Increased Antifungal Activity of *Trichoderma harzianum* Transformants That Overexpress a 33-kDa Chitinase

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ABSTRACT

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Transformants of the biocontrol agent *Trichoderma harzianum* strain CECT 2413 that overexpressed a 33-kDa chitinase (Chit33) were obtained and characterized. Strain CECT 2413 was cotransformed with the *amdS* gene and its own *chit33* gene under the control of the *pki* constitutive promoter from *T. reesei*. Southern blotting indicated that the *chit33* gene was integrated ectopically, mostly in tandem. Some transformants showed the same restriction pattern, indicating preferable sites of integration. There was no correlation between the number of integrated copies

and the level of expression of the *chit33* gene in the transformants. When grown in glucose, the extracellular chitinase activity of the transformants was up to 200-fold greater than that of the wild type, whereas in chitin, the activity of both the transformants and the wild type was similar. Under both conditions, the transformants were more effective in inhibiting the growth of *Rhizoctonia solani* as compared with the wild type. Similar results were obtained when culture supernatants from the transformants and the wild type were tested against *R. solani*.

Additional keywords: antagonism, chitinase overproduction, hydrolases, phytopathogenic fungi, transformation.

Strains of *Trichoderma* are increasingly being used to control soil-borne plant pathogens (22,24,50). The most frequently suggested mechanisms of biocontrol by *Trichoderma* include mycoparasitism, antibiosis, competition for nutrients, or all of the above. *Trichoderma* can inhibit the pathogen by means of antibiotics (51,57) or cell wall-degrading enzymes (CWDEs) (10) such as chitinases (2, 8,30), β -1,3-glucanases (39), proteases (20), mannanases, and other hydrolases (34,51). The relative importance of these two mechanisms in the antagonistic process depends on specific pathogen-host interactions (25,27,28). However, the combination of hydrolytic enzymes and antibiotics of *Trichoderma* has been shown to have an antifungal synergism (53). Involvement of CWDEs in the penetration of the antibiotics into the pathogen has been described (1,39). Furthermore, purified chitinases, as well as genetically engineered *Escherichia coli* and tobacco plants that produce chitinases, protected plants from infection produced by *Cercospora nicotianae* and *Rhizoctonia solani* (46). More recently, transgenic potato and tobacco plants expressing chitinase Chit42 from *Trichoderma* have been shown to be highly tolerant or resistant to foliar pathogens (*Alternaria alternata*, *Alternaria solani*, and *Botrytis cinerea*) and to the soilborne pathogen *R. solani* (40).

The biosynthesis of CWDEs in *Trichoderma* is controlled mainly at the transcriptional level (13,14,19), and the responsible genes are present as single-copy genes (19,37,38). To overproduce these enzymes, their gene copy number has been increased by transformation (23,33). Improvement in biocontrol efficiency of *Trichoderma* by transformation with genes *prb1* (basic protease) and *egl1* (β -1,4-glucanase) has been described by Flores et al. (16) and Migheli et al. (45).

Chitinase Chit33 from *T. harzianum* CECT 2413 is encoded by the *chit33* gene, whose expression is repressed by glucose and

derepressed by chitin, fungal cell walls, or starvation conditions. The main goal of this study was to determine in vitro if improved *R. solani* inhibition could be achieved by overexpressing *chit33* in *T. harzianum* CECT 2413, in order to gain a basic understanding of these transformants for future biocontrol experiments.

MATERIALS AND METHODS

Strains. *T. harzianum* Rifai strain CECT 2413 and *R. solani* Kühn strain CECT 2815 AG4 (5) were obtained from Colección Española de Cultivos Tipo, Burjasot, Valencia, Spain (11). They were maintained on PPG agar medium (2% [wt/vol] dehydrated mashed peeled potatoes [Maggi; Nestlé, Vevey, Switzerland]; 2% [wt/vol] glucose; and 2% [wt/vol] agar).

Media and culture conditions. *T. harzianum* was grown in Czapek salt minimal medium (CZ) (11) supplemented with 5% (wt/vol) glucose or 0.5% (wt/vol) crabshell chitin (Sigma Chemical Co., St. Louis), as previously described (12). Potato dextrose broth (PDB) (Difco Laboratories, Detroit) and potato dextrose agar (PDA) also were used to culture *T. harzianum*.

