* strains were grown on cellophane and dialysis membranes (cutoff, 10 kDa) to allow the diffusion of *Trichoderma* extracellular compounds into the medium. After the removal of the membranes containing the mycelia, the effect of such hydrolytic enzymes plus metabolites (cellophane) or only metabolites (dialysis) on the growth of two different plant-pathogenic fungi was determined.

Table 3 summarizes the percentages of growth inhibition of *B. cinerea* and *R. solani* by *T. arundinaceum* Ta37 (wild type), disruptant strain TaΔTri4, silencing transformant strain TaS4-9, and *T. harzianum* T34, which was used as a control in the antifungal assays. It was observed that the inhibition values for growth on cellophane were always similar to or higher than the values for growth on dialysis membranes. The level of inhibition of the growth of *B. cinerea* caused by Ta37, similar to that observed with *T. harzianum* T34, was higher than that caused by strains TaΔTri4 and TaS4-9 on both cellophane and dialysis membranes, and lower values were obtained with TaΔTri4 than with TaS4-9. Thus, on cellophane, the percentages of inhibition of Ta37 against *B. cinerea* were approximately 11 and 4% higher than those measured with TaΔTri4 and TaS4-9, respectively, whereas on dialysis membranes, the inhibition values of Ta37 were 22 and 6% higher than those of TaΔTri4 and TaS4-9, respectively.

Strains Ta37 and TaS4-9 showed identical inhibition values against *R. solani* on both cellophane and dialysis membranes at 96 h of growth. However, significant differences between the wild type and the *tri4* disruptant strain on dialysis membranes were

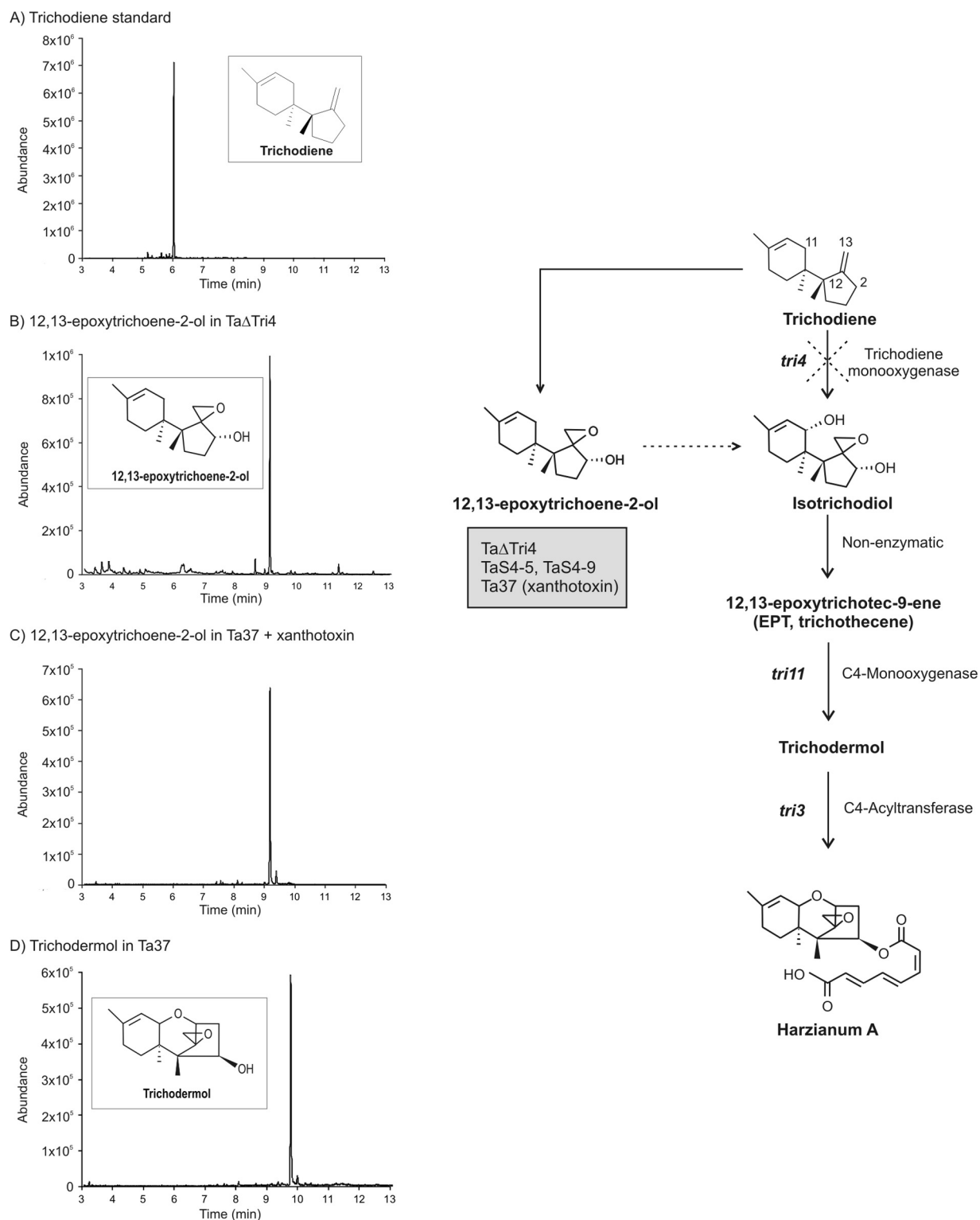
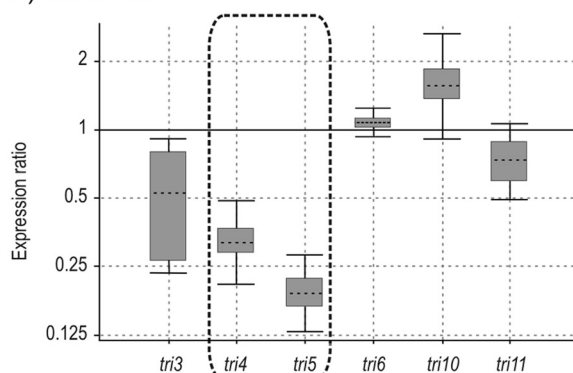


FIG 1 (Left) Ion chromatograms of the trichodiene standard (A), extracted Ta Δ Tri4 supernatant (B), supernatant extracted from xanthotoxin-treated strain Ta37 (C), and supernatant extracted from Ta37 without xanthotoxin (D). (Right) Schematic HA biosynthetic pathway. Dotted lines indicate the production of 12,13-epoxytrichoene-2-ol from trichodiene by the strains within the gray box. Note that this compound was detected in Ta37 cultures only after xanthotoxin treatment.

obtained at this growth time (Table 3). These differences can be better visualized by photographs that were taken after 10 days of growth, in which it is even possible to see differences between Ta37 and TaS4-9 (Fig. 3).

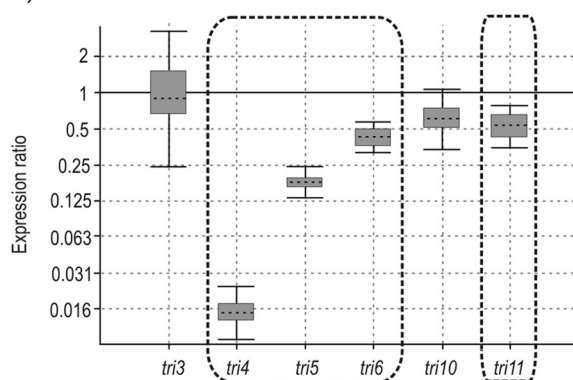
These results show that the disruption of *tri4* reduced the bio-control activity of *T. arundinaceum* against *B. cinerea* and *R. solani*. In addition, this level of reduction was higher than that found with TaS4-9, which could be the result of the different levels of HA

A) TaΔTri4.



