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Synthesis of a *Trichoderma* Chitinase Which Affects the *Sclerotium rolfsii* and *Rhizoctonia solani* Cell Walls

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ABSTRACT. A *Trichoderma* sp. isolate, hereafter called T_6, produces a 46-kDa endochitinase (CHIT 46) which had been shown to drastically affect *in vitro* the cell walls of the phytopathogens *Sclerotium rolfsii* and *Rhizoctonia solani*. We attempted to gain insight into its properties. The CHIT 46 N-terminal amino acid sequence shares a very high homology with other fungal chitinases. Western blot analysis using polyclonal antibodies anti-CHIT 46 revealed that this enzyme is immunologically distinct from other proteins produced by the same *Trichoderma* isolate T_6, but is immunologically identical with proteins having equivalent molar mass, probably chitinases, produced by other *Trichoderma* spp. isolates. In addition, the antibodies revealed also that a substantial amount of this enzyme is secreted into the culture medium 2 d after the *Trichoderma* isolate T_6 comes into contact with chitin.

Considerable attention has been focused over the past 20 years on the isolation of fungal antagonist that could be as effective as a pesticide in repressing fungal pathogens (Benhamou and Chet 1996). The proposed mechanisms resulting in biocontrol are competition for substrate (Sivan and Chet 1989), ability to colonize the niche favored by the pathogen, antagonism by antibiotics (Ghisalberti *et al.* 1990; Schirmböck *et al.* 1994) and action of cell-wall-degrading enzymes (Singh and Faull 1990). Indirect evidence suggested that *T. harzianum* antagonizes first and foremost by antibiosis leading to cell death, followed by degradation of the cell wall by chitinolytic enzymes (Belanger *et al.* 1995).

The chitinolytic enzyme activity induced by chitin in *T. harzianum* comprises at least seven enzymes identified as four endochitinases (CHIT 52, CHIT 42, CHIT 33 and CHIT 31), two N-acetylglucosaminidases (CHIT 102 and CHIT 73) and one exochitinase (CHIT 40) (Haran *et al.* 1996). We have recently demonstrated that the *Trichoderma* sp. isolate T_6 produces a 46-kDa chitinase, hereafter called CHIT 46, which disrupts the cell wall of the phytopathogen *Sclerotium rolfsii in vitro* (Lima *et al.* 1997). In agreement with this result is the fact that purified endochitinases and exochitinases (chitobiosidase) from *T. harzianum* strain P1 inhibited spore germination and germ-tube elongation of different fungal species (Lorito *et al.* 1993). Fungal chitinolytic enzymes and/or genes encoding them may, therefore, be useful for the production of transgenic microorganisms with superior biocontrol capability and the development of transgenic plants with high resistance to plant-pathogenic fungi (Lorito *et al.* 1993). Here we report on the synthesis of the CHIT 46 by the *Trichoderma* sp. isolate T_6 and several other *Trichoderma* isolates, and on the homology of its N-terminal amino acid sequence with other chitinases.

MATERIALS AND METHODS

Microorganisms, enzyme production, purification and sequencing. T. harzianum 39.1, hereafter called Tc, was obtained from the collection of the *Microbial Genetics and Biochemistry Group of the University of Nottingham* (UK). The other *Trichoderma* isolates 13/523×609 w5, 2/523×609 w5, CNP 17, 29/523×609 w5, T_{25}-Sami, 8/523×609 w5, hereafter called T_2, T_3, T_4, T_5, T_6, and T_7, respectively, and *Metarhizium anisopliae* were obtained from the collection of the *Centro Nacional de Pesquisas de Monitoramento e Avaliação de Impacto Ambiental* (EMBRAPA/CNPMA, Brasil). All microorganisms were maintained on agar medium. Enzyme production and assays, purification of the CHIT 46 chitinase produced by the *Trichoderma* sp. isolate T_6 and determination of its N-terminal amino acid sequence were reported earlier (Lima *et al.* 1997). Comparison of the N-terminal amino acid sequence of the CHIT 46 with the complete sequences of other chitinases available in the Swiss-Prot databank was done using the BLAST algorithm (Altschul *et al.* 1990).

Anti-CHIT 46 polyclonal antibody production. Three doses (30 μg each) of purified CHIT 46 were injected intraperitoneally at 15-d intervals. The first injection was given with complete Freund's adjuvant, the second with incomplete Freund's adjuvant and the third without any adjuvant. The animals were bled 45 d after the first injection and the sera were collected by centrifugation after blood clotting.