Transformation media were those described by Penttilä et al. (48). Transformants were selected on AA medium, which was a CZ-modified medium in which NH_4Cl was replaced by 10 mM acetamide (Aldrich Chemical Co., Steinheim, Germany) or acrylamide (Boehringer Mannheim Biochemicals, Indianapolis, IN) and supplemented with 12.5 mM CsCl and 2% agar. AA medium amended with 0.1% Triton X-100 (Sigma Chemical Co.) was called AAT medium.

All transferred cultures were washed with 2% (wt/vol) MgCl_2 , followed by distilled water, and harvested by vacuum filtration through Whatman #1 filter paper (Whatman International, Ltd., Maidstone, England).

Plasmids. The plasmids used in these studies were p3SR2 and pLMRS3-CHIT33. Plasmid p3SR2 (29) carries an *Aspergillus nidulans amdS* gene that codes for an acetamidase that was the selection marker. The *chit33* gene was previously cloned from *T.*

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harzianum CECT 2413 and codes for a 33-kDa chitinase (37). The expression plasmid pLMRS3-CHIT33 was constructed using pLMRS3 (41) and contain the *chit33* gene cDNA under the control of the constitutive *pki* (pyruvate kinase) promoter from *T. reesei*. Plasmids were constructed and isolated following standard protocols (52), maintained in *E. coli* DH5 α , and kept at -20°C until use.

Transformation procedure. *T. harzianum* was cotransformed with the plasmids p3SR2 and pLMRS3-CHIT33 (Fig. 1). Preparation and transformation of protoplasts were carried out according to Penttilä et al. (48). Cotransformation was conducted with a 1:10 (p3SR2/pLMRS3-CHIT33) plasmid ratio, and 200- and 500- μl aliquots of the transformed protoplasts were added to the top agar and overlaid on selective medium plates. Original transformants were transferred onto selective AAT media to obtain colonies from which two repeated selective and nonselective cycles were carried out to obtain homokaryons. Stable transformants were those that retained the selection marker without selective conditions. The stability of the homokaryotic transformants was checked by four cycles of sporulation on nonselective PPG medium, followed by a single cycle on selective AAT medium.

Protein isolation. Cultures were carried out as described previously (12). Briefly, 1.0×10^6 spores per ml were inoculated into PDB medium and incubated for 48 h at 22°C in a rotary shaker at 200 rpm (New Brunswick Scientific Co., Inc., Chicago). The mycelium was recovered by vacuum filtration, resuspended in CZ medium supplemented with either crabshell chitin (0.5% wt/vol) or glucose (5% wt/vol), and further incubated for 48 h. The cultures were filtered through Whatman #1 filter paper. Mycelia were frozen and kept at -80°C until used for RNA isolation, and the culture filtrates were used for chitinase assay and western blot analysis. The 100-ml filtrates from glucose-supplemented cultures were brought to 80% saturation with $(\text{NH}_4)_2\text{SO}_4$ at 4°C . The resultant precipitates were collected by centrifugation at $20,000 \times g$ for 20 min (model J2-21M; Beckman Instruments, Inc., Palo Alto, CA), resuspended in 1 ml of distilled water, and then dialyzed prior to western blotting. The filtrates from chitin-supplemented cultures were used for either chitinase activity or western blot assays without being concentrated. Protein concentration was measured according to Bradford (6), using the Bio-Rad dye reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (Sigma Chemical Co.) as a standard.

DNA manipulations. Genomic DNA was extracted from mycelia grown in PDB medium using a standard protocol modified with additional phenol extraction steps (32). Ten micrograms of DNA was digested with *EcoRI* and *PstI* according to the instruc-

tions of the supplier (Boehringer Mannheim Biochemicals) and fractionated in 0.7% agarose gels at 2 V cm^{-1} for 15 h. After electrophoresis, DNA fragments were capillary blotted onto nylon membranes (Hybond N; Amersham International plc., Buckinghamshire, England) and ultraviolet cross-linked (RPN 2500; Amersham International plc.). *chit33* and *amdS* probes were random prime-labeled with ^{32}P -dCTP (oligolabeling kit; Pharmacia Biotech Europe GmbH, Uppsala, Sweden). Labeled probe was separated from free ^{32}P -dCTP using Sephadex G-50 (Sigma Chemical Co.) column chromatography. High-stringency hybridizations and washes were done according to Sambrook et al. (52). Hybridization signals were detected by exposing membranes to X-ray films (X-OMAT; Eastman Kodak Co., Rochester, NY) for 1 to 2 days at -80°C with an intensifying screen. To calculate the plasmid copy number integrated in the transformants' genome, we used the presence of the native *chit33* gene within each lane as an internal copy control. The quantification of the radioactivity, measured as counts per minute, present in the bands corresponding to both the native and the transforming *chit33* gene was determined with electronic autoradiography equipment (Instant Imager 2024; Packard Instruments Co., Meriden, CT). The plasmid copy number was then calculated by comparing the radioactivity incorporated in the endogenous *chit33* gene copy with the transforming one(s) within the same lane.

