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REVIEW ARTICLE

Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*

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Overview

The many achievements of modern agriculture notwithstanding, certain cultural practices have actually enhanced the destructive potential of crop diseases caused by fungi. These practices include the use of genetically uniform crop plants in continuous monoculture, the use of plant cultivars susceptible to pathogens, and the use of nitrogenous fertilizers at concentrations that increase disease susceptibility. Plant-disease control has thus become heavily dependent on fungicides to combat the wide variety of fungal diseases that threaten agricultural crops (Waard *et al.*, 1993). Studies aimed at replacing pesticides with environmentally safer methods are currently being conducted at many research centres. Biological control is a potent means of reducing the damage caused by plant pathogens and is environmentally nonhazardous. Although commercialized systems for the biological control of plant diseases are few, intensive activity is currently being geared towards the development of an increasing number of biocontrol agents.

Potential agents for biocontrol activity are rhizosphere-compatible fungi and bacteria which exhibit antagonistic activity towards plant pathogens. Before biocontrol can become an important component of plant-disease management, it must be effective, reliable, consistent and economical. To meet these criteria, superior strains exhibiting improved biocontrol activity as well as an expanded host range must become available (Goldman *et al.*, 1994; Harman *et al.*, 1989). This goal requires extensive study of the molecular and cellular biology of the antagonistic interactions between the biocontrol agent and the phytopathogenic fungi. The acquired knowledge can be used for genetic manipulations to improve existing biocontrol agents.

Trichoderma spp. are common fungi, found in almost any soil. Members of this genus are antagonistic to other fungi, including plant-pathogenic species. Possible mechanisms involved in *Trichoderma* antagonism are: (a) antibiosis, whereby the fungi produce volatile or non-volatile antibiotics (Dennis & Webster, 1971a, b); (b) competition, when space or nutrients (i.e. carbon, nitrogen, microelements) are limiting factors (Schippers *et al.*, 1987; Weller, 1988); (c) mycoparasitism, whereby *Trichoderma* attacks another fungus by excreting lytic enzymes (such as proteases, glucanases and chitinases) that enable it to degrade the latter's cell walls and utilize its nutrients (Chet, 1990; Cook & Baker, 1983; Elad *et al.*, 1982; Geremia *et al.*, 1993; Ridout *et al.*, 1988). Parasitism by *Trichoderma* spp. is destructive, causing the death of the host fungus (Barnett & Binder, 1973). The cell-wall-degrading enzymes are induced in *Trichoderma* during the parasitic interaction (Cherif & Benhamou, 1990; Elad *et al.*, 1983; Goldman *et al.*, 1992; Limón *et al.*, 1995; Lora *et al.*, 1994), and have been shown to have a direct antifungal effect (Lorito *et al.*, 1993). Via these mechanisms, *Trichoderma* antagonizes other fungi, thereby serving as a potential biological control agent of plant diseases (Baker, 1987; Chet, 1987, 1990).

Hydrolytic enzymes of *Trichoderma harzianum* involved in mycoparasitism

Chitinases

Chitin, an unbranched homopolymer of 1,4-β-linked *N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant polymer in nature, after cellulose. It does not occur in plants, vertebrates or prokaryotes, but is abundant as a structural polymer in most fungi and insects, including those that are agricultural pests (Havukkala, 1991). Chitinases are chitin-degrading enzymes, and their role in biological control and plant-defence mechanisms is now under extensive study (Broglie *et al.*, 1991; Jach *et al.*, 1995; Melcher *et al.*, 1994; Schlumbaum *et al.*, 1986; Shapira *et al.*, 1989).

A considerable amount of recent research has been aimed

Table 1. Summary of *T. harzianum* chitinolytic activities induced by chitin

Designation	Apparent mol. mass (kDa)	Activity	Strain	Reference
CHIT 102	102–118	*N*-Acetylglucosaminidase	TM	Haran *et al.* (1995)
			39.1	Ulhoa & Peberdy (1991)
CHIT 73	73	*N*-Acetylglucosaminidase	TM	Haran *et al.* (1995)
			P1	Lorito *et al.* (1994)
CHIT 52	52	Endochitinase	TM	Haran *et al.* (1995)
CHIT 42	40–42	Endochitinase	CECT 2413	De La Cruz *et al.* (1992)
			39.1	Ulhoa & Peberdy (1992)
			P1	Harman *et al.* (1993)
CHIT 40	40	Exochitinase (chitobiosidase)	P1	Harman *et al.* (1993)
CHIT 33	33–37	Endochitinase	CECT 2413	De La Cruz *et al.* (1992)
			TM	Haran *et al.* (1995)
CHIT 31	31–33	Endochitinase	CECT 2413	De La Cruz *et al.* (1992)
			TM	Haran *et al.* (1995)

at elucidating the chitinolytic system of *Trichoderma* spp. Most of these studies have been performed with *T. harzianum*, an effective biocontrol agent of several economically important plant-pathogenic fungi. *T. harzianum* attacks pathogens by excreting lytic enzymes, including glucanases, chitinases, proteases and lipases, which enable it to degrade host cell walls and thus reduce disease incidence (Chet *et al.*, 1993; Elad *et al.*, 1982; Lorito *et al.*, 1993).

Chitinolytic enzymes were divided by Sahai & Manocha (1993) into three principal types: (a) 1,4-β-*N*-acetylglucosaminidases (EC 3.2.1.30), which split the chitin polymer into GlcNAc monomers in an exo-type fashion; (b) endochitinases (EC 3.2.1.14), which cleave randomly at internal sites over the entire length of the chitin microfibril; and (c) exochitinases (EC 3.2.1.14), which catalyse the progressive release of diacetylchitobiose in a stepwise fashion such that no monosaccharides or oligosaccharides are formed. Harman *et al.* (1993) termed an enzyme exhibiting this activity 'chitobiosidase'.