SDS-PAGE and Western-blot protein analysis. Electrophoresis in SDS-PAGE was done under denaturing conditions (Laemmli 1970) in a Mini Gel System (*Sigma*, St. Louis, MO, USA). After electrophoresis the gels were silver-stained according to Blum *et al.* (1987), or used to transfer the proteins to a nitrocellulose sheet using a LKB Multifor II System (*Pharmacia*). For immunodetection of the proteins, the nitrocellulose filters were blocked for 3 h at room temperature in TBS buffer (25 mmol/L Tris-HCl, pH 7.5 plus 150 mmol/L NaCl) containing 5 % (*W/V*) skimmed milk. Anti-CHIT 46 antiserum (1:200) was added and the mixture was incubated with shaking overnight at 4 °C. The filters were then washed with Tris–NaCl and incubated at 4 °C for 3 h with peroxidase-conjugated anti-mouse IgG diluted 1:500 in 2 % (*W/V*) skimmed milk. After washing five times with PBS buffer, the membranes were incubated with naphthol color-developing reagent according to the manufacturer (*Pierce*, Rockford, IL, USA) instructions.

RESULTS AND DISCUSSION

The CHIT 46 is an endochitinase produced by the *Trichoderma* sp. isolate T_6 growing in liquid medium containing chitin as inducer. Previous biochemical and scanning-electron-microscopic studies convincingly showed that this enzyme can drastically affect the *S. rolfsii* cell wall (Lima *et al.* 1997). These results further support the participation of hydrolytic enzymes on the antagonistic properties of *Trichoderma* species against phytopathogenic fungi.

The N-terminal amino acid sequence of the CHIT 46 showed 96, 96, 89 and 88 % homology, respectively, with the amino acid sequence of the chitinase from *T. harzianum* P1, *T. harzianum* IMI 206040, *T. harzianum* CECT 2413, and *Aphanocladium album* ETH M 483. However, no homology at all was shared by the CHIT 46 and the *T. harzianum* CECT 2413 33-kDa chitinase (Table I). Interesting, according to Limón *et al.* (1995) there is a single copy of *chit33* in the genome of *T. harzianum*, and no other genes showing more than 80 % of similarity to *chit33* are present. In contrast to the mechanisms controlling expression of *T. harzianum* CECT 2413 *chit33* gene, the *chit42* gene encoding *T. harzianum* CECT 2413 42-kDa chitinase (Garcia *et al.* 1994) is weakly derepressed indicating the existence of independently regulated chitinase genes in *T. harzianum* CECT 2413.

Table I. Comparison of the N-terminal amino acid sequences of CHIT 46 and chitinases from other *Trichoderma harzianum* strains and *Aphanocladium album*

Fungi	Chitinase *M*, kDa	N-Terminal	Homology %	References
T. harzianum P1	42	ASGYANAVYF^{10}TNWGIYGRNF^{20}QPQNLVAS	96	Hayes *et al.* 1994
T. harzianum IMI 206040	42	ASGYANAVYF^{10}TNWGIYGRNF^{20}QPQNLVAS	96	Carsolio *et al.* 1994
T. harzianum CECT 2413	42	ANGYANSVYF^{10}TNWGIYDRNF^{20}QPADLVAS	89	Garcia *et al.* 1994
T. harzianum CECT 2413	33	LTALASLLAL^{10}VPSALAGWNV^{20}NSKQNIAV	0	Limón *et al.* 1995
Trichoderma sp. T25/(T_6)	46	ASGYTNAVYF^{10}TNWGIYGRNF^{20}QPQDLVAS	100	Lima *et al.* 1997
A. album ETH M 483	39	GSGFANAVYF^{10}TNWGIYGRNF^{20}QPADLPAS	88	Kunz *et al.* 1992

Polyclonal antibodies raised in mice against the purified CHIT 46 reacted specifically with the isolated enzyme (*not shown*) and with the enzyme present in the culture fluid transferred from SDS-PAGE to a nitrocellulose membrane (Fig. 1). No cross-reaction (Fig. 1 *bottom*, lane F) with another protein present in the culture fluid (Fig. 1 *top*) was observed, indicating that *Trichoderma* CHIT 46 is encoded by a specific gene and probably no other posttranslationally modified variants of this chitinase are produced by this isolate. Antibodies raised against the CHIT 42, CHIT 37 and CHIT 33 were also specific and did not immunologically cross-react (De La Cruz *et al.* 1992). Nevertheless, in addition to the fact that it shares a very high homology with other chitinases (Table I), the CHIT 46 is antigenically

similar to several chitinases produced by other *Trichoderma* isolates and also by fungi belonging to other genera as revealed by Western-blot analysis (Fig. 1 *bottom*).

