Differential Expression of *Trichoderma harzianum* Chitinases During Mycoparasitism

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ABSTRACT

Haran, S., Schickler, H., Oppenheim, A., and Chet, I. 1996. Differential expression of *Trichoderma harzianum* chitinases during mycoparasitism. Phytopathology 86:980-985.

The chitinolytic system of the biocontrol agent *Trichoderma harzianum* is made up of two β-1,4-*N*-acetylglucosaminidases and four endochitinases. The expression of the various *N*-acetylglucosaminidases and endochitinases during mycoparasitism was found to be regulated in a very specific and finely tuned manner that was affected by the host. When *T. harzianum* was antagonizing *Sclerotium rolfsii*, an *N*-acetylglucosaminidase of 102 kDa (CHIT 102) was the first to be induced. As early as 12 h after contact, its activity diminished, and another *N*-acetylglucosaminidase of 73 kDa (CHIT 73) was expressed at high levels. However, when *T. harzianum* was antagonizing *Rhizoctonia solani*, the chitinase expression patterns differed considerably. Twelve hours after contact, CHIT 102 activity was elevated, and the activities of three additional endochitinases at 52 kDa (CHIT 52), 42 kDa (CHIT 42), and 33 kDa (CHIT 33) were detected. As the antagonistic interaction proceeded, CHIT 102 activity decreased, whereas the activity of the endochitinases gradually increased. The differential expression of *T. harzianum* chitinases may influence the overall antagonistic ability of the fungus against a specific host.

Additional keywords: biological control, lytic enzymes, plant pathogens.

Trichoderma harzianum Rifai, a filamentous soil fungus, is an effective biocontrol agent of several economically important plant-pathogenic fungi. *Trichoderma* spp. attack the pathogens by excreting lytic enzymes including β-1,3-glucanase(s), proteinase(s), and chitinases, enabling them to degrade host cell walls and thus reduce disease incidence (7,13,14,23). There has been a considerable amount of recent research aimed at elucidating the chitinolytic system of *T. harzianum* (8,15,17,24,31,32,33,34). The chitinolytic system of *T. harzianum* strain TM was found to have six distinct chitinolytic enzymes: two were identified as β-1,4-*N*-acetylglucosaminidases (EC 3.2.1.30) that hydrolyze chitin to *N*-acetylglucosamine (GlcNAc) monomers in an exotype fashion (CHIT 102 and CHIT 73), and four were characterized as endochitinases (EC 3.2.1.14) that cleave randomly at internal sites over the entire length of the chitin microfibril (CHIT 52, CHIT 42, CHIT 33, and CHIT 31) (15). Harman et al. (17) reported the purification of a 40-kDa enzyme from *T. harzianum* strain P1 that released dimeric units from chitin and was termed chitobiosidase. The genes coding for CHIT 42 and CHIT 33 were recently cloned and sequenced (4,12,18,21). No significant homology was detected between these genes (21).

Most of the studies on the expression and regulation of these lytic enzymes were performed in liquid culture supplemented with different carbon sources, e.g., chitin, purified fungal cell walls, glucose, or GlcNAc (9,12,13,15,21,28,32,34). Elad et al. (11) found that *Trichoderma* isolates produce chitinases and glucanases when grown on live mycelium of *Sclerotium rolfsii* Sacc. and *Rhizoctonia solani* Kühn in soil. High β-(1,3) glucanase and chitinase activities were also detected when *T. harzianum* parasitized these pathogens in dual agar cultures (10). Carsolio et al. (4) showed that CHIT 42 expression is strongly enhanced during direct interaction of the mycoparasite with *R. solani*.

On the other hand, the differential expression of the chitinolytic system of *Trichoderma* during mycoparasitic interactions has only recently been studied. Inbar and Chet (19) used direct confrontation between *Trichoderma* and *S. rolfsii* to identify specific chitinases expressed during parasitism. They detected a change in the chitinolytic enzyme profile during the interaction between the fungi. Before coming into contact with each other, both fungi contained a protein with constitutive β-1,4-*N*-acetylglucosaminidase activity. After contact, the chitinolytic activity of *S. rolfsii* disappeared, whereas that of the *Trichoderma N*-acetylglucosaminidase (CHIT 102) greatly increased. As the interaction proceeded, the activity of CHIT 102 diminished concomitantly with the appearance of CHIT 73.