1,4-β-N-Acetylglucosaminidases. Two 1,4-β-*N*-acetylglucosaminidases have been reported to be excreted by *T. harzianum*. Ulhoa & Peberdy (1991) described the purification of one of these from *T. harzianum* strain 39.1. They estimated its native molecular mass to be 118 kDa by gel filtration, whereas by SDS-PAGE this value was 66 kDa. They therefore suggested that the active form was a homodimer. Haran *et al.* (1995) reported an *N*-acetylglucosaminidase of 102 kDa (CHIT 102) which was expressed by *T. harzianum* strain TM when grown on chitin as the sole carbon source. They assumed it to be essentially the same enzyme described by Ulhoa & Peberdy (1991) and the different estimates of its molecular mass were suggested to be the result of the different procedures used.

The other 1,4-β-*N*-acetylglucosaminidase, purified from *T. harzianum* strain P1, was estimated to be of 72 kDa and had a pI of 4·6 (Lorito *et al.*, 1994). Haran *et al.* (1995) reported a 73 kDa glucosaminidase (CHIT 73) that was expressed and excreted when *T. harzianum* strain TM was grown on chitin as the sole carbon source, but was not detected when the fungus was grown on glucose. The activity of CHIT 73 was found to be heat-stable.

Endochitinases. Four endochitinases have been reported to be expressed by *T. harzianum*. Haran *et al.* (1995) reported an endochitinase of 52 kDa (CHIT 52). The enzyme was excreted into the growth medium when *T. harzianum* (strain TM) was grown on chitin as the sole carbon source, and it was highly sensitive to heat treatment.

An endochitinase of 42 kDa (CHIT 42) was reported by De La Cruz *et al.* (1992), Haran *et al.* (1995) and Harman *et al.* (1993). The pI value of the endochitinase purified from *T. harzianum* strain P1 was approximately 3·9 and showed optimum activity at pH 4·0 (Harman *et al.*, 1993). The pI value of CHIT 42 purified from *T. harzianum* strain CECT 2413 was 6·2 (De La Cruz *et al.*, 1992). Ulhoa & Peberdy (1992) reported a similiar endochitinase of 40 kDa from *T. harzianum* strain 39.1, which exhibited optimum activity at pH 4·0.

Two endochitinases, estimated at 37 kDa and 33 kDa, were expressed by *T. harzianum* strain CECT 2413 when grown on chitin as the sole carbon source. Their pI values were 4·6 and 7·8, respectively (De La Cruz *et al.*, 1992). Haran *et al.* (1995) detected two similiar endochitinases of 33 kDa (CHIT 33) and 31 kDa (CHIT 31), expressed by *T. harzianum* strain TM.

Exochitinases (chitobiosidases). A chitobiosidase of 40 kDa (CHIT 40) was reported to be secreted by *T. harzianum* strain P1 when grown on crab-shell chitin as the sole carbon source. This enzyme was glycosylated, and had a pI value of approximately 3·9 (Harman *et al.*, 1993). A summary of the chitinolytic activities of *T. harzianum* is presented in Table 1.

The chitinolytic enzymes were induced and excreted during growth of *T. harzianum* strain TM on liquid

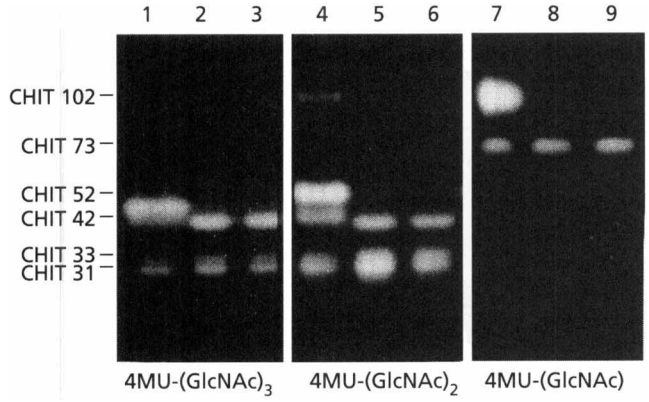

Fig. 1. Detection of extracellular chitinolytic activity of proteins produced by *T. harzianum* strain TM when grown on chitin as the sole carbon source. Lanes 1–9 contain 20 μg extracellular protein, renatured following their separation by SDS-PAGE. Temperature treatments prior to loading on the gel were: lanes 1, 4 and 7, room temperature; lanes 2, 5 and 8, 3 min at 55 °C; lanes 3, 6 and 9, 3 min at 100 °C. Chitinolytic activity was detected using 4-methylumbelliferyl β-D-*N,N′,N″*-triacetylchitotriose [4-MU-$(GlcNAc)_3$] (lanes 1–3), 4-methylumbelliferyl β-D-*N,N′*-diacetylchitobioside [4-MU-$(GlcNAc)_2$] (lanes 4–6), or 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide [4-MU-(GlcNAc)] (lanes 7–9) as the substrate (authors' unpublished data).

medium containing chitin as the sole carbon source (Fig. 1). Only CHIT 102 was expressed intracellularly at a low constitutive level when *Trichoderma* was grown on glucose, but none of the chitinolytic enzymes were secreted under these conditions (Haran *et al.*, 1995).