RNA isolation and analysis. Total cellular RNA was isolated using the acid phenol method (9). Mycelia were lysed in a mini-bead-beater (Biospec Products, Bartlesville, OK) using five 2.3-mm-diameter steel beads. Ten-microgram samples were loaded onto 2.2 M formaldehyde, 1.2% (wt/vol) agarose gels and run at 7 V cm^{-1} for 2 h. RNA was blotted and fixed as described for DNA. Hybridizations and detection were carried out as described above.

Chitinase activity. Chitinase activity (EC 3.2.1.14) in cultures of *Trichoderma* strains with chitin was assayed according to Boller et al. (4) using colloidal chitin, prepared as previously described (3), as a substrate. The released *N*-acetyl-glucosamine (GlcNAc) was measured using GlcNAc as a standard, according to Reissig et al. (49). One unit (U) of chitinase was defined as the amount of enzyme releasing 1 μmol of GlcNAc per min. Specific activity was expressed in units per microgram of protein. Chitinase activity in glucose-containing media was measured with 4-methylumbelliferyl- β -D-*N,N',N'',N'''*-tetraacetylchitotetraoside (mU-(glNAc) $_4$), a substrate more sensitive than colloidal chitin, in order not to concentrate the filtrates. Fifty microliters of culture filtrate from a culture grown in CZ medium containing 5% (wt/vol) glucose for 48 h at 22°C was mixed with 50 μl of 250 mM mU-(glNAc) $_4$ in 0.1 M sodium

Fig. 1. Maps of plasmids p3SR2 and pLMRS3-CHIT33 used for transformation of *Trichoderma harzianum* CECT 2413. Plasmid p3SR2 (29) carries an *Aspergillus nidulans amdS* gene coding for an acetamidase that was the selection marker. The *chit33* gene was previously cloned from *T. harzianum* CECT 2413 and encodes a 33-kDa chitinase (37). The expression plasmid pLMRS3-CHIT33 was constructed using pLMRS3 (41) and contains the *chit33* cDNA under the control of the constitutive *pki* (pyruvate kinase) promoter from *T. reesei*. Relevant restriction sites and their relative positions are indicated.

citrate buffer, pH 3.0, and incubated at 30°C for 30 min. The reaction was then diluted into 2.9 ml of 0.5 M glycine-NaOH buffer, pH 10.4. The liberated 4-methylumbelliferone (Umb) was measured (excitation at 350 nm, emission at 440 nm) with a fluorescence spectrophotometer (LS-3B; Perkin-Elmer Ltd., Beaconsfield,

Buckinghamshire, England). One unit of chitinase activity was defined as the amount of enzyme releasing 1 nmol of Umb per min. Specific activity was expressed in units per microgram of protein. Chitinase activity was measured in two different experiments and each experiment included three replicates.

Gel electrophoresis and immunoblotting. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12% polyacrylamide gels according to Laemmli (35). Low-range protein markers (Bio-Rad Laboratories) were used as a molecular mass standard. Proteins were electrotransferred from the gels to nitrocellulose membranes (Millipore Corp., Bedford, MA) as described by Towbin et al. (54), using a Mini Trans-blot cell (Bio-Rad Laboratories). The membranes were blocked for 2 h with a solution of 5% (wt/vol) skim milk in 15 mM Tris-HCl, pH 7.4, with 200 mM NaCl (TBS). Filters were then incubated overnight at room temperature with polyclonal rabbit antibody prepared to Chit33 (37) and diluted 1:500 in blocking solution. Washes were done using TBS buffer plus 0.05% (wt/vol) Tween 20. Goat anti-rabbit immunoglobulin G antibody conjugated to peroxidase (Sigma Chemical Co.) was the secondary antibody. Detection was with 4-chloro-1-naphthol (Sigma Chemical Co.) and H₂O₂.

