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Characterization of *ech-42*, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism

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ABSTRACT **A gene (*ech-42*; previously named *ThEn-42*) coding for one of the endochitinases produced by the biocontrol agent *Trichoderma harzianum* IMI206040 was cloned and characterized. Expression of the cDNA clone in *Escherichia coli* resulted in bacteria with chitinase activity. This chitinase has been shown to have lytic activity on *Botrytis cinerea* cell walls *in vitro*. The *ech-42* gene was assigned to a double chromosomal band (chromosome V or VI) upon electrophoretic separation and Southern analysis of the chromosomes. Primer extension analysis indicated that transcription of the gene begins preferentially 109 bp upstream of the translation initiation codon. Expression of *ech-42* was strongly enhanced during direct interaction of the mycoparasite with a phytopathogenic fungus when confronted *in vitro* and by growing it in minimal medium containing chitin as sole carbon source. Similarly, light-induced sporulation resulted in high levels of transcript, suggesting developmental regulation of the gene. The implications of these findings are discussed.**

The soil fungus *Trichoderma harzianum* has been shown to act as a mycoparasite against a range of economically important aerial and soil-borne plant pathogens, being successfully used in the field and greenhouse (1–3). Different factors involved in the antagonistic properties of *Trichoderma* have been identified, including antibiotics (4–8) and hydrolytic enzymes such as 1,3-β-glucanases, proteases, and chitinases (9).

The interaction between *Trichoderma* and its host is first detectable as chemotropic growth of hyphae of the mycoparasite toward its host (10, 11). When the mycoparasite reaches the host, its hyphae often coil around it or are attached to it by forming hook-like structures (12–16). After these interactions, the mycoparasite penetrates the host mycelium, apparently by partially degrading its cell wall (12, 13). Microscopic observations (6, 10, 13, 17–19) suggest that *Trichoderma* spp. produces and excretes mycolytic enzymes, as indicated by localized cell wall lysis at points of interaction. The susceptible host hyphae show rapid vacuolation, collapse, and disintegration (10). Thus, the mycoparasitic process involves (*i*) chemotropic growth of *Trichoderma*, (*ii*) recognition of the host by the mycoparasite and attachment, (*iii*) excretion of extracellular enzymes, (*iv*) hyphae penetration, and (*v*) lysis of the host.

Chitin and 1,3-β-glucan are the two major structural components of the cell wall of many plant pathogenic fungi. Therefore, it is expected that the 1,3-β-glucanases, chitinases, and proteases produced extracellularly by *Trichoderma* (18, 20–23) play an important role in biocontrol. Biological control of some soil-borne fungal diseases has been correlated with chitinase production (24). Bacteria producing chitinases and/or glucanases exhibit antagonism *in vitro* against fungi (25, 26). Inhibition of fungal growth by plant chitinases and dissolution of fungal cell walls by a streptomycete chitinase and a 1,3-β-glucanase have also been demonstrated (27, 28).

Purification and characterization of three chitinases (33, 37, and 42 kDa) from *T. harzianum* has been reported by De la Cruz and co-workers (29). The purified 42-kDa chitinase alone hydrolyzed *Botrytis cinerea* purified cell walls *in vitro*, but this effect was heightened in the presence of either of the other two chitinases (29).

In this paper, we describe the cloning and characterization of an endochitinase gene of *T. harzianum (ech-42)*, whose expression is strongly enhanced during mycoparasitism, as well as induced by chitin and light-induced sporulation.§

MATERIALS AND METHODS

Strains and Plasmids. *T. harzianum* strain IMI206040 was used throughout this work. *E. coli* JM103 (30) was used for DNA manipulation and generation of single-stranded DNA. Plasmids were pBluescript (Stratagene) and pSPORT (BRL) as well as pPCRII (Invitrogen) when cloning PCR-generated fragments.

cDNA Library. For construction of the cDNA library *T. harzianum* (IMI206040) was inoculated (10^6 conidia per ml) in minimal medium [MM; 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.9 g of K_2HPO_4, 0.2 g of KCl, 1.0 g of NH_4NO_3, 2 mg of $FeSO_4 \cdot 7H_2O$, 2 mg of $ZnSO_4 \cdot 7H_2O$, 2 mg of $MnCl_2 \cdot 7H_2O$, and 2.0 g of asparagine per liter (pH 5.5)] supplemented with 2% glucose. Mycelia were grown on a rotary shaker for 48 h at 28°C and collected by filtration, washed with sterile water, and transferred to MM-containing cell walls (0.2%) of the phytopathogenic fungus *Rhizoctonia solani* as sole carbon source (31). Incubation was continued for an additional 24 h; mycelia were recovered and frozen in liquid nitrogen. Total RNA was extracted according to Jones *et al.* (32). Poly(A)$^+$ RNA was isolated by the mRNA isolation system (BRL). cDNA synthesis, ligation into plasmid pSPORT, and transformation of *E. coli* DH5α were performed with the Super-Script plasmid system (BRL) following the manufacturer's instructions.