To the best of our knowledge, two genes encoding endochitinases have been cloned. A cDNA clone of CHIT 42 was isolated from *T. harzianum* strain P1 by Hayes *et al.* (1994). The entire sequence, designated ThEn-42, consisted of 1554 bp, with an ORF encoding a putative protein of 424 amino acids. The deduced size of the mature endochitinase encoded by ThEn-42 was 42·66 kDa, which approximated to the measured value of 41 kDa. Garcia *et al.* (1994) also cloned a cDNA encoding CHIT 42. Analysis of the N-terminal amino acid sequence of the chitinase, and comparison with that deduced from the nucleotide sequence, revealed post-translational processing of a putative signal peptide of 34 amino acids cleaved in two steps. Carsolio *et al.* (1994) isolated and sequenced a genomic clone of the same gene. 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. Expression of the cDNA clone in *Escherichia coli* confirmed that the gene encodes a chitinase with activity on 4-methylumbelliferyl-β-D-*N,N′,N″*-triacetylchitotriose. A gene for another endochitinase of 33 kDa was recently cloned from *T. harzianum* strain CECT 2413 by Limón *et al.* (1995). The cDNA encodes a protein of 321 amino acids, which includes a putative signal peptide of 10 amino acids. The *chit33* gene appears as a single copy in the *T. harzianum* genome.

Glucanases

β-Glucans are homopolymers of D-glucose linked in a β configuration. Some are relatively simple molecules, consisting of linear chains of glycosyl residues joined by a single linkage type, whereas others are more complex and can consist of a variety of linkages in either linear or branched chains. Although many fungi synthesize β-glucans, either extracellularly or cytoplasmically (Faro, 1972; Zevenhuizen & Bartnicki-Garcia, 1970), they are generally located in the cell wall. The role of the fungal β-glucans is diverse, depending on their size, structure, physical and chemical properties, and most importantly, their location. The primary role of cell-wall β-glucans in fungi is as structural polymers, maintaining rigidity and conferring protection (Wessels & Siestma, 1981). This is generally achieved through the assistance of other cell-wall components, most commonly chitin, but also α-glucans and assorted homo- and heteropolysaccharides. However, the nature and location of the wall β-glucans suggest that they may also be degraded and used as nutritional sources, after exhaustion of external nutrients, or for changes in the cell-wall composition during morphogenesis (Pitson *et al.*, 1993).

The production of β-glucan-degrading enzymes is a characteristic attributed to a wide variety of organisms. Fungi are the main producers, with β-glucanases found in most isolates examined (Reese & Mandels, 1963).

β-Glucanases can act via two possible mechanisms, identified by the products of hydrolysis: (a) exo- or endwise-splitting and (b) endo- or random-splitting (Duncan *et al.*, 1956; Pitson *et al.*, 1993). Exo-β-glucanases hydrolyse the β-glucan chain by sequentially cleaving glucose residues from the non-reducing end. Consequently, the sole hydrolysis product is a monomer, usually glucose (Pitson *et al.*, 1993; Yamamoto & Nevins, 1983). Endo-β-glucanases cleave β-linkages at random sites along the polysaccharide chain, releasing smaller oligosaccharides (Pitson *et al.*, 1993), and may be categorized into two groups according to the hydrolysis product: those principally releasing oligosaccharides during hydrolysis, and those rapidly producing glucose and disaccharides (Fleet & Phaff, 1981). Synergistic action between at least two enzymes with different modes of action is common in fungi that degrade β-glucans (Copa-Patino *et al.*, 1990).

β-Glucan-hydrolysing enzymes are classified according to the type of β-glucosidic linkage(s). Due to the abundance and importance of cellulose, the homopolymer of 1,4-β-glucan, it is no surprise that most of the research has been concentrated on investigating 1,4-β-glucans and their degradation. Functionally complete cellulase enzyme systems can be produced by a large variety of microorganisms, such as aerobic and anaerobic bacteria, and white-rot, soft-rot and anaerobic fungi. Among the best-characterized and most widely studied of these systems are the inducible cellulases of *Trichoderma*, particularly those of *Trichoderma reesei*. Kubicek *et al.* (1993) reviewed the cellulase system of *Trichoderma* spp., which consists of three general classes of enzymes: (a) 1,4-β-D-glucan cellobiohydrolases (CBH, EC 3.2.1.91), which cleave

Table 2. Characterization of purified glucanases produced by *T. harzianum*

Strain	Glucanase type	Apparent mol. mass (kDa)	Optimum			Reference
			pI	pH	Temp. (°C)	
TMI 60622	Exo-1,3-β-	31	–	4·6	–	Kitamoto *et al.* (1987)
Undefined	Exo-1,3-β-	40	7·8	–	–	Dubourdieu *et al.* (1985)
P1	Endo-1,3-β-	78	6·2	4·5–5·5	40	Lorito *et al.* (1994)
CECT 2413	Endo-1,6-β-	43	5·8	–	–	De La Cruz *et al.* (1995)

cellobiosyl units from the non-reducing end of cellulose chains; (b) endo-1,4-β-D-glucanases [EG, cellulase: 1,3-(1,3;1,4)-β-D-glucan 3(4) glucanohydrolase, EC 3.2.1.4], which cleave internal glucosidic bonds; and (c) 1,4-β-D-glucosidase (cellobiase; β-D-glucoside glucohydrolase, EC 3.2.1.21), which cleaves cello-oligosaccharides to produce glucose. At least two cellobiohydrolases (CBH I and CBH II), two or more endoglucanases (EG I and EG III), and one β-glucosidase operate synergistically in the degradation of cellulose by this fungus.