Gene	Expression ratio	P(H1)
tri3	0.463	0.321
tri4	0.320*	0.000
tri5	0.191*	0.000
tri6	1.082	0.336
tri10	1.569	0.336
tri11	0.731	0.344

B) TaS4-9.



Gene	Expression ratio	P(H1)
tri3	0.894	0.336
tri4	0.015*	0.000
tri5	0.180*	0.000
tri6	0.430*	0.000
tri10	0.606	0.344
tri11	0.526*	0.000

FIG 2 Effect of *tri4* disruption or silencing on relative *tri* gene expression levels. (A) Comparison between the *tri* gene expression levels of TaΔTri4 and those of Ta37 determined by using REST2009 software. (B) TaS4-9 versus Ta37. Dotted-line squares indicate gene expression levels statistically different from those under the control conditions ($n = 3$; $P < 0.05$).

production in each of the mutants. Under the conditions used in this experiment, TaS4-9 produced just slightly lower levels of HA than the wild-type strain, while TaΔTri4 did not produce HA. To test the inhibitory activity of defined amounts of HA, an antibiogram assay was used. An extracted Ta37 sample was analyzed by HPLC, and just traces of other compounds apart from HA were detected. The culture supernatant was diluted so that 2.4, 1.2, 0.6, and 0.3 mg HA/ml were tested on the growth of *B. cinerea*. After 1 week of incubation, the diameter of the zone of inhibition was measured, 4.47 ± 0.09 , 4.27 ± 0.09 , 4.1 ± 0.08 , and 3.87 ± 0.09 cm for 2.4, 1.2, 0.6, and 0.3 mg HA/ml, respectively. Acetonitrile

TABLE 2 Chitinase activity against *p*-NP-β-D-glucosaminide measured in supernatants of strains Ta-pUR5750, TaS4-9, and TaΔTri4 at four times^a

Strain	Mean chitinase activity (nmol/min/mg dry wt) against <i>p</i> -NP-β-D-glucosaminide ± SD			
	24 h	48 h	72 h	96 h
Ta-pUR5750 ^b	0.05 ± 0.0 ^a	0.84 ± 0.07 ^a	2.17 ± 0.06 ^a	4.65 ± 0.96 ^a
TaS4-9	0.41 ± 0.25 ^a	3.66 ± 0.11 ^b	6.97 ± 0.25 ^b	11.61 ± 1.41 ^b
TaΔTri4	0.20 ± 0.13 ^a	3.29 ± 0.23 ^b	4.89 ± 0.13 ^c	11.90 ± 0.57 ^b

^a For each time point, values followed by different letters are significantly different ($P < 0.05$ by a Duncan test).

^b Control strain.

did not block *B. cinerea* growth (see Fig. S3 in the supplemental material).

(ii) **Direct confrontation assays.** Plate confrontation experiments between Ta37 or TaΔTri4 and the pathogens *B. cinerea*, *R. solani*, *M. roridum*, and *F. sporotrichioides* were carried out at 28°C, and plates were photographed after 10 days of incubation (see Fig. S4 in the supplemental material). In all cases, the four

TABLE 3 Percent growth inhibition of *Botrytis cinerea* and *Rhizoctonia solani* by hydrolytic enzymes/metabolites from *T. arundinaceum* Ta37, the *tri4* transformants TaΔTri4 and TaS4-9, and *T. harzianum* T34 grown on cellophane or dialysis membranes with a 10-kDa cutoff for 2 days^a

Strain	Mean % growth inhibition ± SD			
	<i>B. cinerea</i> (72 h)		<i>R. solani</i> (96 h)	
	Cellophane	Dialysis	Cellophane	Dialysis
Ta37 ^b	100.0 ^a ± 0.0	88.8 ^a ± 0.3	100.0 ^a ± 0.0	100 ^a ± 0.0
TaΔTri4	89.2 ^b ± 1.8	66.3 ^b ± 5.9	96.6 ^b ± 2.2	75.1 ^b ± 8.2
TaS4-9	96.0 ^c ± 1.6	82.9 ^a ± 2.8	100.0 ^a ± 0.0	100.0 ^a ± 0.0
T34 ^c	100.0 ^a ± 0.0	87.1 ^a ± 1.0	96.0 ^b ± 0.0	78.4 ^b ± 0.9

^a For each column, values followed by different superscript letters are significantly different ($P < 0.001$).

^b Wild-type strain.

^c Control.

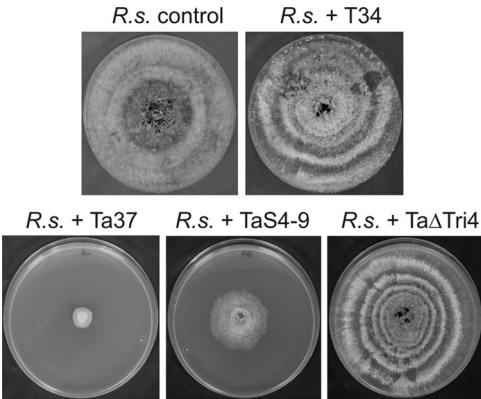


FIG 3 Fungal growth inhibition of *R. solani* after 10 days of incubation on plates prepared by growing *Trichoderma* strains for 2 days on cellulose membranes. As a control, a plate without previous *Trichoderma* growth (*R.s.* control) was included.

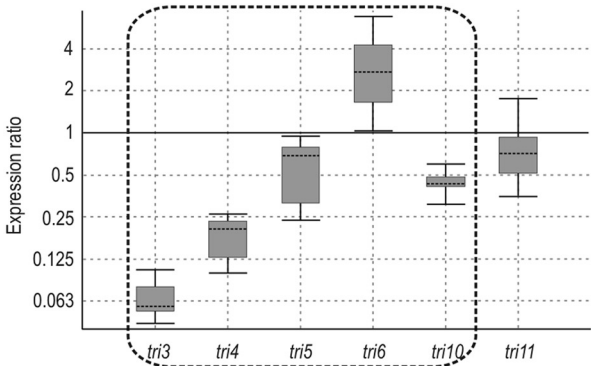
pathogens on control plates of PDA or MEA completely covered the surface after this incubation time. In the confrontation plates, *Trichoderma* strains overgrew the colonies of *B. cinerea*, *R. solani*, and *F. sporotrichioides* and surrounded the colonies of *M. roridum*. Both *Trichoderma* strains (wild-type strain Ta37 and the TaΔTri4 mutant) were able to inhibit the growth of the four pathogens tested, reducing the colony diameters of *B. cinerea* and *M. roridum*

to no more than 25 mm. Significant differences were observed only between the inhibition effects of Ta37 and TaΔTri4 against *B. cinerea* and *R. solani* in this assay.