PCR Amplification. PCR was carried out on *T. harzianum* genomic DNA. The PCR cycle was as follows: 1 min at 95°C, 2 min at 50°C, and 2 min at 72°C for 30 rounds. Extension period was 7 min at 72°C. Primers were 5′-CTTGTAGTC-CCAAATACCGTTCTCCCA-3′ and 5′-GCAAACGCCGT-CTACTT CACCAACTGG-3′.

***ech-42* Expression.** *T. harzianum* was initially grown as described above (cDNA library) but for 12 h followed by harvesting and washing (50 mM NaOAc). Mycelia were re-

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Abbreviations: IPTG, isopropyl β-D-thiogalactoside; MUChT, 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotriose; MU, methylumbelliferone.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. X79381).

suspended in MM without glucose, grown for another 12 h, collected, and washed. Equal amounts of mycelia were transferred to MM supplemented with either 0.75% colloidal chitin or 2% glucose. Mycelia were harvested at 0, 12, 24, 48, and 72 h and washed with TES buffer (50 mM Tris·HCl/50 mM NaCl/5 mM EDTA, pH 8.0). Culture media were also collected, and both media and mycelia were frozen in liquid nitrogen.

Direct Confrontation Assays. Confrontation assays *in vitro* between *T. harzianum* and *R. solani* were established as follows. Agar plugs cut from growing colonies of each fungi were placed, on opposite sides, in 9-cm plates containing MM with 2% agar plus 0.3% glucose and covered with sterile cellophane sheets. Control plates were inoculated with only *R. solani* or *T. harzianum*. The fungi were allowed to grow at 28°C, and mycelia were collected before they touched each other (i1), when they were just touching (i2), and when the interaction zone was ≈1 cm wide (i3). At stages i1 and i2, only *Trichoderma* mycelia 0.5 cm away from the original inoculum and without spores were collected; at stage i3, all mycelia in the area of interaction were recovered. Equivalent zones were harvested from control plates. All plates were manipulated and grown in the dark. Additional *T. harzianum* plates were incubated in the light, and mycelia were collected at the same times.

DNA/RNA Manipulations. All DNA manipulations were performed by standard techniques (30) or according to the manufacturer's recommendations. DNA probes were labeled with a random primer kit (Boehringer Mannheim). Sequencing was carried out by the method of Sanger (33). Southern and Northern blots were performed according to standard procedures (30) under high-stringency conditions.

Chitinase Assays. Chitinase activity was determined in the supernatants of the *T. harzianum* cultures obtained as described (see *ech-42 Expression*) and in the crude extracts of *E. coli* DH5α containing the clones quiAc38 and quiAc41. *E. coli* were grown at 37°C in Luria–Bertani (LB) medium plus 50 μg of carbenicillin per ml until OD_{600} = 0.5; then isopropyl β-D-thiogalactoside (IPTG) was added to 1 mM (final concentration) and incubated for 3 h. Controls were grown without the inducer. Cells were collected by centrifugation, resuspended in chitinase reaction buffer (ChR buffer; 50 mM NaOAc/0.15 mg of phenylmethylsulfonyl fluoride per ml/10 mM EDTA, pH 5.0), disrupted by sonication, and centrifuged; the supernatants were recovered to determine activity. Either 10 or 100 ng of total protein was resuspended in 100 μl of ChR buffer. One microliter of 4-methylumbelliferyl β-D-*N*,*N*′,*N*″-triacetylchitotriose (MUChT) (Sigma) (2.5 mg/ml) was added; the reaction mixtures were incubated at 40°C for 10–20 min, stopped by adding 1.9 ml of 0.2 M Na_2CO_3, and kept at 4°C. Fluorescence was read in a TKO 100 fluorometer (Hoefer). Activity is expressed in pmol of methylumbelliferone (MU) per min per μg of protein.