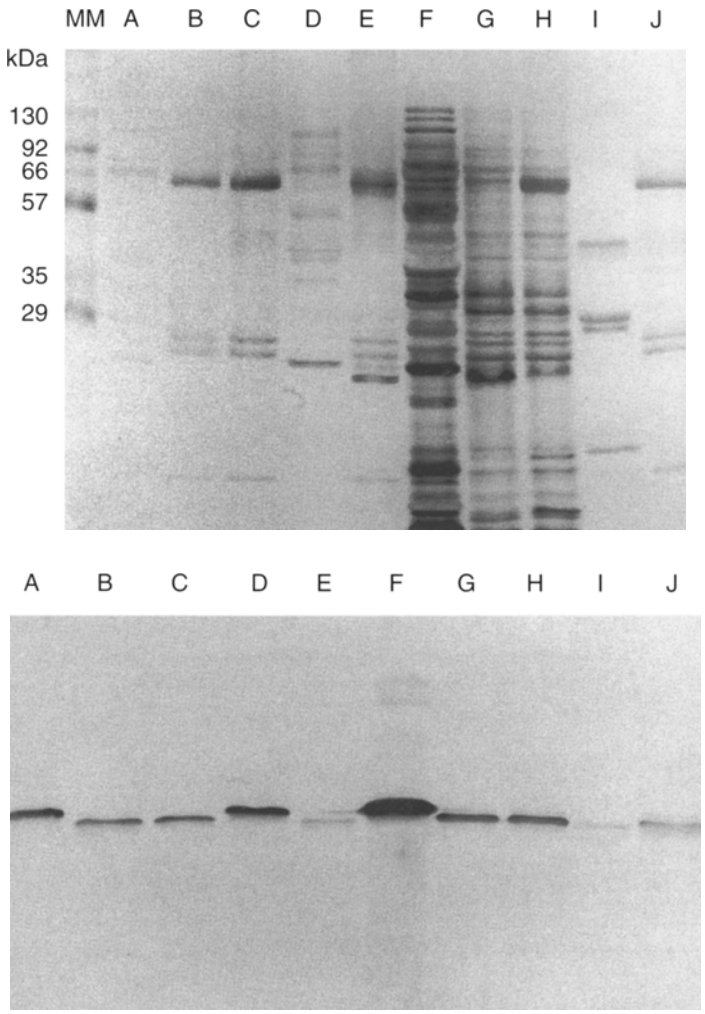

Fig. 1. SDS-PAGE (*top*) and Western blot (*bottom*) analysis of the proteins present in the culture supernatant of *Trichoderma* isolates Tc (A), T_2 (B), T_3 (C), T_4 (D), T_5 (E), T_6 (F), JM_{28} (G), JM_{29} (H), T_7 (I) and *Metarhizium anisopliae* (J) grown in liquid medium containing chitin. Molar mass standards (MM). Samples of culture medium containing about 100 µg proteins were precipitated with 5 % TCA, resuspended in sample buffer and applied to the gels. Electrophoresis was run at room temperature.

The time course of synthesis of CHIT 46 by *Trichoderma* isolate T_6 in the presence of chitin was studied using polyclonal antibodies. Several proteins were secreted as early as 12 h after the *Trichoderma* T_6 mycelium was transferred to chitin-containing medium as revealed by staining of the proteins with silver (Fig. 2 *top*). Nevertheless, the polyclonal antibodies did not react with any protein present in the culture fluid collected before 2 d of growth but did recognize the CHIT 46 in the 2-d-old or older cultures (Fig. 2 *bottom*). Previous studies showed that upon contact with its host, *T. harzianum* mycelium coils around or grows along the host hyphae and forms hook-like structures that aid in penetrating the host's cell wall (Elad *et al.* 1983). In *T. harzianum* strain T-Y, this reaction has been found to be specific and lectin–saccharide interactions were assumed to mediate this recognition and attachment (Haran *et al.* 1996). Induction of CHIT 102 in *T. harzianum* was shown to be an early event elicited by a recognition signal (*i.e.* lectin–saccharide interaction). In a dual culture of *T. harzianum* and *S. rolfsii*, CHIT 102 activity was the first to be induced, but as the interaction proceeded the activity of the CHIT 102 diminished simultaneously with the appearance of CHIT 73 (Haran *et al.* 1996). Inter-