Test for inhibition in broth cultures. *T. harzianum* wild-type strain, T6, and T30 were grown in 200 ml of PDB medium with shaking (220 rpm) at 22°C. After 2 days, *T. harzianum* was washed, filtered, and weighed. *R. solani* was cultured on five petri dishes with PPG for 7 days; mycelia were scraped from the agar and fragmented in a glass homogenizer (Pobel, Madrid, Spain). The mycelial fragments of *R. solani* were incubated for 4 days in PDB medium where they grew into small spherical masses. *R. solani* mycelium was harvested, washed by centrifugation at 14,000 × g for 20 min, and weighed. Mycelia of *T. harzianum* (wild-type strain, T6, or T30) and *R. solani* were cocultured in a 2:1 ratio (4.5 and 2.2 g wet weight, respectively). The cocultures were grown in 100 ml of CZ medium containing 2% (wt/vol) glucose, as well as in CZ medium containing 0.5% chitin (wt/vol), for 6 days with shaking (220 rpm) at 22°C. As controls, *R. solani* was grown in CZ medium with either 2% (wt/vol) glucose or 0.5% (wt/vol) chitin. Samples of *R. solani* were taken from each culture at different time intervals and transferred to *R. solani*-selective medium (PPG + 5 ppm of benomyl). The radius of each colony was measured every 12 h until it covered the surface of the plate. The radial growth of each colony was plotted as a function of time, and the slope of this line was defined as a growth rate. The relative growth rate (*R*) in the presence of each *T. harzianum* strain was calculated as follows:

$$R = \frac{\text{growth rate of } R. \text{ solani from cocultures with } T. \text{ harzianum}}{\text{growth rate of } R. \text{ solani from control cultures}} \times 100$$

Fig. 2. Southern blot analysis showing integration of *amdS* and *chit33* genes into the genome of the *Trichoderma harzianum* transformants. **A**, Genomic DNA was digested with *EcoRI*, which cuts twice in plasmid pLMRS3-CHIT33. DNA was hybridized with an *EcoRI-SalI* fragment from plasmid p3SR2 that contains the *amdS* gene. Arrow shows fragments corresponding to the integrated plasmid. **B**, Ten micrograms of genomic DNA was digested with *PstI*, which cuts only once in plasmid pLMRS3-CHIT33, and probed with *chit33* cDNA.

Fig. 3. Southern blot analysis showing the number of copies of the *chit33* gene integrated into the genome of the transformants and DNA rearrangements. This blot was used for quantification of the number of copies of the *chit33* gene integrated in the genome of the transformants. Genomic DNA was digested with *EcoRI*, which cuts twice in plasmid pLMRS3-CHIT33. Gene *chit33* was used as a probe and arrows show fragments corresponding to the promoter plus the *chit33* open reading frame. The upper arrow corresponds to the wild-type *chit33* copy and the lower one shows a band corresponding to the *cbhII* promoter plus *chit33* cDNA.

Fig. 4. Northern blot analysis of RNA isolated from mycelia of *Trichoderma harzianum* CECT 2413 and transformants showing transcription levels of the *chit33* gene. To obtain mycelia, 1×10^6 spores per ml of each strain were inoculated into potato dextrose broth medium and incubated for 48 h at 22°C. Mycelia were recovered by filtration, transferred to Czapek salt minimal medium supplemented with either 5% (wt/vol) glucose or 0.5% (wt/vol) chitin, and further incubated for 48 h. RNA was isolated from the mycelia harvested by filtration. **A**, Hybridization of total cellular RNA (10 µg) with the full-length *chit33* gene cDNA probe. **B**, Methylene blue staining of the filter is shown as a loading control.

Treatments were arranged in a completely randomized design. The experiment was repeated twice and each experiment included three replicates.

Test for inhibition on agar plates. To confirm the results from the studies described above, 5-mm-diameter mycelial discs of *T. harzianum* wild-type strain, T6, or T30 and of *R. solani* were placed on opposite sides (1 cm from the edges) of PPG agar plates and incubated at 22°C. When the mycelia of the *T. harzianum* strains and *R. solani* were in contact, the area of overlap was examined both visually and microscopically and the size of the lysed area was measured.