Primer Extension. Primer-extension analysis of the *T. harzianum ech-42* gene was performed according to Goldman *et al.* (31) after the method of Ausubel *et al.* (34). The oligonucleotide used was 5′-CCGTGGCCCTGCTTGCT-GCGCTGC-3′, complementary to the *ech-42* mRNA between nt 23 and 46 downstream of the ATG (where A = +1).

Contour-Clamped Homogeneous Field Electrophoresis (CHEF) Gel Conditions. CHEF analysis was performed with the CHEF mapper (Bio-Rad) under the following conditions: 0.65% Megarose, 168-h running time (0.25× Tris borate/EDTA buffer; TBE), 1800- to 5400-s switching intervals with linear ramping, 120° constant angle, 1.3 V/cm, and 12°C constant temperature.

RESULTS

To isolate the *T. harzianum* chitinase genes, oligonucleotide primers that code for conserved amino acid sequences were designed based on a reported *T. harzianum* endochitinase gene (*ThEn-42*) sequence (35) and a comparison of a series of DNA sequences of bacterial and fungal origin available in the GenBank data base. PCR amplification was performed on genomic DNA generating two main products that were cloned and sequenced. One of them (900 bp) showed high homology to the endochitinase sequence reported (35) and to other chitinase sequences and was used as a probe to isolate both cDNA and genomic clones. Four cDNA clones were obtained from the cell wall-induced cDNA bank (see *Materials and Methods*); two (quiAc38 and quiAc41) were ≈1550 bp, differing slightly at the 5′ end.

Several independent clones were isolated from a genomic bank in λEMBL3, all containing the same 2.3-kb *Sal* I hybridizing fragment, which was sublconed into the *Sal* I site of pBluescript (Stratagene). Both cDNA and genomic clones