Relative expression levels of Ta37 *tri* genes in the interaction region of confrontation assays against *B. cinerea* and *R. solani*. Both structural *tri* genes (*tri3*, *tri4*, *tri5*, and *tri11*) and regulatory *tri* genes (*tri6* and *tri10*) were monitored for gene expression during confrontation assays against *B. cinerea* and *R. solani*. Relative expression was determined by comparing fungal gene expression in the confrontation assay to that of the wild-type strain growing alone. During confrontation with *B. cinerea*, only *tri6* was upregulated in Ta37 (Fig. 4A), by a mean factor of 2.7 ($P = 0.03$). *tri11* expression was not significantly affected by the interaction with *B. cinerea*, while *tri3* expression was highly repressed, as shown by the expression ratio of 0.062 ($P = 0.00$). During confrontation with *R. solani*, all the *tri* genes in Ta37, except *tri10*, were upregulated ($P = 0.00$) (Fig. 4B). These results suggest that the production of harzianum A should be stimulated by the interaction of Ta37 with *R. solani*.

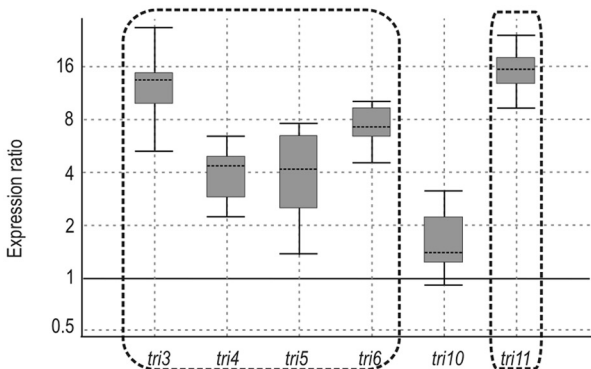
Effect of silencing or disruption of the *tri4* gene on plant growth and plant defense responses. (i) **Effect of *T. arundinaceum* on growth of tomato plants in hydroponic cultures.** The *T. arundinaceum* wild-type strain and the *tri4*-disrupted transformant did not significantly affect the growth of tomato seedlings in hydroponic cultures, as measured by the parameters stem length, root length, and stem diameter. However, an analysis of *tri* gene

A) Ta37 v.s. *B. cinerea*.



Gene	Expression ratio	P(H1)
<i>tri3</i>	0.062*	0.000
<i>tri4</i>	0.178*	0.011
<i>tri5</i>	0.536*	0.000
<i>tri6</i>	2.678*	0.032
<i>tri10</i>	0.439*	0.000
<i>tri11</i>	0.761	0.338

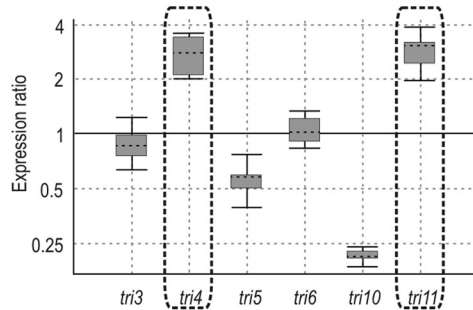
B) Ta37 v.s. *R. solani*.



Gene	Expression ratio	P(H1)
<i>tri3</i>	12.469*	0.000
<i>tri4</i>	3.970*	0.000
<i>tri5</i>	4.031*	0.000
<i>tri6</i>	7.526*	0.000
<i>tri10</i>	1.587	0.101
<i>tri11</i>	15.124*	0.000

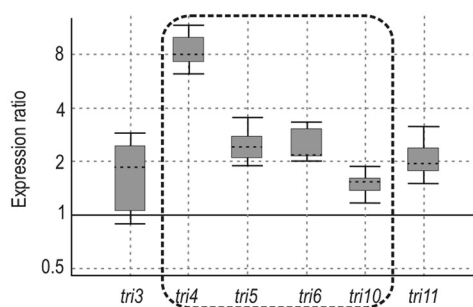
FIG 4 Relative *tri* gene expression levels of Ta37 in confrontation assays against *B. cinerea* (A) and *R. solani* (B), determined by using REST2009 software. Dotted-line squares indicate gene expression levels statistically different from those under the control conditions (strain Ta37 grown without the pathogen) ($n = 3$; $P < 0.05$).

A) Ta37 gene expression in hydroponic cultures.



Gene	Expression ratio	P(H1)
<i>tri3</i>	0.871	0.496
<i>tri4</i>	2.754*	0.000
<i>tri5</i>	0.565	0.093
<i>tri6</i>	1.051	0.596
<i>tri10</i>	0.216	0.093
<i>tri11</i>	2.880*	0.000

B) TaΔTri4 gene expression in hydroponic cultures.



Gene	Expression ratio	P(H1)
<i>tri3</i>	1.695	0.188
<i>tri4</i>	8.393*	0.000
<i>tri5</i>	2.517*	0.000
<i>tri6</i>	2.471*	0.045
<i>tri10</i>	1.509*	0.000
<i>tri11</i>	2.091	0.096

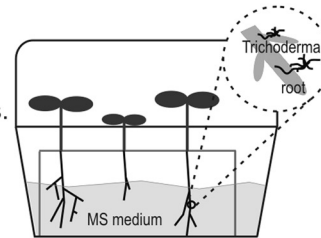


FIG 5 Effect of the presence of tomato roots on relative *tri* gene expression levels of Ta37 (A) and TaΔTri4 (B). Data were analyzed with REST2009 software. Dotted-line squares indicate gene expression levels statistically different from those under the control conditions ($n = 3$; $P < 0.05$). A schematic representation of a hydroponic culture is shown.

expression in the wild-type strain showed that the interaction with the plants affects mainly the expressions of *tri4* and *tri11*, which were 2.7- and 2.9-fold upregulated ($P = 0.00$), respectively (Fig. 5A). In the case of the TaΔTri4 transformant, the *tri4*, *tri5*, *tri6*, *tri10*, and *tri11* genes were all significantly upregulated, by factors of 8.4 ($P = 0.00$), 2.5 ($P = 0.00$), 2.5 ($P = 0.04$), 1.5 ($P = 0.00$), and 2.1 ($P = 0.09$), respectively (Fig. 5B). It must be taken into account that the TaΔTri4 transformant contains a truncated copy of the *tri4* gene, and the promoter region is still functional in the transformant chromosome; thus, it is also possible for us to determine the effect of such a disruption on the induction of *tri4* expression.