Since 1,3-β-glucan is one of the main structural components of deuteromycete cell wall (Bartnicki-Garcia, 1968), the extracellular 1,3-β-glucanase has been thought to be involved in mycoparasitism (Ridout *et al.*, 1988). Enzymes with activity against 1,3-β-glucosidic linkages have been described in fungi, bacteria, actinomycetes, algae, molluscs and higher plants (Pitson *et al.*, 1993). Although possibly less common, enzymes with activity against 1,6-β-glucosidic linkages have also been detected in many fungi and bacteria.

A number of *Trichoderma* isolates secrete 1,3-β- and 1,6-β-glucanases (Del Rey *et al.*, 1979). Along with chitinases and proteases, they are involved in the cell lysis of several phytopathogenic fungi during the mycoparasitic process (Elad *et al.*, 1982). Since chitin and β-glucan are embedded in a matrix of amorphous material, successful cell-wall degradation may depend on the activity of more than one enzyme. Furthermore, chitin seems to be protected by β-glucan, and is not readily accessible to chitinases (Cherif & Benhamou, 1990). It is thus likely that chitinase activity is preceded by, or coincides with, the hydrolytic activity of other enzymes, especially 1,3-β- and 1,6-β-glucanases. Simultaneous induction of both chitinase and 1,3-β-glucanase was reported in bean plants infected by *Fusarium solani*. Both enzymes were required for fungal cell-wall lysis and growth inhibition of the pathogen (Mauch *et al.*, 1988a, b).

β-Glucanases from many fungi have been extensively characterized, and the results have led to an appreciation of their vastly different physicochemical properties (Pitson *et al.*, 1993). A summary of characterized β-glucanases from *T. harzianum* is presented in Table 2.

The importance of β-glucanases in plant-defence mechanisms against fungal attack has been very well established (Jach *et al.*, 1995), and many glucanase-encoding genes have been identified and cloned. Although the involvement of glucanases in the mycoparasitic process and in biological control is well documented, direct evidence for their role has only recently been demonstrated. Lorito *et al.* (1994) purified a glucan 1,3-β-glucosidase from *T. harzianum* strain P1 to homogeneity. The purified enzyme inhibited spore germination and germ-tube elongation of *Botrytis cinerea* with 50%-effective dose (ED_{50}) values of 94.5 and 90 μg ml^{-1}, respectively. For complete inhibition, 200–600 μg ml^{-1} were needed.

T. harzianum strain CECT 2413 was found to produce at least two 1,6-β-glucanases (De La Cruz *et al.*, 1995). One of these 1,6-β-glucanases, of 43 kDa, was purified to homogeneity. When the protein was combined with other *T. harzianum* cell-wall-degrading enzymes, such as 1,3-β-glucanases and chitinases, it hydrolysed filamentous fungal cell walls and inhibited the growth of the fungi tested (*B. cinerea*, *Gibberella fujikuroi*, *Phytophthora syringae* and *Saccharomyces cerevisiae*). To the best of our knowledge, only one gene encoding β-glucanase has been cloned from *T. harzianum*. Lora *et al.* (1995) described genomic and cDNA clones encoding the above 1,6-β-endoglucanase gene. The deduced protein sequence was found to have limited homology with other β-glucanases.

Proteases

Besides chitin and glucan, the skeleton of filamentous fungal cell walls contains lipids and proteins (Hunsley & Burnett, 1970). Fungal proteases may therefore play a significant role in the cell-wall lysis that occurs during pathogen–host interactions. Sivan & Chet (1989) found that pretreating hyphal walls of *Fusarium oxysporum* with proteolytic enzymes increases their susceptibility to lysis by the chitinase and 1,3-β-glucanase of *T. harzianum*. They suggested that a protein or protein-like constituent(s) is involved in the resistance of *Fusarium* cell walls to lytic enzymes. More recently, Geremia *et al.* (1993) identified an alkaline proteinase (Prb1) from *T. harzianum* strain IMI 206040. These authors suggested that Prb1 is involved in mycoparasitism, since it is induced by autoclaved mycelia, fungal cell-wall preparations or chitin, and repressed by glucose (Fig. 2). The gene (*prb1*) encoding this proteinase was cloned and characterized, and the induction of the enzyme was found to be due to an increase in the corresponding mRNA level.