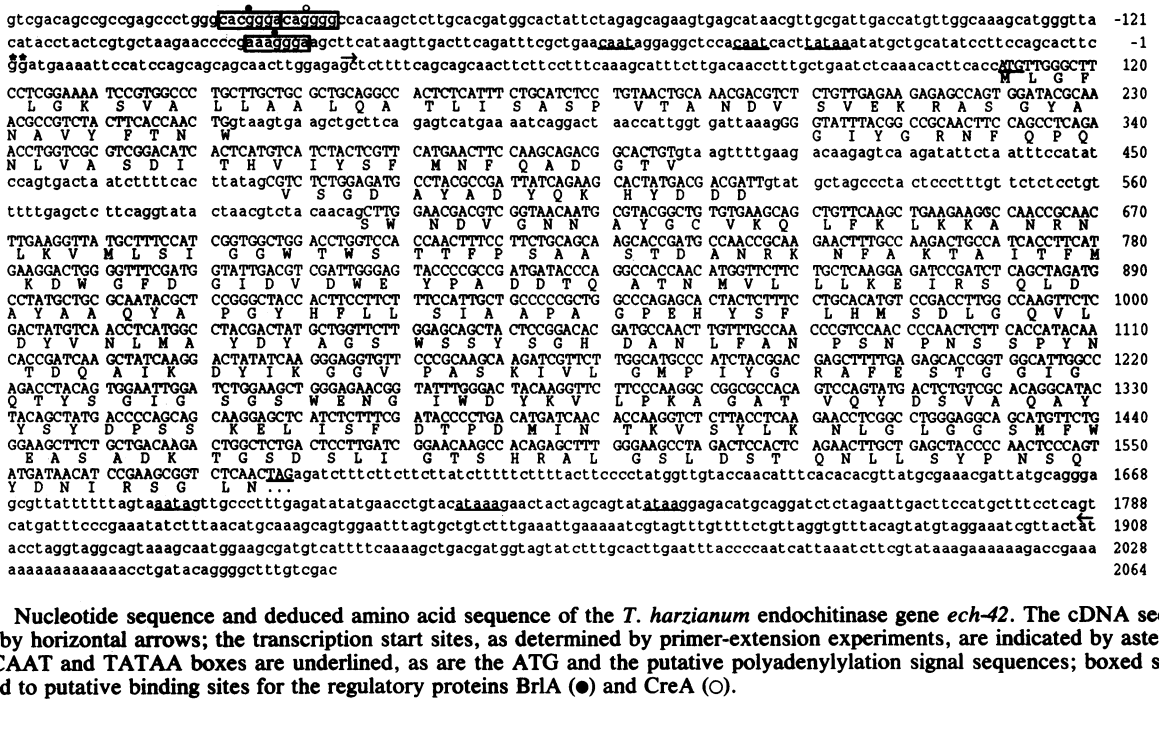

FIG. 1. Nucleotide sequence and deduced amino acid sequence of the *T. harzianum* endochitinase gene *ech-42*. The cDNA sequence is delimited by horizontal arrows; the transcription start sites, as determined by primer-extension experiments, are indicated by asterisks; the putative CAAT and TATAA boxes are underlined, as are the ATG and the putative polyadenylylation signal sequences; boxed sequences correspond to putative binding sites for the regulatory proteins BrlA (●) and CreA (○).

were completely sequenced and aligned (Fig. 1). Primer-extension analysis indicates that transcription of the gene begins preferentially at a G residue located 109 bp upstream from the translation initiation codon, with one alternative transcription initiation site also at a G residue but 110 bp upstream of the ATG (data not shown). Comparison of the genomic and cDNA sequences revealed the presence of three short introns (56, 69, and 70 bp), a 209-bp 3′ untranslated region, and a 240-bp promoter region where the TATA box and two CAAT boxes were found at the appropriate distances. Two other regulatory motifs were identified in the control region of the gene, one corresponding to two BrlA binding boxes, which have been proposed to modulate the expression of several light-induced genes involved in sporulation in *Aspergillus nidulans* (36), and the other to a CreA binding motif present in the 5′ regions of several genes regulated by catabolic repression (37, 38).

Computer alignment of the deduced amino acid sequence with other chitinase sequences was performed with the GENEWORKS program. When *ech-42* was compared with the previously reported sequence (*ThEn-42*; ref. 35), only 5 amino acids differed at positions 75, 76, 78, 121, and 137. A very high homology (73% identity) was also found between the *T. harzianum* chitinase (Ech-42) and Chi1, a chitinase produced by the fungus *Aphanocladium album* (39). In addition, regional homology with various domains of several fungal and bacterial chitinases was found (data not shown).

The vector (pSport1) used in the construction of the cDNA library allows IPTG-induced expression of the clones as β-galactosidase fusions. Sequencing of the two cDNA clones isolated indicated that insertion of quiAc41 had resulted in an in-frame fusion of the chitinase with β-galactosidase, whereas insertion of quiAc38 had not. Therefore, these two clones were used to verify that the cloned gene actually codes for a chitinase by performing chitinase activity assays. Cultures of *E. coli* carrying the clones quiAc38 and quiAc41 were grown and induced with IPTG; controls were noninduced. Chitinase activity was detected only in the protein extracts of induced bacteria carrying the quiAc41 clone (Table 1). No activity was detected in the noninduced cultures or in the one carrying the out-of-frame protein fusion. These data corroborate that *ech-42* encodes a chitinase.

Chromosomes of the *T. harzianum* strain IMI206040 were prepared as described (40) and separated by CHEF, resulting in five bands corresponding to the seven chromosomes identified previously (ref. 40; Fig. 2, lane A). In the Southern analysis the quiAc38 probe hybridized to a double chromosomal band where chromosomes V and VI comigrate (lane B). Southern blots of total *T. harzianum* DNA probed with quiAc38 under high-stringency conditions indicated that *ech-42* is present as a single copy gene (data not shown), confirming the results reported (35).

T. harzianum cultures were grown in MM with 0.75% chitin (Fig. 3, lanes Ch) or 2% glucose (lanes G) as sole carbon sources. Preliminary experiments showed that in cultures grown on 2% glucose chitinase activity is inhibited, so this condition was used as a control. Mycelia and culture media were collected after 12, 24, 48, or 72 h. A Northern assay with

Table 1. Chitinase activity in *E. coli*

	MU, pmol per min per μg of protein	
	− IPTG	+ IPTG
quiAc38	0.22	0.50
quiAc41	0.97	10.03

Total protein extracts from IPTG-induced (+) and noninduced (−) cultures were used for chitinase activity assays with a fluorogenic substrate (MUChT).