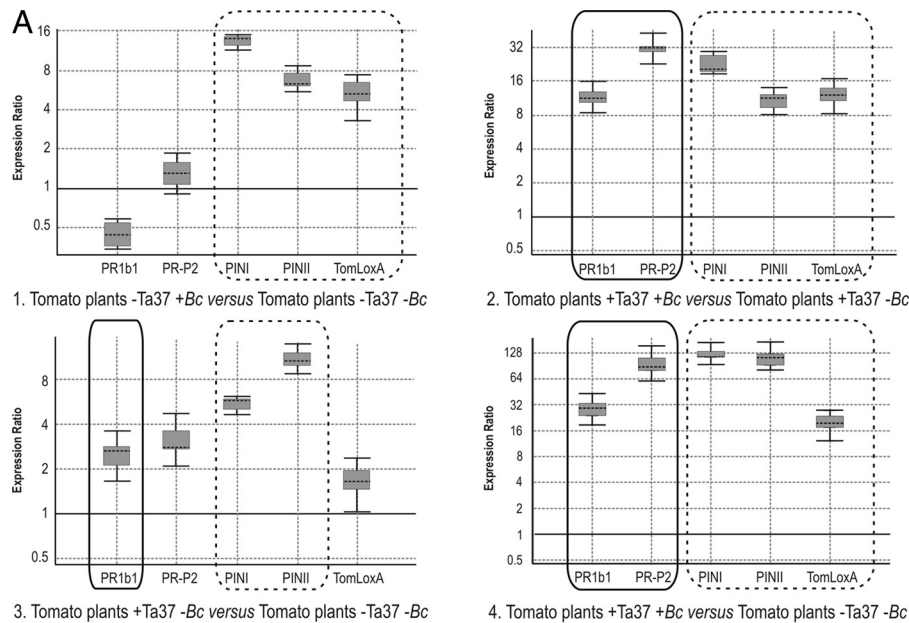
(ii) **Analysis of expression of defense-related genes in plants obtained from seeds treated with wild-type *T. arundinaceum* strain IBT 40837 or the *tri4*-disrupted transformant.** As in the above-described results for the hydroponic cultures, wild-type *T. arundinaceum* and the *tri4*-disrupted transformant did not significantly affect tomato growth in a greenhouse when the parameters stem length, root length, and stem diameter were measured. It was described previously that the biocontrol effect of *Trichoderma* sp. on plants can be due to several factors, including secondary metabolite production, plant growth promotion, and resistance induction in plants (21). Thus, we investigated the expression levels of several defense-related genes in leaves from tomato plants that were treated with strain Ta37 or TaΔTri4. We analyzed markers of both the salicylic acid (SA) (*PR1b1* and *PR-P2*) and jasmonate (JA) (*PINI*, *PINII*, and *TomLoxA*) pathways under the following four conditions: tomato plants plus *B. cinerea* versus tomato plants (condition 1), tomato plants plus *Trichoderma* and *B. cinerea* versus tomato plants plus *Trichoderma* (condition 2), tomato plants plus *Trichoderma* versus tomato plants (condition 3),

and tomato plants plus *Trichoderma* and *B. cinerea* versus tomato plants (condition 4).

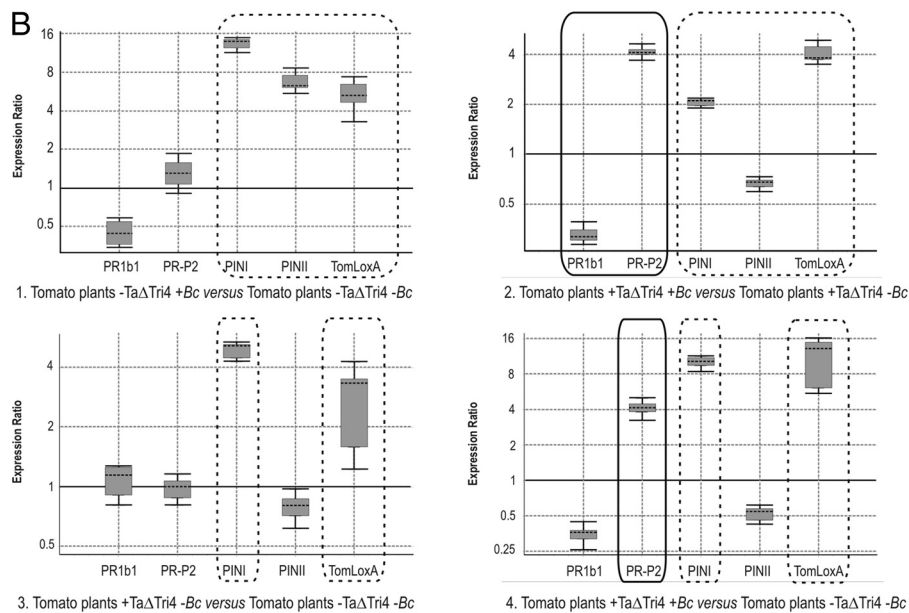
As shown in Fig. 6A, Ta37 alone (condition 3) induced the expressions of *PR1b1* (SA) and *PINI* and *PINII* (JA). *B. cinerea* alone (condition 1) induced *PINI*, *PINII*, and *TomLoxA*, all genes in the JA pathway. *B. cinerea* induced *PINI* at a higher ratio than did Ta37 alone. A comparison of relative gene expression levels in tomato plants treated with Ta37 and then infected with *B. cinerea* versus Ta37-inoculated plants that were not infected with *B. cinerea* (condition 2) showed that all tested genes were upregulated. Finally, in plants treated with Ta37 and infected with *B. cinerea* (condition 4), all the analyzed genes were drastically upregulated versus nontreated, noninfected plants.

In the case of strain TaΔTri4, which does not produce HA, the relative gene expression results (Fig. 6B) indicated that the level of induction of the expression of genes involved in the SA and JA signaling pathways was significantly lower than that obtained when strain Ta37 was used. Some genes that were upregulated in interactions with strain Ta37 were downregulated in strain TaΔTri4. Thus, the *PINII* gene was downregulated by a factor of 0.63 ($P = 0.02$) in plants treated with TaΔTri4 and infected with *B. cinerea* (condition 2) (Fig. 6B), while the corresponding condition with strain Ta37 showed this gene to be upregulated by a factor of 10.27 (condition 2) ($P = 0.00$) (Fig. 6A).

The greatest differences in relative gene expression levels obtained between the plants treated with Ta37 and those treated with TaΔTri4 were observed under condition 4, when plants obtained from seeds treated with these strains and infected with *B. cinerea* were compared with non-*Trichoderma*-treated and non-*B. cinerea*-infected plants (condition 4) (Fig. 6). Under these condi-



		① -Ta37+Bc/ -Ta37-Bc		② +Ta37+Bc/ +Ta37-Bc		③ +Ta37-Bc/ -Ta37-Bc		④ +Ta37+Bc/ -Ta37-Bc	
	Gene	Expression ratio	P(H1)	Expression ratio	P(H1)	Expression ratio	P(H1)	Expression ratio	P(H1)
SA	PR1b1	0.445	0.068	11.519*	0.000	2.503*	0.000	28.835*	0.000
	PR-P2	1.305	0.134	31.029*	0.000	2.990	0.063	92.761*	0.000
JA	PINI	13.506*	0.000	22.112*	0.016	5.541*	0.043	122.512*	0.000
	PINII	6.637*	0.024	10.268*	0.000	10.931*	0.031	112.233*	0.000
	TomLoxA	5.207*	0.019	12.224*	0.000	1.606	0.064	16.630*	0.030



		① -TaΔTri4+Bc/ -TaΔTri4-Bc		② +TaΔTri4+Bc/ +TaΔTri4-Bc		③ +TaΔTri4-Bc/ -TaΔTri4-Bc		④ +TaΔTri4+Bc/ -TaΔTri4-Bc	
	Gene	Expression ratio	P(H1)	Expression ratio	P(H1)	Expression ratio	P(H1)	Expression ratio	P(H1)
SA	PR1b1	0.445	0.068	0.300*	0.044	1.082	0.628	0.325	0.100
	PR-P2	1.305	0.134	4.792*	0.042	0.969	0.730	4.645*	0.029
JA	PINI	13.506*	0.000	2.345*	0.033	5.244*	0.014	12.296*	0.000
	PINII	6.637*	0.024	0.635*	0.024	0.765	0.066	0.486	0.081
	TomLoxA	5.207*	0.019	5.127*	0.009	2.292*	0.000	11.750*	0.000

tions, all the analyzed genes involved in SA and JA pathways were expressed at higher levels in plants treated with strain Ta37 than in those treated with strain Ta Δ Tri4.