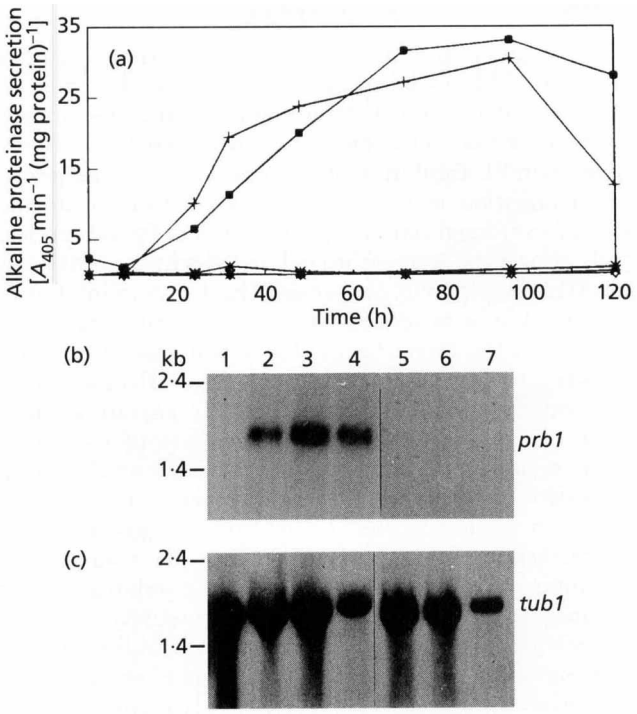

Fig. 2. Induction of *T. harzianum* alkaline proteinase and its encoding gene (*prb1*) during simulated mycoparasitism. (a) Secretion of the alkaline proteinase. *T. harzianum* was cultured in the presence of glucose (×), *R. solani* cell walls (■), chitin (+), *R. solani* cell walls and glucose (◇), or chitin and glucose (*). Samples were collected at the indicated times. (b) and (c) Northern analysis. Total RNA extracted from mycelia cultured in the presence of *R. solani* cell walls without glucose (lanes 2–4) or with glucose (lanes 5–7) at 24 h (lanes 2 and 5), 48 h (lanes 3 and 6), and 72 h (lanes 4 and 7). RNA (20 μg) from mycelia prior to induction was used as a control (lane 1). The RNA was electrophoresed and blotted using standard procedures. Induction of the *prb1* was demonstrated by hybridization of a 500 bp *Pst*I fragment of the proteinase-encoding gene (*prb1*) cDNA with the RNA blot (b). An internal control was performed (c) using the *Trichoderma viride* tubulin-1-encoding gene (*tub1*) cDNA as a probe (Geremia *et al.*, 1993).

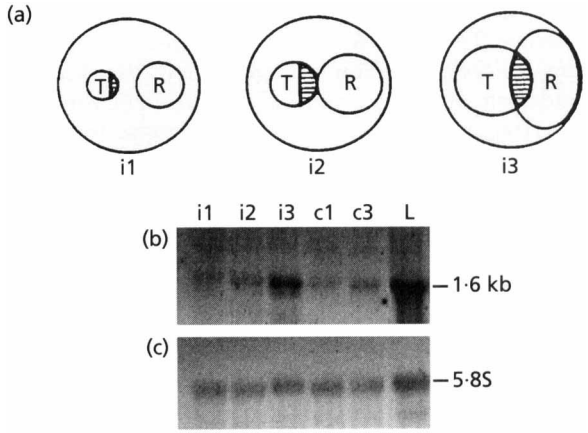

Fig. 3. Expression of *T. harzianum* endochitinase gene (*ech-42*) during mycoparasitism. *T. harzianum*, strain IMI 206040, and *R. solani* were grown in dual culture and mycelia were collected at different stages of the interaction. (a) Schematic representation of the three different stages at which the samples (shaded area) were taken. The outer circle represents the plate; the 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, when *Trichoderma* is overgrowing *Rhizoctonia*. (b) Northern blot analysis of the expression of *ech-42*, using a cDNA clone of *ech-42* as the 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) An internal control was performed using human 5·8S rRNA as the probe. Samples were as in (b). Migration position of the rRNA is indicated (Carsolio *et al.*, 1994).

Lytic enzymes of *T. harzianum* and biological control

Regulation during mycoparasitism

Upon contact with the 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 rather specific, and lectin–carbohydrate interactions were assumed to mediate the recognition and attachment between *Trichoderma* and soil-borne plant-pathogenic fungi. Recently, Inbar & Chet (1994) isolated and purified a new lectin from the culture filtrate of the soil-borne plant pathogenic fungus *Sclerotium rolfsii*. The authors developed a biomimetic system based on nylon fibres designed to imitate the pathogen hyphae. The purified lectin, bound to the surface of these nylon fibres, specifically induced mycoparasitic behaviour in *T. harzianum*. The fungus formed tightly adhering coils, which were significantly more frequent with the purified-agglutinin-treated fibres than with untreated controls, or with those treated with non-agglutinating extracellular proteins from *Scler. rolfsii*. Inbar & Chet (1995) further showed that the induction of a 102 kDa glucosaminidase (CHIT 102) is an early event elicited by the recognition signal (i.e. lectin–carbohydrate interactions). When *T. harzianum* was grown in dual culture with *Scler. rolfsii*, CHIT 102 activity was the first to be induced. As the interaction proceeded, the activity of CHIT 102 diminished concomitantly with the appearance of another glucosaminidase of 73 kDa (CHIT 73).

Carsolio *et al.* (1994) reported strong enhancement of CHIT 42 expression during interactions of *T. harzianum* strain IMI 206040 with *Rhizoctonia solani* (see Fig. 3). Schirmböck *et al.* (1994) reported the induction of a 40 kDa chitobiosidase, a 41 kDa endochitinase and a 1,3-β-glucanase in *T. harzianum* strain ATCC 36042 when the fungus was grown on cell walls of *B. cinerea*. These enzymes were not detected when *T. harzianum* was grown on glucose. De La Cruz *et al.* (1993) also reported the induction of chitinase when *T. harzianum* strain CECT 2413 was grown on cell walls of *B. cinerea*.

Lora *et al.* (1994) investigated changes in gene-expression

patterns elicited by chitin in the same strain of *T. harzianum*. They speculated that oligosaccharides containing GlcNAc, which are generated by the partial degradation of fungal cell walls, act as elicitors which might trigger a general antifungal response in *T. harzianum*. However, Limón *et al.* (1995) reported differences in the gene expressions of CHIT 42 and CHIT 33, suggesting independent regulation of each of these endochitinases.