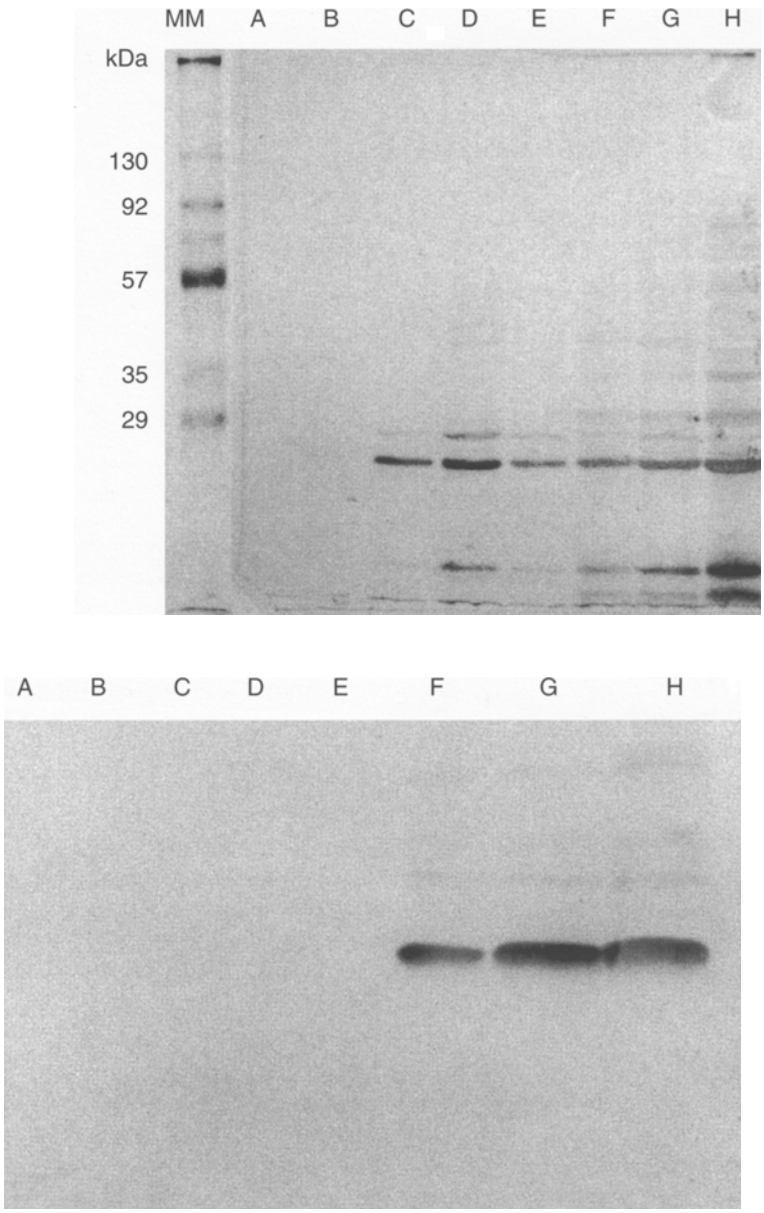

Fig. 2. SDS-PAGE (*top*) and Western blot analysis (*bottom*) of the proteins present in the culture supernatant of *Trichoderma* sp. isolate T_6 growing in chitin-containing medium for zero (A), 6 h (B), 12 h (C), 24 h (D) 36 h (E), 48 h (F), 60 h (G) and 72 h (H). Molar mass standards (MM). Samples of about 100 µg proteins were applied to the gel. See legend to Fig. 1.

action of *T. harzianum* strain IMI 206040 with *R. solani* resulted in a high expression of CHIT 42 (Carsolio *et al.* 1994). A 40-kDa chitobiosidase, a 41-kDa endochitinase and a 1,3-β-glucanase were produced by *T. harzianum* strain ATCC 36042 growing on cells of *B. cinerea* (Bélanger *et al.* 1995), but not on glucose (Schirmböck *et al.* 1994). Oligosaccharides containing GlcNAc, which are generated by partial degradation of fungal cell walls, are speculated to act as an elicitor which might trigger a general antifungal response in *T. harzianum* (Lora *et al.* 1994). As the CHIT 46 appeared in the culture fluid only 2 d after the beginning of cultivation (Fig. 2 *bottom*) it is clear that synthesis of this enzyme is a late induced event. Indeed, substantial amounts of chitinase activity are only found in the culture medium 2 d after *Trichoderma* T_6 starts to grow on chitin (Lima *et al.* 1997). Its is, therefore, likely that in nature the CHIT 46 acts as a phytophatogen cell-wall-degrading factor following recognition and interaction of *Trichoderma* with the phytophatogen, and enzyme induction.

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