The development of a simple and sensitive assay for the simultaneous detection of the various chitinolytic activities allowed us to follow the temporal expression of chitinases during mycoparasitism of *T. harzianum* on the plant pathogens *S. rolfsii* and *R. solani*. Our results show that the pattern of chitinases expressed by *T. harzianum* during the parasitic action is specifically affected by these hosts and that the regulation of the various components of its chitinolytic system is a finely tuned process.

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MATERIALS AND METHODS

Culture of organisms. *Trichoderma harzianum* strain T-Y was grown on potato-dextrose agar (PDA) (Difco Laboratories, De-

troit). *R. solani* and *S. rolfsii* type A, ATCC 26325, were maintained on synthetic medium (SM) described by Okon et al. (27).

Dual cultures were carried out as described by Inbar and Chet (19). Fungi were grown on a cellophane membrane covering a 90-mm petri dish that contained SM amended with 0.2% (wt/vol) glucose. Plates were inoculated with two PDA strips (3 × 15 mm), each carrying mycelium from one of the fungi. These strips were placed parallel to each other, 6 cm apart, and incubated at 28°C in the dark.

Enzyme production. At the indicated time, mycelium from the interaction zone where *T. harzianum* parasitized *S. rolfsii* or *R. solani* was collected aseptically and washed with sterile, distilled water. This mycelium was then homogenized in sterile, distilled water by Ultra-Turrax (TP 18/10; IKA-WERK, Staufen, Germany) for 3 min at 4°C and sonicated for 3 min at 4°C with 50% pulses using a small microtip (Sonicator model W-375; Heat Systems-Ultrasonics Inc., Farmingdale, NY). Finally, the mycelium was homogenized for 3 min at 4°C with a Heidolph RZR-50 homogenizer (Heidolph Elektro, Kelheim, Germany) and centrifuged for 20 min at 20,000 × *g* at 4°C. The supernatant was collected and dialyzed against distilled water (4 × 5 liters) for 24 h at 4°C. For a positive control, intracellular proteins of *T. harzianum* grown for 48 h on SM supplemented with chitin as the sole carbon source were isolated. Mycelium was washed three times with 200 ml of phosphate-buffered saline, pH 7.3 (1), filtered through Whatman No. 1 filter paper, homogenized, and then sonicated (as before) and centrifuged for 30 min at 20,000 × *g* at 4°C. The supernatant was dialyzed, and proteins were kept at –20°C until use.

Protein concentration was determined according to Bradford (3) using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Richmond, CA) and bovine serum albumin as a protein standard.

Identification of enzymatic activities. Proteins were prepared in Laemmli buffer (20) without 2-mercaptoethanol. Samples were not boiled before loading. The denatured protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 1.5-mm gels with 4% acrylamide (stacking gel) and 10% acrylamide (separating gel) in a Mighty Small II protein electrophoresis cell (Hoefer Scientific Instruments, San Francisco). Enzymes were reactivated by removing SDS, following the casein/EDTA procedure (25) modified by Haran et al. (15). Chitinolytic enzymes were detected in situ using two highly sensitive substrates that produce a fluorescent product following enzymatic hydrolysis: 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MU-GlcNAc) (Sigma Chemical Co., St. Louis) and 4-methylumbelliferyl-β–D-*N,N'*-diacetylchitobioside (4-MU-$[GlcNAc]_2$) (Sigma Chemical Co.). These compounds function as dimeric and trimeric substrates, respectively, with the 4-methylumbelliferyl group linked by β-1-4 linkage to the GlcNAc oligosaccharides. Only 4-methylumbelliferone when hydrolyzed from the GlcNAc oligosaccharides is a fluorescent product. Enzyme activity was detected on gels using a modification of the procedure described by Tronsmo and Harman (30); an overlay gel of 1% agarose (low gel temperature; Bio-Rad Laboratories) containing 100 mM sodium acetate, pH 4.8, was mixed with both enzyme substrates (300 μg/ml of 4-MU-[GlcNAc] and 300 μg/ml of 4-MU-$[GlcNAc]_2$). After overlay, gels were incubated at 25°C until bands were evident under UV light (302 nm). This procedure enabled the simultaneous detection of all the chitinolytic activities of *T. harzianum* described in Haran et al. (15).