Maximal 1,3-β- and 1,6-β-glucanase-specific activities were detected in media supplemented with purified cell walls from either *B. cinerea* or *Sacc. cerevisiae*, or pustulan (1,6-β-glucan) and nigeran (1,3-α-glucan alternating with 1,4-α-glucan) (De La Cruz *et al.*, 1993). However, Tangarone *et al.* (1989) reported excretion of glucanase from *Trichoderma longibrachiatum* grown on a D-glucose medium, and De La Cruz *et al.* (1993) and Lorito *et al.* (1994) reported excretion of the enzyme from *T. harzianum* grown on a chitin medium. These results indicate that 1,3-β-glucan is not required for glucanase synthesis. A possible explanation is that glucanases are produced constitutively, due to their involvement in fungal growth and differentiation, as has previously been suggested by Pitson *et al.* (1993). Alternatively, simultaneous induction with chitinolytic enzymes may occur. Concurrent induction of chitinases and glucanases has been described in plants as a response to infection by microbial pathogens. The two enzymes have also been shown to exhibit synergistic antifungal activity (Lorito *et al.*, 1994). Since the substrates of both classes of enzymes frequently occur together in nature, as in fungal cell walls, a combined induction of glucosidases and chitinases may be ecologically relevant.

The activity of an alkaline proteinase gene (*prb1*) was suggested by Geremia *et al.* (1993) to play a key role in mycoparasitism. Flores *et al.* (1996) determined its induction in mycoparasitic-like situations. They found that this gene was induced as early as 4 h after the fungus was transferred to cultures containing *R. solani* cell walls. To establish whether the induction observed with cell walls correlated with the actual phenomenon (fungus–fungus interaction), the authors performed direct confrontation assays between *T. harzianum* strain IMI 206040 and *R. solani*. Induction of *prb1* mRNA was observed in the presence of *R. solani*.

In an attempt to identify genes specifically expressed by the same strain of *T. harzianum* during growth on cell walls of *R. solani*, Vasseur *et al.* (1995) used differential screening of an induced cDNA library. They reported the expression of two cDNA clones that encode putative mycoparasitism-related proteins of 62853 and 37010 Da. They suggested the use of this methodology for the identification of genes involved in mycoparasitism.

These results suggest that a recognition event initiates a set of specific mycoparasitic responses, consisting of morphological as well as biochemical changes: coiling and appressorium formation, induction of unique combinations of chitinolytic enzymes, and induction of other cell-wall-hydrolysing enzymes such as glucanases and proteases.

Synergism of hydrolytic enzymes

The antifungal activity of cell-wall-degrading enzymes has recently been studied by several authors. Lorito *et al.* (1993) tested antifungal activity of purified endochitinase and exochitinase (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 levels of chitin in the cell wall of the target fungi. These chitinolytic enzymes appeared to be biologically more active than enzymes from other sources and more effective against a wider range of fungi. Combining the activities of the endochitinase and exochitinase (chitobiosidase) resulted in a synergistic increase in antifungal activity. The authors suggested that mixtures of hydrolytic enzymes with complementary modes of action may be required for maximum efficacy and that correct combinations of enzymes may increase *in vitro* antifungal activity. Lorito *et al.* (1994) reported the purification of two additional cell-wall-degrading enzymes from the same strain of *T. harzianum*: an *N*-acetyl-β-glucosaminidase and a glucan 1,3-β-glucosidase. Using the above bioassays, they found a synergistic inhibitory effect on *B. 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.

A similar phenomenon has been reported in plants. Using transgenic tobacco, Jach *et al.* (1995) showed that when a heterologous class-II chitinase was co-expressed with a heterologous class-II 1,3-β-glucanase, the transgenic tobacco plant exhibited significantly enhanced protection against fungal attack as compared to protection levels obtained when either heterologous gene was expressed alone.

Synergism of lytic enzymes and other antifungal compounds

Schirmböck *et al.* (1994) reported the parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, formed by *T. harzianum* strain ATCC 36042 when grown on cell walls of *B. cinerea*. Purified trichorzianines A_1 and B_1, as well as purified exochitinase (chitobiosidase), endochitinase, or glucan 1,3-β-glucosidase, inhibited spore germination and hyphal elongation only at concentrations higher than those observed in culture supernatants. However, when the enzymes and the peptaibols were tested together, a synergistic antifungal interaction was observed and the ED_{50} values were in the range of those determined in the culture supernatants. When endochitinase and exochitinase (chitobiosidase) from *T. harzianum* were combined with a biocontrol strain of *Enterobacter cloacae*, antifungal activities were increased synergistically (Lorito *et al.*, 1993).

Synergistic activity might serve as a tool to reduce the use of hazardous chemical fungicides. A synergistic effect was

found when commercial fungicides against *B. cinerea* were mixed with any of the chitinolytic or glucanolytic enzymes, and antifungal efficacy was significantly enhanced (Lorito *et al.*, 1994). Dose–response curves were determined for each combination of toxin and enzyme, and in all cases the ED_{50} values of the mixtures were substantially lower than those of each compound alone. The level of synergism appeared to be higher when enzymes were combined with toxins having primary sites of action associated with membrane structure, as compared to pesticides having multiple or cytoplasmic sites of action. The use of hydrolytic enzymes to enhance the antifungal ability of fungitoxic compounds could reduce the impact of some chemical pesticides on plants and animals.

Heterologous genes as a tool for improving biocontrol activity

Successful biocontrol is dependent upon several factors, including the development of superior biocontrol strains (Chet *et al.*, 1993). To this aim, one of the most attractive approaches is the production of transgenic strains with genetic components conferring improved antifungal features.