DISCUSSION

Trichoderma is an amazing fungus because it can produce extracellular hydrolytic enzymes to act as a biocontrol agent and produce phytotoxic mycotoxins. However, *T. arundinaceum* is a poorly studied species that has not been described previously as a biocontrol strain. It produces the trichothecene harzianum A. HA has not been evaluated for phytotoxicity, although its toxicity against eukaryotic cells is well known (30).

In this study, a *tri4* disruption mutant was generated to evaluate the role of HA in biocontrol. Previous studies have shown that *tri4* controls the addition of oxygens at C-2, C-12, and C-11, converting trichodiene to isotrichodiol (11). Disruption mutant strain Ta Δ Tri4 was expected to accumulate trichodiene, as this is the case for *Fusarium Tri4* mutants (27). Instead, 12,13-epoxytrichoene-2-ol (52, 59) accumulated in cultures of the mutant strain. Interestingly, this compound also accumulated in wild-type *T. arundinaceum* cultures that had been treated with low concentrations of the P450 inhibitor xanthotoxin. The addition of xanthotoxin to *Fusarium* cultures causes the accumulation of trichodiene (3, 22). These results suggest that *tri4* is partially functional in the mutant and can control C-2 and C-12 oxygenations. However, although the disruption strategy allowed the transcription of the 5' end of the gene, it prevented the synthesis of a complete copy of the *tri4* transcript and, therefore, a functional Tri4 protein. The oxygenation of trichodiene may be due to one or two P450 oxygenases that efficiently convert any trichodiene into 12,13-epoxytrichoene-2-ol. In *Fusarium*, other P450 enzymes can add oxygens to the C-11 or the C-2 position of trichodiene or trichodiene derivatives (34, 51). Although no trichodermol was detected by GCMS analysis of Ta Δ Tri4 extracts, HPLC analysis detected trace amounts of HA in some Ta Δ Tri4 cultures. This leaky-mutant phenomenon has been observed for blocked mutants of *Fusarium*. For example, trace amounts of the trichothecene T-2 toxin were detected in *Tri3* mutants of *F. sporotrichioides* (35). The trace amount of HA suggests that there may be another P450 that can convert 12,13-epoxytrichoene-2-ol into isotrichodiol albeit with a lower efficiency. Any isotrichodiol formed could then be converted to HA by the Tri11 and Tri3 enzymes.

As we observed, Tri4-overexpressed transformants do not increase levels of harzianum A production (data not shown). Note that *tri4* is expressed at one of the highest levels of all the *T. arundinaceum tri* genes at 24 h of growth (see Fig. S5 in the supplemental material); thus, the level of *tri4* gene transcription is probably not a limiting step of biosynthesis. The other biosynthetic genes, such as the *tri3*, *tri5*, *tri6*, and *tri11* genes, are expressed at lower levels than *tri4*, and they may represent a limitation of HA biosyn-

thesis at the transcriptional level. Similar to this result, the overexpression of the *tri5* gene in *T. arundinaceum* IBT 40837 did not result in a statistically significant increase in the level of HA production in comparison with that of the wild-type strain (M. G. Malmierca, unpublished data). However, the overexpression of this gene in *T. brevicompactum* IBT 40841 resulted in the production of increased levels of trichodermin (50). These differences in the results between *T. brevicompactum* and *T. arundinaceum* led us to propose an additional hypothesis by which the limiting steps in trichodermin and HA biosynthesis, respectively, are probably different. Thus, the results for *T. brevicompactum* indicated that the *tri5*-encoded protein would be a limiting step in trichodermin biosynthesis, since the overexpression of this gene resulted in an increase in the level of trichodermin biosynthesis (50), while the results obtained with *T. arundinaceum* indicated that the bottleneck in HA biosynthesis would be another step(s) of its biosynthesis. Taking into account that the only difference in the trichodermin and HA biosynthetic pathways is the acylation by esterification of the C-4 oxygen of trichodermol, possibly by Tri3, with an octatriendiol or acetyl moiety in *T. arundinaceum* or *T. brevicompactum*, respectively (Fig. 1), the biosynthesis of HA would most likely require another enzyme, possibly a polyketide synthase, for the synthesis of the octatriendiol moiety, as indicated previously by Cardoza and coworkers (11). One possibility would then be that the limiting step of HA biosynthesis would be octatriendiol biosynthesis rather than the *tri5*- or *tri4*-encoded steps.

All the *tri* genes of *T. arundinaceum* include in their promoter regions hypothetical binding sites for the *tri*-positive regulator Tri6 (42). Thus, the regulation of the expressions of all these genes might be coordinated (11). However, the *tri* genes are expressed at different levels, similar to those described previously for the sterigmatocystin/aflatoxin (ST/AF) biosynthetic genes in *Podospira anserina*, *Aspergillus flavus*, and *A. nidulans* (7, 41). These genes are regulated by the transcriptional regulator AflR and also have different levels of expression (44).

The Ta Δ Tri4 and TaS4-9 transformants differed drastically in their levels of HA production, but they had similarly increased levels of chitinase activity compared to that of the control strain, which may explain why the Ta Δ Tri4 transformant still had significant biocontrol activity. Thus, although HA has been shown to be involved in the biocontrol/mycoparasitic activity of *T. arundinaceum* (see Fig. S3 in the supplemental material), the chitinase and other extracellular hydrolytic activities obviously also contribute to its mycoparasitic activity. It is also apparent that the differences in the biocontrol activities between strains Ta Δ Tri4 and TaS4-9 are due to their different levels of HA production, since they both have similar levels of chitinase activity. To help explain the increase in chitinase activity in the mutants, perhaps the levels of trichodiene and/or 12,13-epoxytrichoene-2-ol produced in the *tri4*-disrupted and -silenced transformants act as regulators of the

FIG 6 (A) Relative expression levels of defense-related genes belonging to the SA and JA pathways in 4-week-old tomato plants infected (+Bc) or not (–Bc) with *B. cinerea*, when seeds were coated with Ta37. The expression ratio data were obtained and represented by using REST2009 software. Black-line squares (SA genes) and dotted-line squares (JA genes) indicate gene expression levels statistically different from those under the control conditions ($n = 3$; $P < 0.05$). (B) Relative expression levels of defense-related genes belonging to the SA and JA pathways in 4-week-old tomato plants infected (+Bc) or not (–Bc) with *B. cinerea* when seeds were coated with Ta Δ Tri4. The expression ratio data were obtained and represented by using REST2009 software. Black-line squares (SA genes) and dotted-line squares (JA genes) indicate gene expression levels statistically different from those under the control conditions ($n = 3$; $P < 0.05$). Note that the top left panels of panels A and B correspond to different repetitions of the same experiment. Thus, they are almost identical, as they represent the effect of *B. cinerea* when *Trichoderma* is not present (control condition).

expression of the gene(s) encoding chitinase or other enzymes involved in the mycoparasitic-biocontrol process. Although the trichothecene deoxynivalenol (DON) has been shown to repress the expression of the *nag1* gene, encoding an *N*-acetyl- β -D-glucosaminidase, one of the most important cell wall-degrading enzymes of *Trichoderma atroviride* P1 (32), the effects of other trichothecenes on chitinase genes are unknown. If the trichothecene intermediates of *T. arundinaceum* do have a direct effect on the production of its own chitinases, this helps to demonstrate the uniqueness of this species. It has combined the production of a trichothecene, normally seen only in plant pathogens, with the production of chitinases, normally seen in beneficial microorganisms that control microbial plant pathogens. Also, *T. arundinaceum* can apparently induce the expression of its own chitinase genes when HA levels are reduced, perhaps in an effort to maintain its environmental niche, in a response similar to that observed previously for *T. atroviride* P1 in confrontation experiments against *Fusarium* strains with a reduced level of DON production (32).