To test for inhibition without hyphal contact, petri dishes of PDA were covered with cellophane discs (Scotch, Cergy, Pontoise, France) and inoculated in the center of the plate with 5 µl of a conidial suspension (10⁸ spores per ml) of the *T. harzianum* wild-type strain, T6, or T30. After 4 days of incubation at 22°C, the cellophane discs were removed from the plates. A single 5-mm-diameter mycelial disc of *R. solani* was placed in the center of the plate. As a control, a disc of *R. solani* was grown on a PDA plate. *R. solani* mycelial growth was measured every 24 h for 5 days, and the relative growth rate was expressed as follows:

$$R = \frac{\text{growth of } R. \text{ solani on } T. \text{ harzianum preinoculated plates}}{\text{growth of } R. \text{ solani on PDA plates}} \times 100$$

Treatments were arranged in a completely randomized design. The experiment was repeated twice and each experiment included three replicates.

Statistical analysis. When tests for inhibition in broth culture were carried out, differences in inhibition of *R. solani* by *T. harzianum* strains were tested by a one-way analysis of variance (ANOVA). As this activity was monitored periodically, time was used as a covariant in the model. Relative growth rate data were subjected to angular transformation prior to analysis (58). Pairwise comparisons were carried out using an honestly significant difference Tukey's test for unequal sample sizes.

In tests of inhibition without hyphal contact, differences in the effect of *Trichoderma* transformants and wild type on the growth rate of *Rhizoctonia* were determined by linear regression analyses after logarithmic transformation; slopes were pairwise compared by means of analyses of covariance (ANCOVA) (58).

To test differences in chitinase activity among *Trichoderma* strains, a one-way ANOVA was done, followed by a Scheffe's test for pairwise comparisons. Data were previously checked for normality using a Shapiro-Wilk test.

RESULTS

Isolation of transformants. *T. harzianum* strain CECT 2413 was cotransformed with the plasmids p3SR2 (29) and pLMRS3-

CHIT33 (Fig. 1). Transformants were isolated on selective media with acetamide as the nitrogen source at a frequency of 40 transformants per microgram of DNA. Among 300 transformants, 80 were selected on the basis of their ability to grow on selective media. The selected stable transformants for the *amdS* marker (T2, T6, T30, T51, T52, and T65) also contained copies of the *chit33* gene and were chosen for further study (described below). Transformant T1, which lost ectopic copies of *amdS* and *chit33*, was chosen as a nonstable control.

Southern analysis. All of the stable transformants showed the presence of the *amdS* gene when DNA was digested with *EcoRI* and hybridized with an *amdS* probe (Fig. 2A). When the same blot was hybridized using *chit33* as a probe, all the stable transformants also showed the presence of *chit33* (Fig. 3). These results indicated that all of the stable transformants for *amdS* were also transformants for *chit33*. Moreover, when DNA from transformants T2, T6, T30, and T65 was digested with *PstI*, which linearizes plasmid pLMRS3-CHIT33, an intense band of 6 kb (the size of the plasmid) was detected when hybridized with *chit33* (Fig. 2B), indicating a tandem integration event. Less intense bands of different sizes appeared, corresponding either to different sites of single-copy insertion or flanking regions of the tandem integrations. All of the stable transformants showed two to three different integrations in the genome when DNA digestion was carried out with *BglII*, an enzyme that does not cut inside pLMRS3-CHIT33 (data not shown). *EcoRI* digestion was expected to produce a hybridizing band of 2 kb, corresponding to the promoter plus *chit33*, in addition to the band corresponding to the wild-type copy (Fig. 3). Nevertheless, extra hybridizing fragments of different sizes appeared, indicating that rearrangements might have occurred during the stabilization process (Fig. 3).

Transformants were separated into two groups with regards to *chit33* integration. One group included transformants for which the vector was not inserted in tandem (T51 and T52) and showed different band patterns (Fig. 2B). The other group included transformants for which the vector was integrated in tandem (T2, T6, T30, and T65) and showed a similar band pattern (Fig. 2B). Similar results were obtained when other restriction enzymes (i.e., *KpnI*) were used (data not shown). Surprisingly, the same groups appeared for the *amdS* gene (Fig. 2A), indicating a preferable integration site for both genes.

The number of copies of the *chit33* gene integrated in the transformants' genome (Fig. 3) was 5 to 6 copies for T2 and T65, 8 to 12 copies for T6 and T30 and T51, and 3 to 4 copies for T51 and T52.