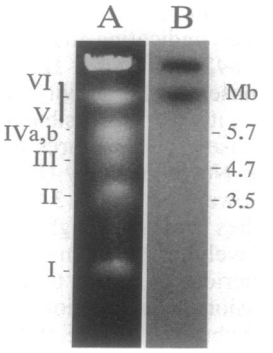

FIG. 2. Chromosomal localization of *ech-42*. Lane A, ethidium bromide staining of the *T. harzianum* chromosomal bands separated by CHEF. Roman numerals refer to the number of the chromosome as previously assigned (40). Lane B autoradiography of the Southern analysis performed on the chromosomal bands in lane A after hybridization with the ^{32}P-labeled *ech-42* cDNA clone. Size and position of migration of the *Schizosaccharomyces pombe* chromosomes used as molecular size standards are indicated.

total RNA of these samples was carried out, hybridizing with the cDNA clone quiAc38. When *Trichoderma* was grown on chitin, the *ech-42* mRNA was strongly expressed at 12 h, reaching a peak at 24 h, and diminishing after 48 h. With glucose, low-level expression was detected after 48 h (Fig. 3*B*). Chitinase activity was determined in the supernatants of the cultures and the activity detected in the chitin-induced cultures increased with time, reaching a maximum at 48 h

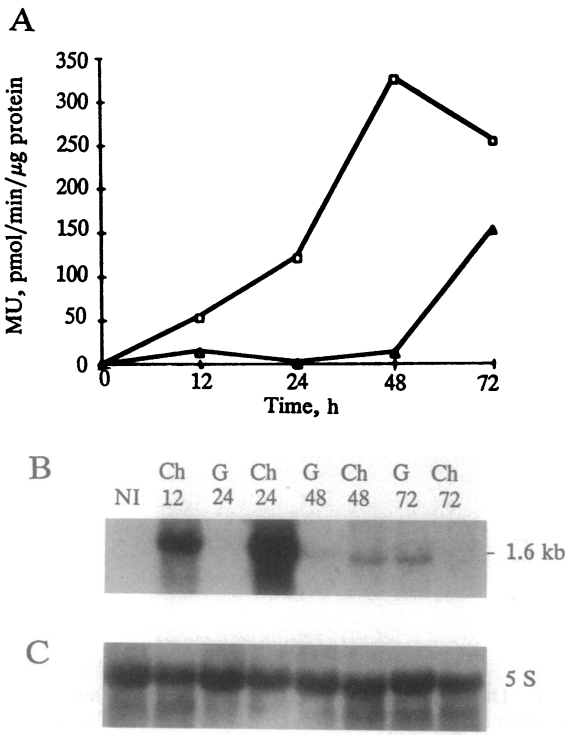

FIG. 3. Chitin induction of *ech-42*. *T. harzianum* was grown in MM with either chitin or glucose as sole carbon source. Culture filtrates and mycelia were collected at 12, 24, 48, and 72 h and analyzed. (*A*) Chitinase activity was measured by degradation of the fluorogenic substrate MUChT. Activity is expressed in pmol of MU per min per μg of protein. △, Glucose; □, chitin-grown cultures. (*B*) Fifteen micrograms of total RNA from each sample was subjected to Northern analysis, with the *ech-42* cDNA clone as probe. NI, noninduced culture; Ch, induced; G, glucose-grown culture. Size and position of migration of the *ech-42* messenger are indicated. (*C*) As in *B* except that a human 5S rRNA clone was used as a probe. Position of migration of the 5S rRNA is indicated.

(Fig. 3*A*). The kinetics of mRNA levels and chitinase activity follow similar curves, indicating that the activity detected could be due to *ech-42* expression. However, the contribution of other chitinases to the activity cannot be ruled out since in these assays it was not possible to distinguish them.

Although in glucose-grown cultures a faint band can be seen at 48 h in the Northern assay (Fig. 3*B*), and enzyme activity increases toward 72 h (Fig. 3*A*), neither mRNA nor activity ever reaches levels as high as in chitin-induced cultures. The low level of expression of *ech-42* and chitinase activity after long periods of growth on glucose could be due to glucose derepression, as no additional glucose was added. The same blot was hybridized to a 5S rRNA-specific probe in order to normalize the quantity of RNA per lane (Fig. 3*C*).