There are many genes involved in plant pathogenesis by a fungus, and it is not known exactly how *B. cinerea* or *R. solani* infects and invades plant tissue. It is possible that *T. arundinaceum* could act as a biocontrol agent of these two pathogens by producing the mycotoxin HA, which can then reduce the growths of these pathogens, as has been shown in the present work. In addition, we have observed in confrontation assays that *B. cinerea* repressed most of the *T. arundinaceum tri* genes, possibly as a defense mechanism to reduce the level of HA production. However, in the case of *R. solani*, the situation seems to be reversed, as the expressions of all the *T. arundinaceum tri* genes assayed, except *tri10*, were induced. It is possible that an unknown stimulant produced by *R. solani* caused the increase in *T. arundinaceum* gene expression levels as a mechanism for this fungus to outcompete *R. solani*. Therefore, it is possible that HA might be acting as an inhibitor of certain fungi but not others. At this point, it is important to take into account that the disruption of the *tri4* gene reduced growth inhibition against *R. solani* to a lesser extent than against *B. cinerea*.

The interaction of *Trichoderma* with tomato plants in hydroponic cultures might be expected to affect seedling growth due to the presence of HA. However, no significant effect on the measured growth parameters of the wild-type-treated plants or the Ta Δ Tri4-treated plants was observed. This suggests that HA does not negatively affect growth under the tested conditions. When *tri* gene expression in these Ta37-treated plants was analyzed, increases in the levels of transcription of the *tri4* and *tri11* genes were observed; however, the level of HA production might not be increased, since the *tri5* gene is just slightly downregulated, by a factor of 0.56 ($P = 0.09$). In addition, the lack of increased levels of *tri6* transcription suggests that the increase in the expression levels of the *tri4* and *tri11* genes is the result of a mechanism independent of Tri6. In the case of the Ta Δ Tri4 transformant, mycelia of this strain recovered from roots of tomato plants in hydroponic cultures showed an increase in the expression level of the *tri4* gene which was higher than that observed for the wild-type tomato cultures. This could be due to a response of the fungal cell, produced as result of the lack of Tri4 activity, to compensate for the increase in levels of trichodiene. In Ta Δ Tri4 mycelia recovered from hydroponic plant cultures, the *tri6* expression level was also increased, perhaps in response to the lack of HA production or due to some other regulatory factor.

In the development of a hypothesis as to the mechanism by which *T. arundinaceum* may serve as a biocontrol agent against the invasion by phytopathogenic fungi on plants, we have shown that the production of HA by *T. arundinaceum* does not affect tomato seedling growth (i.e., it is not phytotoxic under our test conditions) and that it slows the growth of the pathogenic fungi *B. cinerea* and *R. solani*. We have also shown that the relative expression levels of the *T. arundinaceum tri* genes respond differently when exposed to *B. cinerea* and *R. solani*. To expand on our hypothesis, we looked at the expression of plant genes that are involved in the plant defense SA and JA pathways in tomato plants grown in a greenhouse for 4 weeks from seeds that were treated or not with strain Ta37 and infected or not with the phytopathogen *B. cinerea*. This test should indicate whether the presence of strain Ta37 increases the intensity of the plant response against *B. cinerea*, in a sensitizing effect similar to that previously described for biocontrol *Trichoderma* strains (1, 13). We found an increase in the level of the SA response (condition 4) (Fig. 6A) when tomato plants inoculated with Ta37 were infected with *B. cinerea*, compared with the level of expression observed for plants infected with *B. cinerea* but not treated with Ta37. When strain Ta Δ Tri4 was used, a much lower level of induction of these responses was observed, indicating that HA could have a relevant function in the sensitization of the plant against phytopathogenic fungi. This new role of HA was further confirmed by comparing the induction of the JA- and SA-related genes in Ta37-treated plants infected with *B. cinerea* versus plants without *Trichoderma* treatment and infected with *B. cinerea*. The difference between these conditions showed a sensitizing effect of Ta37, reflected in an upregulation of all the studied defense-related genes. In contrast, when this comparison was performed with strain Ta Δ Tri4 (Ta Δ Tri4-treated plants infected with *B. cinerea* versus plants not treated with Ta Δ Tri4 and infected with *B. cinerea*), only the genes *PR-P2* and *TomLoxA* were slightly upregulated, indicating that when HA is not produced, the sensitizing function carried out by *T. arundinaceum* is almost entirely lost. Therefore, HA contributes as a necessary partner to plant sensitization against fungal pathogens. We have proposed a network interaction among the tomato plant, the biocontrol agent *T. arundinaceum*, and the phytopathogen *B. cinerea* (Fig. 7), where *T. arundinaceum* sensitizes the tomato plant by inducing plant defense genes, which are in turn induced faster and to higher levels when the pathogen *B. cinerea* invades.

As also indicated in Fig. 7, in addition to HA, other metabolites and/or enzymes produced by *Trichoderma* may participate in plant sensitization. Thus, the production of at least five different peptaibiotics, including alamethicins, trichocopactin, trichobrevins, trichocryptins, and trichoferin, and six different hydrophobins was described previously for different *T. arundinaceum* HA producer strains (14), which, together with HA and probably other unknown metabolites, would participate in the induction of the plant defense-related genes and or in the interaction with fungal pathogens. For instance, in a recent work, the involvement of hydrophobins of *Metarhizium brunneum* in the virulence of this fungus against *Spodoptera exigua* (beet armyworm) was shown (46), which contributes to support the hypothesis of the involvement of these kinds of proteins of *T. arundinaceum* in the *Trichoderma-Botrytis*-plant interaction.

Additionally, a recent review of plant-beneficial effects of different *Trichoderma* strains, most of them known as biocontrol agents, summarized different proteins (cellulase, polygalacturo-

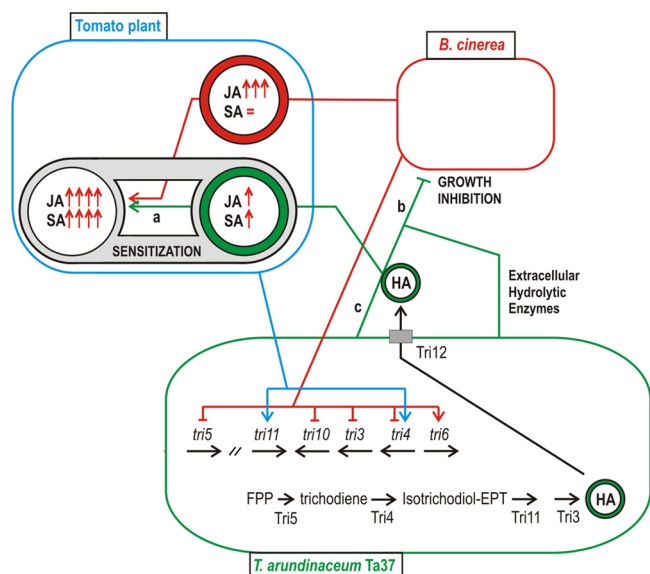


FIG 7 Schematic representation of the network of interactions established among Ta37, *B. cinerea*, and tomato plants deduced from the present work. Arrows indicate response stimulation or gene upregulation, and blunt-ended lines indicate gene repression or growth inhibition. Red, blue, and green lines indicate interactions mediated by *B. cinerea*, tomato plant, and strain Ta37, respectively. a, sensitizing effect of Ta37-pretreated tomato plants mediated by HA; b, coupled action of HA and extracellular hydrolytic enzymes to inhibit *B. cinerea* growth; c, other metabolites produced by *T. arundinaceum* that, in addition to HA, would also affect its interaction with plants and with its fungal targets.

nase, ceratoplatanins, cellulose-binding-domain proteins, and nonactive xylanase) and secondary metabolites (peptaibiotics, pyrones, pyridines, and butenolides) that are able to induce plant defenses (21).