Purification of enzymes and preparation of antibodies. A β-1,4-*N*-acetylglucosaminidase of 73 kDa (CHIT 73), described by Haran et al. (15), was partially purified as follows: *T. harzianum* was grown for 2 days on liquid SM supplemented with chitin and no glucose. Protein extracts were prepared by concentrating the growth medium to recover the enzymatic activities. The growth medium was filtered through Whatman No. 1 filter paper, and 4 mM leupeptin (Sigma Chemical Co.) and 0.2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.) were added. The supernatant was dialyzed and concentrated in Micro-ProDiCon membranes (molecular weight cut-off: 25,000) (Spectrum Medical Industries, Inc., Houston) against distilled water at 4°C in a Micro-ProDiCon negative-pressure micro protein dialysis/concentrator, as described by the supplier (Spectrum Medical Industries, Inc.). Proteins were kept at –20°C until use. Since CHIT 73 activity was found to be heat-stable (15) and boiling improved its separation from other proteins, protein extracts were boiled for 3 min and separated in a Mighty Small II (10 cm long) protein electrophoresis cell (Hoefer Scientific Instruments) by 10% SDS-PAGE. The activity of CHIT 73 was reactivated by removing SDS, following the casein/EDTA procedure previously described, and detection was performed using 4-MU-GlcNAc as the substrate. CHIT 73 activity was visualized under UV light, verifying the exact position of the protein band. The portion of the gel containing CHIT 73 was removed using a clean scalpel, and the overlaying substrate-containing agarose was discarded. To separate the protein from the casein that became integrated in the gel during SDS removal, a second electrophoresis was performed. Gel slices containing CHIT 73 were incubated in Laemmli buffer (20) without 2-mercaptoethanol for 4 min at room temperature and placed on SDS-polyacrylamide gels for reelectrophoresis. After separation, proteins were stained with Coomassie brilliant blue G-250 as described by Neuhoff et al. (26). CHIT 73 appeared as a sharp band of the expected size, whereas the casein appeared as a band with an apparent molecular mass of about 30 kDa (data not shown).

An endochitinase of 33 kDa (CHIT 33) was purified from *T. harzianum* protein extracts as follows: culture filtrate was lyophilized to reduce volume (15- to 25-fold) and then dialyzed overnight at 4°C against 50 mM sodium acetate buffer (pH 5.5). The dialyzed enzyme solution was applied to an SP-Sepharose column (HiTrap SP; Pharmacia Biotechnology Inc., Uppsala, Sweden) previously equilibrated with 50 mM sodium acetate buffer (pH 4.4). The column was thoroughly washed using this buffer and then eluted with 1 M sodium chloride. CHIT 33 passed through the column and was separated from other contaminating proteins. CHIT 33-rich fractions were combined and applied to a Q-Sepharose column (HiTrap Q; Pharmacia Biotechnology Inc.) previously equilibrated with 50 mM sodium acetate buffer (pH 6.3). The column was thoroughly washed and then eluted with a continuous linear gradient of 200 ml of 50 mM sodium acetate buffer (pH 6.3) and 200 ml of the same buffer containing 80 mM sodium chloride at a flow rate of 60 ml h^{-1}. CHIT 33 was eluted at 20 mM sodium chloride. The fractions containing CHIT 33 were pooled, lyophilized, and electrophoresed on 10% SDS-PAGE (data not shown).

Antibodies against the purified *N*-acetylglucosaminidase (CHIT 73) and the endochitinase (CHIT 33) were prepared in separate rabbits (Biological Services, Weizmann Institute of Science, Rehovot, Israel) using standard techniques (16).

Immunological analysis. Proteins were prepared in Laemmli buffer (20) without 2-mercaptoethanol and were boiled for 3 min before loading. Protein extracts were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. Western blot analysis was performed as described by Ausubel et al. (1), using rabbit polyclonal antibodies raised against a 41-kDa endochitinase of *T. harzianum* strain P1, kindly provided by G. E. Harman (17); rabbit polyclonal antibodies were raised against a β-1,4-*N*-acetylglucosaminidase of 73 kDa (CHIT 73) or against a 33-kDa endochitinase (CHIT 33), both from *T. harzianum* strain TM (15). Detection was performed by enhanced chemiluminescence as described by the manufacturer (Amersham International, Buckinghamshire, United Kingdom).

All experiments were repeated at least three times. Representative results are shown.