Pattern of *chit33* mRNA and protein accumulation. Northern blot analysis showed the absence of *chit33* mRNA in the wild type in glucose-containing media, whereas all of the transformants had high levels of *chit33* mRNA. The maximum level was found in transformants T6 and T30 (Fig. 4). When glucose was replaced by chitin as the carbon source, all of the transformants showed *chit33*

Fig. 5. Western analysis of *Trichoderma harzianum* CECT 2413 and the transformants. *T. harzianum* wild type and the transformants were cultured as described in Figure 4. Twenty-four and twelve micrograms of extracellular protein from glucose-containing and chitin-containing cultures, respectively, were loaded in the gels. Protein excreted in the presence of glucose was concentrated 100 times after (NH₄)₂SO₄ precipitation.

RNA accumulation and the maximum levels were reached by transformants T6 and T2.

The extracellular proteins were assayed by western blots under the conditions described above. In 100-fold concentrated supernatants from glucose-grown cultures, a very strong signal was detected in all of the transformants (Fig. 5), while the wild type did not show any signal. In chitin-grown cultures, a signal was displayed both by the wild type and the transformants. The molecular weight of the chitinase overproduced by the transformants was identical to that of the wild type. The main band in glucose-grown cultures was Chit33 protein. The extracellular protein produced by the wild type was about 4.5 mg/liter in glucose-containing media, while the transformants produced it in the range of 4.6 to 26 mg/liter. The amount of extracellular protein ranged from 128 to 162 mg/liter for both the wild type and the transformants in chitin-containing medium.

Chitinase activity in the wild type and the transformants. In cultures of CZ medium containing glucose, all of the transformants had greater (up to 200 times) specific chitinase activities than the wild type. However, the transformants showed similar activity to that of the wild type in cultures of CZ medium with chitin as the carbon source (Table 1).

Inhibition in broth cultures. *R. solani* was cocultured with either the *T. harzianum* wild type or the transformants T6 and T30 in CZ medium supplemented with either glucose or chitin. Spherical samples of *R. solani* were taken periodically and placed on *T. harzianum*-suppressive medium. In samples from chitin-containing medium, the growth rate of *R. solani* was reduced to about 50% of the initial growth (0 h) in 20 h by the transformants, while the same results were obtained in 55 h in the case of the wild type. Similarly, *R. solani* growth was reduced to zero in a shorter period of time by the transformants (40 h) than by the wild type (70 h) (Fig. 6). Both transformants, T6 and T30, reduced similarly the growth rate of *R. solani*. In the samples taken from glucose-containing media, the growth of *R. solani* was not affected by either the wild type or the transformants for up to 45 h. After 45 h, the differences observed between the wild type and the transformants were not significant.

Inhibition on agar plates. When *R. solani* and *T. harzianum* wild type or transformants were grown in the same petri dishes with PPG, they produced a zone of lysis in the *R. solani* mycelia. As shown in Figure 7, *T. harzianum* wild type only produced a faint zone of lysis, whereas the transformants T6 and T30 showed a very large zone of lysis of *R. solani*. Moreover, both transformants

were able to grow and sporulate over *R. solani*. The *R. solani* mycelia were killed, as no growth was observed when discs from the overgrown area were transferred to fresh plates of PPG.

Inhibition without contact. Regression analysis showed that the growth of *R. solani* was significantly inhibited on the plates where T6 or T30 had previously grown (Figs. 8 and 9). Growth inhibition by T6 and T30 was about 37 and 67%, respectively, relative to the control. Inhibition by T6 and T30 was significantly different as compared with that of the wild type and the control. The amount of inhibition caused by both transformants also was significantly different. On the other hand, the growth rate of *R. solani* on PDA plates and on wild-type pregrown plates did not differ significantly (Fig. 8). The differences in their colony radius could be explained by a nutrient depletion in the wild-type plates