However, even though it has been shown that the tomato plants used in this study respond by activating their defense systems against *B. cinerea* at a higher level in the presence of strain Ta37, the lesions caused by *B. cinerea* did not show any reduction in the Ta37-treated plants. This finding is in agreement with previous results (54) showing that the treatment of the tomato line TA209 with *T. atroviride* P1 increased pathogenesis-related (PR) gene expression but had no significant effect on resistance, which led to the conclusion that the effect of the *Trichoderma* interaction on resistance against phytopathogenic fungi depends on the genotype of the treated plant. The importance of this concept was also proven for maize, to the point that there are varieties for which the use of *T. harzianum* T22-based products may be either recommended or counterindicated (19, 20).

In conclusion, trichothecenes are important for the biocontrol activity of *T. arundinaceum*, as their interactions with plants induce the expression of genes related to the defense response in plants. They also participate in sensitizing the plant to respond, inducing the genes involved in the defense response to pathogen attack faster and at higher levels. In addition, both the plants and the phytopathogenic fungi regulate the expression of the *tri* genes of *T. arundinaceum*. Thus, this is the first report of the interaction between the production of trichothecenes and plant defense responses, showing how these processes establish a network of interactions in which each partner regulates the other parts and

showing how secondary metabolites play an essential role in the mechanism of biocontrol (Fig. 7).

ACKNOWLEDGMENTS

We thank Karl Vermillion, USDA/ARS, for doing the NMRs to identify the low-molecular-weight components detected in TaΔTri4 culture broths. We also recognize J. Álvarez from the University of León and J. Teresi from the Bacterial Food-Borne Pathogens and Mycology Unit, USDA/ARS, for their excellent technical assistance.

Research project funding was obtained from Junta de Castilla y León (GR67) and the Spanish Ministry of Science and Innovation (AGL2006-05660, AGL2009-13431-C01, and AGL2009-13431-C02). M. G. Malmierca was granted an FPU fellowship by the Spanish Ministry of Science and Innovation (AP2007-02835).

REFERENCES

- Ahn IP, Lee SW, Suh SC. 2007. Rhizobacteria-induced priming in *Ara-bidopsis* is dependent on ethylene, jasmonic acid, and NPR1. *Mol. Plant Microbe Interact.* 20:759–768.
- Alexander NJ, Hohn TH, McCormick SP. 1998. The *TRI11* gene of *Fusarium sporotrichioides* encodes a cytochrome P-450 monooxygenase required for C-15 hydroxylation in trichothecene biosynthesis. *Appl. Environ. Microbiol.* 64:221–225.
- Alexander NJ, McCormick SP, Blackburn JA. 2008. Effects of xantho-toxin treatment on trichothecene production in *Fusarium sporotrichioides*. *Can. J. Microbiol.* 54:1023–1031.
- Ausubel FM, et al (ed). 1987. Current protocols in molecular biology. John Wiley & Sons, Inc, New York, NY.
- Baldwin C, Bycroft BW, Dewick PM, Gilbert J, Holden I. 1985. Bio-synthesis of trichothecene mycotoxins in *Fusarium culmorum* cultures. *Z. Naturforsch. C* 40:514–518.
- Benítez T, Rincón AM, Limón MC, Codón AC. 2004. Biocontrol mech-anisms of *Trichoderma* strains. *Int. Microbiol.* 7:249–260.
- Bennett JW, Klich M. 2003. Mycotoxins. *Clin. Microbiol. Rev.* 16:497–516.
- Brown DW, McCormick SP, Alexander NJ, Proctor RH, Desjardins AE. 2001. A genetic and biochemical approach to study trichothecene diversity in *Fusarium sporotrichioides* and *Fusarium graminearum*. *Fungal Genet. Biol.* 32:121–133.
- Cardoza RE, Vizcaino JA, Hermosa R, Monte E, Gutiérrez S. 2006. A comparison on the phenotypic and genetic stability of the recombinant *Trichoderma* spp. generated by protoplast- and *Agrobacterium*-mediated transformation. *J. Microbiol.* 44:383–395.
- Cardoza RE, et al. 2007. Partial silencing of a hydroxy-methylglutaryl-CoA reductase encoding gene in *Trichoderma harzianum* CECT 2413 results in a lower level of resistance to lovastatin and a lower antifungal activity. *Fungal Genet. Biol.* 44:269–283.
- Cardoza RE, et al. 2011. Identification of loci and functional character-ization of trichothecene biosynthetic genes in the filamentous fungus *Trichoderma*. *Appl. Environ. Microbiol.* 77:4867–4877.
- Cole RA, Jarvis BB, Schweikert MA (ed). 2003. Handbook of secondary fungal metabolites, vol III, p 199–560. Academic Press, San Diego, CA.
- Conrath U, et al. 2006. Priming: getting ready for battle. *Mol. Plant Microbe Interact.* 19:1062–1071.
- Degenkolb T, et al. 2008. The *Trichoderma brevicompactum* clade: a separate lineage with new species, new peptaibiotics, and mycotoxins. *Mycol. Prog.* 7:177–219.
- De Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG. 1998. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nat. Biotechnol.* 16:839–842.
- Desjardins AE. 2006. *Fusarium* mycotoxins. Chemistry, genetics and biology. American Phytopathological Society, St. Paul, MN.
- Elad Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Prot.* 19: 709–714.
- Harman GE, Howell CR, Viterbo A, Chet I, Lorito M. 2004. *Trichoderma* species—opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2:43–56.
- Harman GE, Petzoldt R, Comis A, Chen J. 2004. Interactions between *Trichoderma harzianum* strain T22 and maize inbred line Mo17 and ef-