RESULTS

Expression of chitinolytic activities. Proteins from *T. harzianum* and *R. solani* or *S. rolfsii* were obtained during their interactions in dual culture and were separated by SDS-PAGE. Chitinolytic activity was detected as described in Materials and Methods. Samples were prepared in Laemmli buffer (20) without 2-mercaptoethanol and were not boiled, since the reducing agent inactivated CHIT 73, CHIT 33, and CHIT 31 (S. Haran, H. Shickler, A. Oppenheim, and I. Chet, *unpublished data*), and boiling inactivated CHIT 102 and CHIT 52 (15). For the Western blot analyses, proteins were incubated in the same buffer as above, but samples were boiled before loading on the gels. Migration distances of CHIT 73, CHIT 42, CHIT 33, and CHIT 31 were not affected by the different heat treatments (15).

The Western blot experiments were performed using antibodies raised against *T. harzianum* chitinases. These antibodies enabled the specific identification of the *T. harzianum* activities and, in some cases, enhanced detection sensitivity.

Expression of chitinolytic enzymes during antagonistic interactions of *T. harzianum* and *R. solani*. Proteins from both *T. harzianum* and *R. solani* were obtained during their interaction in dual culture at 0 h (just before contact) and at 12, 24, and 48 h after contact.

Chitinolytic activities during antagonistic interactions of *T. harzianum* and *R. solani* are presented in Figure 1. Both fungi expressed constitutive chitinolytic activity that could be detected before contact. The constitutive chitinolytic activity of *R. solani* was associated with a band of approximately 115 kDa (lane 1), whereas that of *T. harzianum* corresponded to a 102-kDa band (CHIT 102) (lane 2). Note, that 12 h after contact, CHIT 102 activity was elevated and two other chitinolytic activities, reflected by bands at 52 kDa (CHIT 52) and 33 kDa (CHIT 33), were detected (lane 3). Twenty-four and 48 h after contact, the activity of the *N*-acetylglucosaminidase CHIT 102 and the activities of the endochitinases CHIT 52 and CHIT 33 were evident (lanes 4 and 5).

Western blot analyses were performed using, separately, polyclonal antibodies raised against *T. harzianum* endochitinases CHIT 42 and CHIT 33 and the *T. harzianum* *N*-acetylglucosaminidase CHIT 73. An additional lane that contained intracellular proteins of *T. harzianum* grown for 48 h on chitin as the sole carbon source (Fig. 2A and B) was used as a positive control for the analysis, since it exhibited all six of the chitinolytic activities of *T. harzianum* detected by the assay (15).

When the proteins were probed with anti-CHIT 42 antibodies (Fig. 2A), a positive reaction with CHIT 42 was revealed 24 h after contact between *T. harzianum* and *R. solani* (lane 4). CHIT 42 expression increased 48 h after contact (lane 5).

When the proteins were probed with anti-CHIT 33 antibodies (Fig. 2B), the protein was first detected 12 h after contact between the fungi (lane 3). Its expression increased considerably 24 and 48 h after contact (lanes 4 and 5, respectively). Both anti-CHIT 42 and anti-CHIT 33 antibodies showed some cross-reactivity with *R. solani* and *T. harzianum* proteins extracted before contact (Fig. 2A and B, lanes 1 and 2). However, no chitinolytic activity related to these proteins was detected (Fig. 1, lanes 1 and 2).