Fig. 6. Inhibition of *Rhizoctonia solani* in broth cultures. *Trichoderma harzianum* CECT 2413 and the transformants were inoculated and cultured individually with *R. solani* in Czapek salt minimal medium supplemented with either glucose or chitin. A single colony of *R. solani* was taken from the broth cultures and placed on *T. harzianum*-suppressive agar medium. The radius of the colonies was measured twice daily and the relative growth calculated. Solid bars represent the mean growth ($n = 3$) of *R. solani* when previously cultured in the presence of the wild type; open and grey bars represent the growth of *R. solani* when previously cultured in the presence of transformants T6 and T30, respectively. In medium supplemented with chitin at 45 and 55 h, there was no growth in either treatment with the transformants. In medium with glucose at 45 h, there was no growth in the treatment with T30. Each value is the average of three measurements. Vertical lines represent the standard error of the mean. Significant differences were found ($F = 18.46$, $P < 0.0001$) in chitin between the wild type and the transformants (time was used as a covariable in the model). Tukey's honestly significant difference test for unequal sample sizes (58) showed differences between the wild type and T6 ($P < 0.001$) and the wild type and T30 ($P < 0.001$), but the transformants were not significantly different ($P = 0.35$). No significant differences were found in glucose-containing medium.

TABLE 1. Specific chitinase activity in cultures of the transformants and the *Trichoderma harzianum* wild-type CECT 2413

Strains	Chitinase activity (U/ μ g of protein)	
	Glucose ^a	Chitin ^b
Wild type	15.6 $\pm$ 1.0 ^c x	79.71 $\pm$ 3.61 ^c
T1	39.9 $\pm$ 1.5 x	64.13 $\pm$ 11.34
T2	2,166.8 $\pm$ 114.6 y	77.66 $\pm$ 6.42
T6	2,172.8 $\pm$ 63.3 y	61.39 $\pm$ 3.06
T30	3,454.3 $\pm$ 103.8 z	69.73 $\pm$ 3.28
T51	2,331.5 $\pm$ 66.4 y	66.32 $\pm$ 6.31
T52	2,178.6 $\pm$ 44.3 y	74.93 $\pm$ 9.10
T65	2,503.8 $\pm$ 101.3 y	77.17 $\pm$ 0.83

^a Strains were cultured in Czapek salt minimal medium (CZ) supplemented with 5% glucose for 44 h at 22°C. Fifteen microliters of filtered cultures was assayed with mU-(glNAc)₄ for 30 min at 30°C ($F = 261.32$; $P < 0.0001$). 1 U = 1 nmol of 4-methylumbelliferone released per min.

^b Strains were cultured in CZ medium supplemented with 0.5% chitin for 48 h. Twenty-four micrograms of extracellular protein was assayed using colloidal chitin at 37°C for 1 h by the method of Reissig et al. (49). ($F = 3.79$; $P < 0.01$). 1 U = 1 μ mol of *N*-acetyl-glucosamine released per min.

^c Data represent mean values ($\pm$ standard error) with $n = 6$ for glucose and $n = 4$ for chitin. Mean values in the glucose column followed by the same letter do not differ significantly ($P > 0.05$) according to the Scheffe's *S* test. For chitin, none of the means differed significantly.

in comparison with the PDA plates where no strain was previously grown (Fig. 8).

DISCUSSION

We transformed *T. harzianum* with *chit33*, the gene that codes for the 33-kDa endochitinase Chit33 (37), under the control of a constitutive promoter and obtained stable *chit33* transformants. In some transformants, integration of *chit33* occurred in tandem and at preferential sites in the *T. harzianum* genome. All transformants had higher chitinase activity than the wild type when grown in glucose-containing medium. Although they have different numbers of copies (3 to 12), most transformants had similar levels of chitinase activity. These results are in general agreement with observations by others (33,47,55). This fact may reflect the importance of the chromosomal location of the transforming DNA as it has been described for other genes (56). Other explanations such as transcriptional or translational regulation cannot be ruled out for the different chitinase expression levels (16,21).

In conditions under which most *T. harzianum* hydrolases are produced and secreted, there were no significant differences in chitinase activity between the wild type and the transformants. This is due to the presence of other chitinases that are produced under such conditions (12). Among them, Chit42 has been shown to be responsible for most of the observed extracellular chitinase activity (12,19,37). Therefore, the increase in Chit33 activity, due to the constitutive expression of ectopic copies of *chit33*, might be undetectable in these conditions with regards to the total chitinase activity. Furthermore, secretion could be a limiting step as it has been described for many recombinant proteins expressed in filamentous fungi (47).