For analysis of *ech-42* expression during mycoparasitism, direct confrontation assays were established between *T. harzianum* and *R. solani*. The fungi were inoculated on opposite sides in Petri dishes and grown until different stages of interaction were reached. *T. harzianum* controls without *Rhizoctonia* were grown both in the dark and in the light. Mycelia were collected as indicated in Fig. 4*A*. Total RNA was extracted, and a Northern assay was carried out with these samples, using as probe the *ech-42* cDNA clone. The *ech-42* mRNA reached high levels in the presence of the pathogenic fungus, when the mycelia are in direct physical contact (Fig. 4*B*, lane i3). However, *ech-42* mRNA can be detected at all stages of the interaction as well as in control experiments, indicating a basal level of expression of the gene (Fig. 4*B*). These basal levels of *ech-42* mRNA may be due to the limiting amount of glucose used in these experiments, which would not be expected to cause repression. When *T. harzianum* was grown alone and exposed to light (Fig. 4*B*, lane L), *ech-42* was strongly expressed, but it was expressed only poorly when grown in the dark for the same time (lane c3). Hybridization of the same Northern blot with a 5S rRNA clone shows no significant quantity variations in any of the samples (Fig. 4*C*).

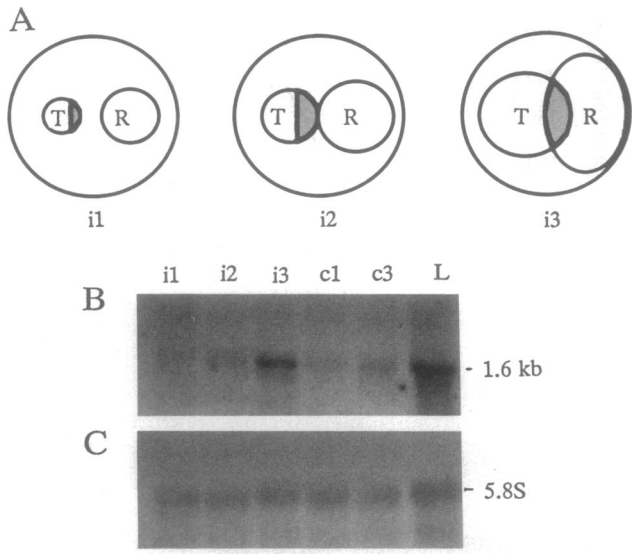

FIG. 4. Direct confrontation assays. *T. harzianum* and *R. solani* were confronted and mycelia were collected at different stages of the interaction. (*A*) Schematic representation of the three different stages at which the samples were taken (shaded areas). Outer circle represents the plate; two inner circles represent the colonies of *R. solani* (R) and *T. harzianum* (T). i1, Interaction before physical contact; i2, interaction at the first direct contact; i3, interaction when *Trichoderma* is overgrowing *Rhizoctonia*. (*B*) Northern analysis of the expression of *ech-42*. cDNA quiAc38 was used as probe. i1, i2, and i3 are as in *A*. RNA from control cultures of *T. harzianum* grown in the absence of *R. solani* are in lanes c1 and c3 (in the dark, equivalent to i1 and i3) and L (with light, equivalent to c1). Migration position of *ech-42* mRNA and its size are indicated. (*C*) As in *B* but with a human 5S rRNA as probe. Migration position of the rRNA is indicated.

DISCUSSION

The mycoparasites *Trichoderma* spp. have been successfully used in biological control of phytopathogenic fungi for >40 years. However, little is known about their mechanism of action, especially at the molecular level. The efforts made to understand this phenomenon have led to cloning of genes related to the mycoparasitic process (23).

In this work, we present data on the cloning of a gene (*ech-42*) that codes for an endochitinase. Comparison of the cDNA and genomic clones revealed that both cDNA clones isolated contain the complete coding sequence and most of the nontranslated message. The calculated size of the predicted product is 46 kDa, but the mature protein has been reported to be 42-kDa, with the N terminus beginning at Ala-36 (35). Computer analysis of the complete polypeptide suggests that the Ech-42 protein is synthesized as a preproenzyme, which undergoes a first processing step of a signal peptide-like sequence between amino acids 23 and 24. The second putative processing site is found between amino acids 34 and 35. This second processing step is most likely carried out by a KEX2-like endoproteinase activity at Lys-33/Arg-34. This type of processing has been shown in the *Aspergillus niger* glucoamylase (41) and has previously been suggested for the *T. harzianum* proteinase Prb1 as well as for the *A. album* chitinase Chi1 (23, 39). Thus, the mature secreted protein would have a calculated molecular mass of 42 kDa, corresponding to the only chitinase reported for *T. harzianum* with the ability to degrade *B. cinerea* cell walls by itself (29). Computer analysis of the deduced amino acid sequence of the Ech-42 protein indicated that this enzyme is related to bacterial chitinase and is highly homologous to a chitinase produced by the fungus *A. album* (73% identity), another effective biocontrol agent. Expression of the cDNA clone quiAc41 in *E. coli* confirmed that the gene codes for a chitinase with activity on MUChT (Table 1).