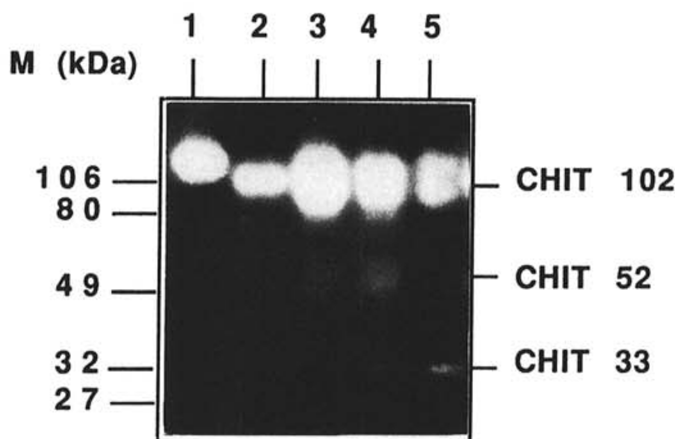

Fig. 1. Expression of chitinolytic activities obtained from dual cultures of *Trichoderma harzianum* and *Rhizoctonia solani* grown on synthetic medium supplemented with glucose (0.2%). A mixture of 4-methylumbelliferyl-*N*-acetyl-b-d-glucosaminide and 4-methylumbelliferyl-b-d-N,N′-diacetylchitobioside was used as a substrate. Detection was performed 20 min after incubation with the substrate. Lanes contained 100-µg proteins renatured following their separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). Lane 1, proteins produced by *R. solani* before coming into contact with *T. harzianum*; lane 2, proteins produced by *T. harzianum* before coming into contact with *R. solani*; and lanes 3, 4, and 5, proteins obtained from the interaction zone 12, 24, and 48 h after contact, respectively.

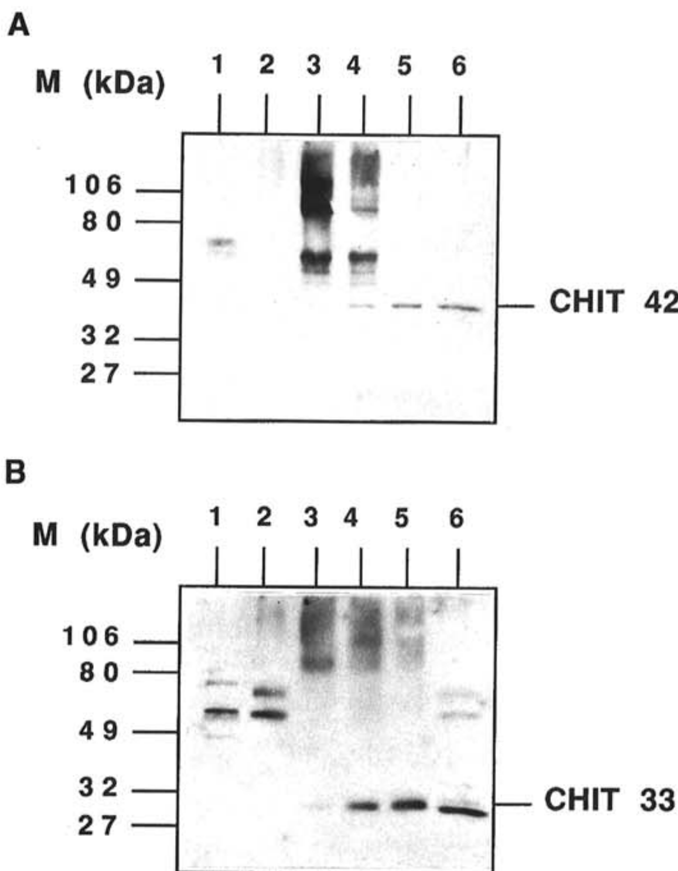

Fig. 2. Western blot analysis of chitinolytic activity during antagonistic interactions of *Trichoderma harzianum* and *Rhizoctonia solani*. **A,** Western blot analysis of electrophoretically separated proteins using rabbit polyclonal antibodies raised against CHIT 42 (a 42-kDa endochitinase from *T. harzianum*). Lanes contained 100-µg proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). Lane 1, proteins produced by *R. solani* before coming into contact with *T. harzianum*; lane 2, proteins produced by *T. harzianum* before coming into contact with *R. solani*; lanes 3, 4, and 5, proteins obtained from the interaction zone 12, 24, and 48 h after contact, respectively; and lane 6, proteins produced by *T. harzianum* grown for 48 h on synthetic medium supplemented with chitin as the sole carbon source. Lane 6 served as a positive control for the analysis, since it exhibited all the chitinolytic activities of *T. harzianum*. **B,** Western blot analysis of electrophoretically separated proteins using rabbit polyclonal antibodies raised against CHIT 33 (a 33-kDa endochitinase from *T. harzianum*). Lanes contained 100-µg proteins as in **A**. When the proteins were probed with antibodies against CHIT 73 (a 73-kDa *N*-acetylglucosaminidase of *T. harzianum*), no positive reaction was observed against CHIT 73 during the parasitic interaction between *T. harzianum* and *R. solani*. The only positive reaction was found in lane 6, which served as a positive control for the analysis (data not shown).