Cell-wall-degrading enzymes have been shown to be strong inhibitors of fungal growth and survival, and can therefore be used to improve biocontrol activities. Shapira *et al.* (1989) demonstrated the involvement of chitinase in the control of *Scler. rolfsii* by genetic engineering techniques: the gene *chiA*, encoding the major chitinase produced by *Serratia marcescens*, was cloned into *E. coli*. The enzyme produced by the cloned gene caused rapid and extensive bursting of *Scler. rolfsii* hyphal tips. This chitinase preparation was also effective in reducing the incidence of diseases caused by *Scler. rolfsii* in bean and by *R. solani* in cotton, under greenhouse conditions.

Sitrit *et al.* (1993) introduced the *chiA* gene from *Serr. marcescens* into the plant symbiont *Sinorhizobium* (*Rhizobium*) *meliloti*, which colonizes alfalfa root nodules. They showed that *Sinorhizobium* colonies harbouring the chitinase gene exhibit antifungal activity during symbiosis on alfalfa roots. The importance of chitinolytic activity in biocontrol has been demonstrated by Chernin *et al.* (1995). Chitinolytic strains of the bacterium *Enterobacter agglomerans* decreased the incidence of disease caused by *R. solani* in cotton by 64–86 %. In contrast, Tn*5* mutants of the bacterium, which were deficient in chitinolytic activity, were unable to protect the plants against the disease.

Enhancement of biocontrol activity by *T. harzianum* (T-35) was attempted by Haran *et al.* (1993). An *Serr. marcescens* chitinase gene was introduced into the *T. harzianum* genome, under the control of a 35S constitutive promoter from cauliflower mosaic virus. The authors expected constitutive elevation of extracellular lytic activity to improve the natural capability of *T. harzianum* to attack pathogens, thereby enabling its consequent use as a superior biocontrol agent. *T. harzianum* protoplasts were co-transformed using two plasmids: (a) pSL3ChiAII, containing a bacterial chitinase gene from *Serr. marcescens* under the control of a constitutive viral promoter and (b) p3SR2, encoding acetamidase cloned from *Aspergillus nidulans*, as a marker for selection after transformation. When grown on glucose, the *T. harzianum* transformants produced and excreted a protein that was detected by SDS-PAGE as a major band of 58 kDa, the size of the *Serr. marcescens*-produced chitinase. The transformants expressed significantly higher chitinase activity than the recipient (wild-type), suggesting that the signals for expression of the introduced gene had been recognized by *T. harzianum*. However, the higher chitinase activity occurred only when the transformants were grown on glucose. When grown in the presence of chitin, both transformants showed lower chitinase activity than the wild-type. Western blot analysis of the proteins excreted by the transformants in the presence of chitin revealed two species of proteins reacting specifically with the polyclonal antibodies against the *Serr. marcescens* chitinase (ChiA). These proteins were of 40 and 18 kDa, relative to the 58 kDa protein of *Serr. marcescens*. These results suggest that under non-inductive conditions, the transformants produced the approximately 58 kDa chitinase encoded by the introduced *chiA* gene, and that induction by chitin caused cleavage of this protein into two fragments of 40 and 18 kDa. The cleavage of foreign proteins by host proteases has previously been reported in *Sacc. cerevisiae* (Bonnet & Spahr, 1990).

The natural chitinolytic system of *T. harzianum* is composed of at least seven distinct enzymes (Table 1). As a result of the cleavage event, additional foreign fragments of 40 and 18 kDa were excreted by the transformed *T. harzianum* during induction. These fragments could have interfered with the natural excretion of the native chitinases, explaining the observed decrease in the chitinase activity of the transformants under inductive conditions.

Fitness evaluations showed that the transformants were not biologically inferior to wild-type *T. harzianum* in biomass production, growth rate or sporulation. Antagonism evaluation using dual cultures showed that the transformants overgrow the pathogen *Scler. rolfsii* at the same rate as wild-type *T. harzianum*. However, while overgrowing the pathogen, the transformants produced wider clear lytic zones relative to the wild-type, probably as a consequence of their higher constitutive chitinase activity (Fig. 4). The higher activity of the chitinase excreted by *T. harzianum* transformants could protect germinating seedlings from certain soil-borne pathogens. Constitutive expression could produce extracellular lytic activity early on, whereas induced enzymes are produced only after the biocontrol agent interacts with the target pathogenic fungus.

The promoters of constitutive genes have proven themselves to be useful parts of expression vectors for genetic engineering in different organisms. Whereas promoters isolated from other organisms can sometimes prove useful, one can never be sure that they will work similarly

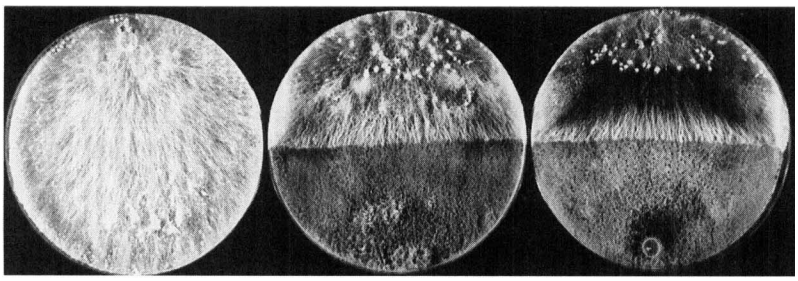

Fig. 4. Mycoparasitic interactions of *T. harzianum* (wild-type) and a transgenic strain of *T. harzianum* expressing a *Serr. marcescens* chitinase gene (*chiA*), with *Scler. rolfsii* in dual cultures. The *Serratia* chitinase gene was introduced into the *T. harzianum* genome, under the control of the 355 promoter from cauliflower mosaic virus, and was constitutively expressed by the transgenic strain. Left to right: *Scler. rolfsii*; dual cultures of *T. harzianum* (wild-type) (middle plate) and a transgenic strain of *T. harzianum* (right plate), respectively (both on the lower half of the plate), with *Scler. rolfsii* (upper half), 7 d after contact (Haran *et al.*, 1993).