- fects of these interactions on diseases caused by *Pythium ultimum* and *Colletotrichum graminicola*. *Phytopathology* 94:147–153.
20. Harman GE. 2006. Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* 96:190–194.
 21. Hermosa R, Viterbo A, Chet I, Monte E. 2012. Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology* 158:17–25.
 22. Hesketh AR, et al. 1990. Isotrichodiol: a post-trichodiene intermediate in the biosynthesis of trichothecene mycotoxins. *J. Chem. Soc. Chem. Commun.* (Camb.) 17:1184–1186.
 23. Hesketh AR, et al. 1991. Biosynthesis of trichothecene mycotoxins: identification of isotrichodiol as a post-trichodiene intermediate. *Phytochemistry* 30:2237–2243.
 24. Hohn TM, Beremand P. 1989. Isolation and nucleotide sequence of a sesquiterpene cyclase gene from the trichothecene-producing fungus *Fusarium sporotrichioides*. *Gene* 79:131–138.
 25. Hohn TM, VanMiddlesworth F. 1986. Purification and characterization of the sesquiterpene cyclase trichodiene synthetase from *Fusarium sporotrichioides*. *Arch. Biochem. Biophys.* 251:756–761.
 26. Hohn TM, McCormick SP, Desjardins AE. 1993. Evidence for a gene cluster involving trichothecene-pathway biosynthetic genes in *Fusarium sporotrichioides*. *Curr. Genet.* 24:291–295.
 27. Hohn TM, Desjardins AE, McCormick SP. 1995. The *Tri4* gene of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase involved in trichothecene biosynthesis. *Mol. Gen. Genet.* 248:95–102.
 28. Jin H-Z, et al. 2007. Harzianum A and B produced by a fungal strain, *Hypocrea* sp. F000527, and their cytotoxicity against tumor cell lines. *J. Asian Nat. Prod. Res.* 9:203–207.
 29. Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, Fujimura M. 2007. Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: pathways, genes and evolution. *Biosci. Biotechnol. Biochem.* 71:2105–2123.
 30. Lee HB, et al. 2005. A new *Hypocrea* strain producing harzianum A cytotoxic to tumour cell lines. *Lett. Appl. Microbiol.* 40:497–503.
 31. Lorito M, Woo SL, Harman GE, Monte E. 2010. Translational research on *Trichoderma*: from 'omics to the field. *Annu. Rev. Phytopathol.* 48:395–417.
 32. Lutz MP, Feichtinger G, Défago G, Brion D. 2003. Mycotoxigenic *Fusarium* and deoxynivalenol production repress chitinase gene expression in the biocontrol agent *Trichoderma atroviride* P1. *Appl. Environ. Microbiol.* 69:3077–3084.
 33. Machida Y, Nozoe S. 1972. Biosynthesis of trichothecin and related compounds. *Tetrahedron* 28:5113–5117.
 34. McCormick SP, Taylor SL, Plattner RD, Beremand MN. 1989. New modified trichothecenes accumulated in solid culture by mutant strains of *Fusarium sporotrichioides*. *Appl. Environ. Microbiol.* 55:2195–2199.
 35. McCormick SP, Hohn TM, Desjardins AE. 1996. Isolation and characterization of *Tri3*, a gene encoding 15-O-acetyltransferase from *Fusarium sporotrichioides*. *Appl. Environ. Microbiol.* 62:353–359.
 36. McCormick SP, Alexander NJ, Proctor RH. 2006. *Fusarium tri4* encodes a multifunctional oxygenase required for trichothecene biosynthesis. *Can. J. Microbiol.* 52:636–642.
 37. McCormick SP, Alexander NJ. 2007. *Myrothecium roridum tri4* encodes a multifunctional oxygenase required for three oxygenation steps. *Can. J. Microbiol.* 53:572–579.
 38. McCormick SP, Alexander NJ, Harris LJ. 2010. *CLM1* of *Fusarium graminearum* encodes a longiborneol synthase required for culmorin production. *Appl. Environ. Microbiol.* 76:136–141.
 39. McCormick SP, Stanley AM, Stover NA, Alexander NJ. 2011. Trichothecenes: from simple to complex mycotoxins. *Toxins* (Basel) 3:802–814.
 40. Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 30:e36. doi:10.1093/nar/30.9.e36.
 41. Price MS, et al. 2006. The aflatoxin pathway regulator AflR induces gene transcription inside and outside of the aflatoxin biosynthetic cluster. *FEMS Microbiol. Lett.* 255:275–279.
 42. Proctor RH, Hohn TM, McCormick SP, Desjardins AE. 1995. *Tri6* encodes an unusual zinc finger protein involved in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. *Appl. Environ. Microbiol.* 61:1923–1930.
 43. Proctor RH, McCormick SP, Alexander NJ, Desjardins AE. 2009. Evidence that a secondary metabolic biosynthetic gene cluster has grown by gene relocation during evolution of the filamentous fungus *Fusarium*. *Mol. Microbiol.* 74:1128–1142.
 44. Rodrigues P, Venancio A, Kozakiewicz Z, Lima N. 2009. A polyphasic approach to the identification of aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* isolated from Portuguese almonds. *Int. J. Food Microbiol.* 129:187–193.
 45. Rubio MB, Domínguez S, Monte E, Hermosa R. 2012. Comparative study of *Trichoderma* gene expression in interactions with tomato plants using high-density oligonucleotide microarrays. *Microbiology* 158:119–128.
 46. Sevim A, et al. 2012. Hydrophobin genes of the entomopathogenic fungus, *Metarhizium brunneum*, are differentially expressed and corresponding mutants are decreased in virulence. *Curr. Genet.* 58:79–92.
 47. Shores M, Harman GE, Mastouri F. 2010. Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48:21–43.
 48. Sivasithamparan K, Ghisalberti EL. 1998. Secondary metabolism in *Trichoderma* and *Gliocladium*, p 139–191. In Kubicek CP, Harman GE (ed), *Trichoderma and Gliocladium*, vol I. Taylor & Francis, London, England.
 49. Sousa S. 2004. Mejora del sistema de expresión en *Trichoderma harzianum* CECT 2413 para la producción de proteínas de interés biotecnológico. Ph.D. thesis. University of Sevilla, Sevilla, Spain.
 50. Tijerino A, et al. 2011. Overexpression of the trichodiene synthase gene *tri5* increases trichodermin production and antimicrobial activity in *Trichoderma brevicompactum*. *Fungal Genet. Biol.* 48:285–296.
 51. Tokai T, et al. 2005. Screening of putative oxygenase genes in the *Fusarium graminearum* genome sequence database for their role in trichothecene biosynthesis. *FEMS Microbiol. Lett.* 251:193–201.
 52. Tokai T, et al. 2007. *Fusarium Tri4* encodes a key multifunctional cytochrome P450 monooxygenase for four consecutive oxygenation steps in trichothecene biosynthesis. *Biochem. Biophys. Res. Commun.* 353:412–417.
 53. Trapp SE, Hohn TM, McCormick SP, Jarvis BB. 1998. Characterization of the gene cluster for biosynthesis of macrocyclic trichothecenes in *Myrothecium roridum*. *Mol. Gen. Genet.* 257:421–432.
 54. Tucci M, Ruocco M, De Masi L, De Palma M, Lorito M. 2011. The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Mol. Plant Pathol.* 12:341–354.
 55. Turgeon BG, Garber RC, Yoder OC. 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. *Mol. Cell. Biol.* 7:3297–3305.
 56. Vandesompele J, et al. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:0034.1–0034.11. doi:10.1186/gb-2002-3-7-research0034.
 57. Vinale F, et al. 2008. *Trichoderma*-plant-pathogen interactions. *Soil Biol. Biochem.* 40:1–10.
 58. Werck-Reichhart D, Feyereisen R. 2000. Cytochromes P450: a success story. *Genome Biol.* 1:3003.1–3003.9. doi:10.1186/gb-2000-1-6-reviews3003.
 59. Zamir LO, Devor KA, Sauriol F. 1991. Biosynthesis of the trichothecene 3-acetyldeoxynivalenol. Identification of the oxygenation steps after isotrichodermin. *J. Biol. Chem.* 266:14992–15000.