A similar assay performed with anti-CHIT 73 antibodies revealed no positive reaction against CHIT 73 during the parasitic interaction between *T. harzianum* and *R. solani* (data not shown).

The use of specific antibodies provided us with a more complete description of the changes occurring during mycoparasitism.

Expression of chitinolytic enzymes during antagonistic interactions of *T. harzianum* and *S. rolfsii*. Proteins from both *T. harzianum* and *S. rolfsii* were obtained from their dual culture at 0 h (just before contact) and at 6, 12, and 24 h after contact.

Chitinolytic activities detected during the antagonistic interaction are shown in Figure 3. Both fungi expressed constitutive chitinolytic activity that could be detected before contact. The constitutive chitinolytic activity of *S. rolfsii* was reflected in a band of approximately 112 kDa (lane 1), whereas that of *T. harzianum* was reflected by a 102-kDa band (CHIT 102) (lane 2). Six hours after contact, the activity of CHIT 102 was still evident (lane 3). Twelve and 24 h after contact, CHIT 102 activity diminished, whereas the activity of CHIT 73 was expressed at high levels (lanes 4 and 5).

These proteins were analyzed by Western blot using, separately, anti-CHIT 73 (Fig. 4), anti-CHIT 42, and anti-CHIT 33 antibodies. An additional lane that contained intracellular proteins of *T. harzianum* grown for 48 h on chitin as the sole carbon source (Fig. 4, lane 6) was used as a positive control for the analysis, since it exhibited all six of the chitinolytic activities of *T. harzianum* detected by the assay (15). This assay demonstrated the presence of CHIT 73 12 and 24 h after contact between *T. harzianum* and *S. rolfsii* (Fig. 4, lanes 4 and 5). The anti-CHIT 73 antibodies cross-reacted with proteins extracted from *S. rolfsii* before contact (Fig. 4, lane 1). The protein of about 112 kDa might represent the pathogens' chitinolytic activity shown in Figure 3 (lane 1). Cross-reactivity was also detected with proteins extracted 12 and 24 h after contact (Fig. 4, lanes 4 and 5). However, these proteins did not show any chitinolytic activity (Fig. 3, lanes 4 and 5). The presence of CHIT 42 and CHIT 33 was not detected during the antagonistic interactions of *T. harzianum* and *S. rolfsii* (data not shown).

The expression pattern detected with the Western blot analyses was the same as that of the chitinolytic activities shown in Figure 3.

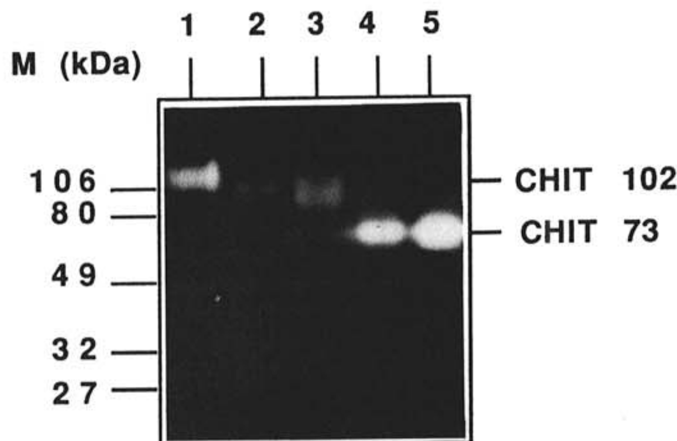

Fig. 3. Expression of chitinolytic activities obtained from dual cultures of *Trichoderma harzianum* and *Sclerotium rolfsii* grown on synthetic medium supplemented with glucose (0.2%), using a mixture of 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide and 4-methylumbelliferyl-β-D-*N*,*N*′-diacetylchitobioside as a substrate. Detection was performed 5 min after incubation with the substrate. Lanes contained 100-μg proteins renatured following their separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). Lane 1, proteins produced by *S. rolfsii* before coming into contact with *T. harzianum*; lane 2, proteins produced by *T. harzianum* before coming into contact with *S. rolfsii*; and lanes 3, 4, and 5, proteins obtained from the interaction zone at 6, 12, and 24 h after contact, respectively.