The overexpression of hydrolases as a means to improve fungal strains has been described (16,21,43–45). However, there have been two main disadvantages to this approach. Many hydrolases have been overexpressed, using strong but regulated promoters that need specific inducers for expression; this is not the optimal situation if the transformants are to be released to control a plant disease (43,44). Secondly, many overproduced hydrolases underwent proteolysis when they were overexpressed in *T. harzianum*. Such is the case with a *Serratia marcescens* chitinase that was proteolysed in the presence of chitin (21). Not only heterologous proteins but also homologous hydrolases underwent proteolysis when they were overexpressed in *T. harzianum* (15,36). In this study, *T. harzianum* overproduces chitinase Chit33 without any specific inducer and does not undergo proteolysis.

Expression of homologous or heterologous genes in *T. reesei* is used for industrial enzyme production (7,17,18,42). The strat-

egy used in this study has shown to give positive results for production of Chit33 without a specific purification process, because *chit33* transformants produced high amounts of Chit33 in glucose-containing cultures where almost no other proteins were excreted.

The transformants T6 and T30 exhibited better antagonistic properties in vitro than the wild type against *R. solani* when the transformants were grown in broth cultures containing chitin, where no increase in chitinase activity was observed. The reason could be that the chitinase activity corresponding to Chit33 is constitutively expressed in the transformants in medium with either glucose or chitin, whereas, in the wild type, Chit33 appeared 24 h after the mycelia was transferred from glucose- to chitin-supplemented media (data not shown). Chit33 transformants can inhibit the growth of

Fig. 8. Effect of metabolites of *Trichoderma harzianum* CECT 2413 on *Rhizoctonia solani* growth. The wild type or the transformants T6 and T30 were grown on potato dextrose agar (PDA) in petri dishes previously covered with cellophane discs. After 4 days, cellophanes were removed and a 5-mm mycelial disc of *R. solani* was placed in the center of the petri dishes. Radial growth of *R. solani* was measured every 24 h. *R. solani* = *R. solani* growth on PDA; *Trichoderma* wild type = *R. solani* growth on PDA where *T. harzianum* CECT 2413 had previously grown; and T6 and T30 = *R. solani* growth on PDA where transformants T6 and T30 had previously grown on the cellophane discs. Each line represents the mean value of six experiments, and vertical bars are the standard errors. Regression lines of *Rhizoctonia* radial growth versus time are shown. Lines with different letters have significantly different slopes ($P < 0.005$). Regression analysis showed significant ($P < 0.001$) R^2 values for *R. solani* (0.983), *Trichoderma* wild type (0.947), T6 (0.960), and T30 (0.958).

Fig. 7. In vitro inhibition of *Rhizoctonia solani* by *Trichoderma harzianum*. Mycelial discs of the wild-type *T. harzianum* (top plate) or transformants T6 (bottom left plate) or T30 (bottom right plate) and mycelial discs of *R. solani* were placed at the opposite sides of PPG petri dishes. Plates were incubated and pictures were taken **A**, after 3 days, when both mycelia were in contact; **B**, after 9 days; and **C**, after 12 days. Each petri dish has *T. harzianum* at the top and *R. solani* at the bottom.

R. solani earlier than the wild type when tested in broth cultures with chitin as the carbon source, while in the presence of glucose, no significant differences were observed between the wild type and the transformants (Fig. 6). Therefore, the expression of Chit33 alone was not enough to inhibit *R. solani* growth, which suggests that synergism with other CWDEs or secondary metabolites is needed. Actually, Chit33 increased the antagonistic activity of the transformants in low nitrogen media, as shown in Figures 8 and 9. This could be attributed to the induction under this condition of other hydrolases (data not shown) or secondary metabolites (26,31). Additionally, the observation that the Chit33 transformants were able to antagonize *R. solani* even without physical contact was also in concordance with secondary metabolites implicated in the inhibition of the pathogen.

In conclusion, we have demonstrated that *chit33 T. harzianum* transformants can inhibit *R. solani* growth under inducing in vitro conditions. However, in vivo, we do not know the antagonistic properties of these transformants. Further biocontrol studies with the *chit33* transformants and different phytopathogens will allow us to assess the value of these new strains for biological control and will provide a better understanding of the role of Chit33 in mycoparasitism.

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Fig. 9. Colonies of *Rhizoctonia solani* grown under the conditions described in Figure 8. Control (C) = growth of *R. solani* on potato dextrose agar (PDA) not previously inoculated; and wild-type (WT) and transformants (T6 and T30) = growth of *R. solani* on PDA petri dishes where either the wild type or transformants previously had grown for 4 days. Pictures were taken after 52 and 100 h of *R. solani* incubation.

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