Southern analysis was performed with the electrophoretically separated chromosomes of *Trichoderma* indicating that the gene is located on either chromosome V or VI (Fig. 2). Interestingly, *prb1*, a *T. harzianum* gene previously reported to be related to mycoparasitism (23) has been assigned to the same chromosomal band (40). Whether these two genes and others related to mycoparasitism are arranged in a cluster remains to be investigated.

Northern analysis of total RNA from *T. harzianum* induced by growing in MM using chitin as sole carbon source showed high levels of *ech-42* messenger 12 h after induction. Expression was maximal at 24 h, decreased sharply by 48 h, and almost disappeared at 72 h (Fig. 3*B*). In contrast, cultures grown on glucose as carbon source had no detectable levels of *ech-42* mRNA at 12 and 24 h, while *ech-42* message could be detected at 48 h of growth, remaining constant until 72 h (Fig. 3*B*). The low levels of expression of *ech-42* in glucose-grown cultures could be explained by the lack of an inducer or the presence of a repressor. Our hypothesis is that both mechanisms could act at the same time. Thus, cultures grown for >24 h would have exhausted the glucose in the medium, leading to low levels of *ech-42* mRNA, although we cannot exclude the possibility of *ech-42* being switched on by aging. However, if our hypothesis is valid, glucose-exhausted cultures would never reach the level of expression of *ech-42* reached by chitin-induced cultures, as was indeed the case (Fig. 3*B*). Furthermore, cultures grown with a combination of chitin and glucose as carbon source had only basal levels of chitinase activity (data not shown) comparable to those reported here for the 12- and 24-h glucose samples (Fig. 3*A*). These data correlate nicely with the fact that levels of

chitinase activity detected at 12 h in the supernatants of cultures grown on chitin were ≈10-fold higher than the levels detected in cultures grown on glucose, and this ratio was at least 100-fold after 24 h of induction. The fact that chitinase activity in the induced cultures reaches its maximum 24 h later than the maximum observed for the *ech-42* message (Fig. 3 *A* and *B*) should be studied in more detail, since the long periods between taking samples could be masking a closer correlation between mRNA levels and activity. It is important to remember, however, that the chitinase activity observed is most probably the product of more than one type of chitinase, while the band seen in the Northern analysis corresponds only to the chitinase encoded by *ech-42*. The relatively high levels of *ech-42* messenger detected in the glucose cultures at 72 h also correlate with activity (Fig. 3*A*).

Direct confrontation assays were designed to investigate the pattern of expression of *ech-42* when *T. harzianum* was interacting with a phytopathogen (*R. solani*). Northern analysis showed that the *ech-42*-mRNA can be detected at low levels in cultures of *Trichoderma* grown in MM with limiting amounts of glucose at early stages of the assay in both the presence and the absence of *Rhizoctonia* (Fig. 4*B*, lanes i1, i2, and c1). A strong induction of the expression of *ech-42* was detected later in the interaction when *Trichoderma* was overgrowing *Rhizoctonia*. This expression was clearly distinguishable from the level of expression reached at the same time by controls where *T. harzianum* was grown alone (compare lanes i3 and c3 of Fig. 4*B*). Cultures exposed to light also showed a strong level of expression of *ech-42* (Fig. 4*B*, lane L) when compared to cultures of the same age kept in the dark (lane c1). Because the cultures exposed to light were sporulating, it is difficult to determine whether it is light, sporulation, or light-induced sporulation that induces *ech-42* expression. However, it is important to note that a perfect BrlA binding box was found in the promoter region of *ech-42* and that BrlA is a regulatory protein controlling light-induced sporulation in *A. nidulans* (36).

From these data, it is evident that *ech-42* expression is regulated in a complex way. Several signals appear to influence its expression: it is highly expressed during mycoparasitism and it is induced by chitin and by light and/or sporulation. In addition, the gene is possibly repressed by glucose.

Further experiments are necessary to fully determine the importance of *ech-42* in mycoparasitism as well as to discern whether its expression is indeed under glucose repression and how light, sporulation, or light-induced sporulation regulates it.

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