DISCUSSION

Direct mycoparasitic activity of fungi of the genus *Trichoderma* has been proposed as one of the major mechanisms involved in their antagonistic activity against phytopathogenic fungi (2,5,6). *Trichoderma* spp. attach to the host hyphae by coiling, hooks, or appressorium-like structures and penetrate the host cell walls by secreting lytic enzymes such as a basic proteinase (13), β-1,3-glucanases, and chitinases (10). Recent studies have reported the purification and characterization of chitinolytic enzymes produced by *T. harzianum* (8,15,17,24,31,33). The chitinolytic system of *T. harzianum* strain TM was found to be composed of six distinct enzymes (15): two β-1,4-*N*-acetylglucosaminidases (CHIT 102 and CHIT 73) and four endochitinases (CHIT 52, CHIT 42, CHIT 33, and CHIT 31). Our results show that each of the specific antibodies raised against CHIT 73, CHIT 42, and CHIT 33 specifically recognized the proteins and did not display cross-reaction with the other chitinolytic enzymes. These results may suggest that each protein is encoded by a different gene. When grown on glucose, only one of the β-1,4-*N*-acetylglucosaminidases, CHIT 102, was expressed. However, when the fungus was grown on chitin as the sole carbon source, all the chitinolytic enzymes were induced simultaneously (15).

The antifungal activity of chitinolytic enzymes has recently been studied by several authors. Lorito et al. (23) tested antifungal activity of purified endochitinase and chitobiosidase produced by *T. harzianum* strain P1. Inhibition of spore germination and germ-tube elongation were used as bioassays to evaluate the level of antifungal activity against different fungal species. Both processes were inhibited in all chitin-containing fungi tested, except *T. harzianum*. The degree of inhibition was found to be proportional to the level of chitin in the cell wall of the target fungus. Combining the activities of the endochitinase and chitobiosidase resulted in a synergistic increase in antifungal activity. The authors suggested that mixtures of hydrolytic enzymes with complemen-

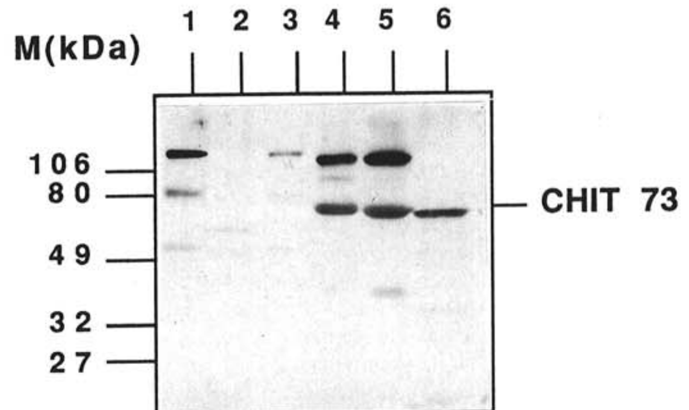

Fig. 4. Western blot analysis of chitinolytic activities during antagonistic interactions of *Trichoderma harzianum* and *Sclerotium rolfsii*. Western blot analysis of electrophoretically separated proteins using rabbit polyclonal antibodies raised against CHIT 73 (a 73-kDa *N*-acetylglucosaminidase of *T. harzianum*). Lanes contained 100-μg proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). Lane 1, proteins produced by *S. rolfsii* before coming into contact with *T. harzianum*; lane 2, proteins produced by *T. harzianum* before coming into contact with *S. rolfsii*; lanes 3, 4, and 5, proteins obtained from the interaction zone 6, 12, and 24 h after contact, respectively; and lane 6, proteins produced by *T. harzianum* grown for 48 h on synthetic medium supplemented with chitin as the sole carbon source. Lane 6 served as a positive control for the analysis, since it exhibited all the chitinolytic activities of *T. harzianum* (15). When these proteins were reacted, separately, with antibodies against CHIT 42 and CHIT 33 (42-kDa and 33-kDa endochitinases, respectively, of *T. harzianum*), no positive reaction was observed against CHIT 42 and CHIT 33 during the parasitic interaction between *T. harzianum* and *S. rolfsii*. The only positive reaction was found in lane 6, which served as a positive control for the analysis (data not shown).