in all physiological or developmental states of the heterologous host. Based on differential screening of an induced cDNA library, Goldman *et al.* (1994) isolated constitutively expressed cDNA clones of *T. harzianum*. One of these cDNA clones corresponded to a gene (*cob4*) that encodes a novel serine + alanine-rich protein. Northern (RNA) blot analysis demonstrated that *cob4* was expressed during growth when glucose or cell walls of a phytopathogenic fungus were provided as the carbon source. They suggested that constitutively expressed genes could provide reliable promoters useful for genetic manipulations.

Most of the work on *T. harzianum* as a biocontrol agent has been performed with phytopathogenic fungi which contain mainly chitin and β-glucan as cell-wall components. However, plant-pathogenic oomycetes contain cellulose as the main cell-wall component (Bartnicki-Garcia, 1968). Migheli *et al.* (1994) attempted a different way of improving *Trichoderma* as a biocontrol agent by using the enzyme 1,4-β-endoglucanase. Hypercellulolytic strains of *T. longibrachiatum* were obtained by co-transformation of plasmid pTLEG12, which contains the *egl1* gene for the 1,4-β-endoglucanase of *T. longibrachiatum* and plasmid pAN7-1, which confers hygromycin B resistance, for selection after transformation (Sanchez-Torres *et al.*, 1994). The transformants were tested for their ability to reduce *Pythium* damping-off on cucumber seedlings, and were shown to be significantly more effective in controlling the disease than the wild-type (disease incidence was reduced from 60 to 28%). These preliminary results suggest that cellulase activity may be involved in the biocontrol of *Pythium ultimum* by *T. longibrachiatum*.

Geremia *et al.* (1993) cloned a gene encoding the basic proteinase *prb1* from *T. harzianum* strain IMI 206040, and suggested that it plays a key role in mycoparasitism. Flores *et al.* (1996) attempted to increase the *prb1* gene dosage by introducing multiple copies of *prb1* into the *Trichoderma* genome. Integration occurred as multiple copies arranged in tandem, as has been previously described (Goldman *et al.*, 1990; Herrera-Estrella *et al.*, 1990). The number of copies was estimated at 2–6 in the various transformants obtained. Densitometric analysis of RNA expression by Northern, and protein expression by Western analyses, indicated that the levels of *prb1* mRNA and protease secreted by the transformants were up to 17- and 20-fold higher than in the wild-type strain, respectively. However, analysis of *prb1* mRNA and Prb1 protein production by each of the transformants indicated that high *prb1* mRNA production does not always result in high protein production. The various transformants showed a gradient of proteinase protein production. The successful overexpression of the *prb1* gene, driven by its own promoter, led the authors to test the effectiveness of the transformants as biocontrol agents (Flores *et al.*, 1996). Greenhouse experiments were performed in which the transformed *T. harzianum* strains were used to protect cotton seedlings from *R. solani*. An up-to-fivefold increase was observed in the biocontrol efficacy of the transformed strains, as compared to the wild-type. However, the best protection was provided by a strain which produced only an intermediate level of proteinase protein. The authors suggested that extreme levels of proteinase protein might cause degradation of other enzymes which are important in the mycoparasitic process. These results demonstrated that the introduction of multiple copies of *prb1* improves the biocontrol activity of *T. harzianum* and showed the importance of the proteinase Prb1 in biological control.

Summary

The 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. Multiple enzyme systems are involved in the parasitic interactions. The chitinolytic system, as well as the glucanolytic system, are composed of enzymes with different modes of action. Only one alkaline proteinase has so far been found to be involved in the process.

The creation of gene-disrupted strains of *T. harzianum* has not been reported to date. Thus, the significance of the individual gene products in the mycoparasitic process was not assessed. However, *in vitro* studies using these enzymes, purified from *T. harzianum*, have shown their direct antifungal effect. Synergism of this effect was reported when combinations of the various enzymes were used.

As a commercial product, wild-type *T. harzianum* preparations are currently making their way to the market. Some formulations are applied as potting mixtures in greenhouses, as seed-coating treatments or as foliar applications. However, their use in commercial agriculture is not yet widespread because, to date, the control of plant diseases with *Trichoderma* has not been as effective and reliable as with synthetic fungicides. Further studies of the right ecological niches and of the mechanisms involved in its mycoparasitic behaviour will improve our ability to use this fungus for effective plant-disease control.

The possibilities for improving biocontrol activity by *T. harzianum* using genetic manipulation techniques are promising. The early studies with transgenic *T. harzianum* transformed to produce increased amounts of specific proteins are encouraging, since even with these preliminary systems, positive results have been obtained. Future research should focus on the development of strains expressing 'multigene' combinations while preserving the intrinsic vigour and the ecological competence of the fungus. Once transgenic *T. harzianum* strains capable of producing highly efficient synergistic combinations of enzymes are produced, much better disease control should be obtained. Transgenic *Trichoderma* therefore offers the potential of substantially reducing the quantity of chemical fungicides required to produce disease-free plants.

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