tary modes of action may be required for maximum efficacy and that correct combinations of enzymes may increase in vitro antifungal activity. Lorito et al. (24) reported the purification of two additional cell-wall-degrading enzymes from *T. harzianum*: an *N*-acetyl-β-glucosaminidase and a β-1,3-glucanase (glucan 1,3-β-glucosidase). Using the above bioassays, they found a synergistic inhibitory effect on *Botrytis cinerea* spore germination and germ-tube elongation when two, three, or four enzymes were applied together. The highest level of antifungal activity was obtained when a solution containing all four cell-wall-degrading enzymes was used. Schirmböck et al. (28) demonstrated the parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics that were elicited in *T. harzianum* by cell walls of *B. cinerea.* They suggested that a cascade of antagonistic events might be regulated by a common mechanism. Changes in *Trichoderma* gene expression patterns, elicited by chitin, were investigated by Lora et al. (22). They speculated that oligosaccharides containing GlcNAc, which are generated by the partial degradation of fungal cell walls, act as elicitors that might trigger a general antifungal response in *Trichoderma.* However, Limón et al. (21) reported differences in gene expression of CHIT 42 and CHIT 33, suggesting independent regulation of each of these endochitinases.

All the studies mentioned previously on the expression and regulation of lytic enzymes were performed in liquid cultures supplemented with different carbon sources. Inbar and Chet (19) took a different approach by studying the induction of specific chitinases in *T. harzianum* during its parasitic interaction with *S. rolfsii.* They found that during the parasitic action towards *S. rolfsii,* two *N*-acetylglucosaminidases (CHIT 102 and CHIT 73) are expressed. These two chitinases were regulated differently. Before coming into contact with *S. rolfsii, T. harzianum* expressed low CHIT 102 constitutive activity, which greatly increased after contact. As the interaction proceeded, CHIT 102 activity diminished concomitantly with the appearance of CHIT 73, which was induced and expressed at high levels up to 48 h after contact.

Trichoderma spp. (especially *T. harzianum* and *T. viride*) exhibit considerable variability among strains with respect to their biocontrol activity and host range (29). An isolate of *T. harzianum,* strain TH 250, produced high levels of chitinase and β-1,3-glucanase when grown on mycelium of *R. solani,* whereas it produced only low levels of these enzymes when grown on mycelium of *S. rolfsii* (11). This isolate efficiently reduced disease incidence in bean seedlings caused by *R. solani.* However, it failed to protect these seedlings from the disease caused by *S. rolfsii.*

In the present work, we found that not only did the level of hydrolytic enzymes differ when *Trichoderma* attacked *R. solani* or *S. rolfsii,* but the expression of the various chitinases during the parasitic interaction was specifically affected by the host.

When *T. harzianum* was antagonizing *S. rolfsii* in dual culture, activity of the *N*-acetylglucosaminidase CHIT 102 was the first to be induced. As early as 12 h after contact, its activity diminished, and another *N*-acetylglucosaminidase, CHIT 73, was expressed. None of the *T. harzianum* endochitinases were detected during the parasitic interaction with *S. rolfsii.* However, when *T. harzianum* was antagonizing *R. solani,* the expression pattern of its chitinases differed considerably. The endochitinase activities of CHIT 52, CHIT 42, and CHIT 33, as well as the exotype *N*-acetylglucosaminidase activity of CHIT 102, were detected. The activity of CHIT 73, which was highly expressed during the parasitic action of *T. harzianum* towards *S. rolfsii,* was not detected during its parasitic action towards *R. solani.*

Our results show that the chitinolytic system of *T. harzianum* is not regulated by a simple "on/off" mechanism. We found that the host affects the regulation of specific chitinases during the mycoparasitic interaction and that the regulation of the various *N*-acetylglucosaminidases and endochitinases is a finely tuned process. In vitro studies using chitinolytic enzymes purified from *T. harzianum* have shown the direct antifungal effect of these enzymes, and synergism of this effect was reported when combinations of enzymes with complementary modes of action were used. The parasitic interaction with *R. solani* involved the expression of both the endochitinase activities and the exotype *N*-acetylglucosaminidase activity. However, during the mycoparasitic interaction with *S. rolfsii,* only the exotype activities of two *N*-acetylglucosaminidases were detected. Endochitinases may, therefore, play a minor role in the mycoparasitic activity of *T. harzianum* on *S. rolfsii.* We suggest that this differential expression of *T. harzianum* chitinases may influence the overall antagonistic ability of the fungus against a specific host.

The development of novel methods for detection of the temporal expression of fungal chitinases may now be applied for the study of additional parasitic interactions of *T. harzianum* with other important plant pathogens.

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