TRICHODERMA: FROM GENES TO BIOCONTROL

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SUMMARY

Species of *Trichoderma* are commercially applied as biological control agents against fungal pathogens. Since this process requires the degradation of the cell-wall of the fungal host, chitinases secreted by *Trichoderma* have been addressed as major determinants of biocontrol activity. In this review we will report on the recent progress in the understanding of chitinase multiplicity in *Trichoderma*, on the outstanding role of one chitinase isoenzyme – the 42-kDa endochitinase – for biocontrol by *T. atroviride* and in rekombinant plants, and on the regulatory circuits governing its expression upon contact of *Trichoderma* with plant pathogenic hosts.

Key words: *Trichoderma*, biocontrol, chitinase, mycoparasitism.

INTRODUCTION

Species of *Trichoderma* have been shown to act, and are commercially applied as biological control agents against fungal pathogens (Chet, 1987; Chet *et al.*, 1998; Harman and Björkman, 1998). Mycoparasitism, a general term to describe the multi-step degradation and final assimilation of phytopathogenic fungi, has been proposed as the major mechanism supporting the antagonistic activity displayed by *Trichoderma*. Since this process requires the degradation of the cell-wall of the fungal host, cell-wall hydrolases secreted by *Trichoderma* (such as chitinases and β-glucanases) have been addressed as major determinants of biocontrol activity (Elad *et al.*, 1982; Hjeljord and Tronsmo, 1998). While it now becomes increasingly evident that biocontrol is the result of various cellular activities and not only enzymatic hydrolysis, recent findings clearly indicate that particularly the chitinases play a major role.

Chitin, the (1-4)-β-linked homopolymer of N-acetyl-D-glucosamine, is one of the most abundant polymers in the biosphere, and chitinolytic enzymes are found among all kingdoms, *i.e.*, protista, bacteria, fungi, plants, invertebrates and vertebrates, including humans (Cabib, 1987; Gooday, 1990; Sakai and Manocha, 1993). Enzymatic degradation of chitin is generally involved in many biological processes, such as autolysis (Vessey and Pegg, 1973), morphogenesis and nutrition (Griffin, 1994), and in addition to mycoparasitism plays also a role in relationships between fungi and other organisms such as plant-fungus and insect-fungus interactions (St. Leger *et al.,* 1986; Mauch *et al.,* 1988). The formation of chitinases by some fungi, including *Trichoderma* spp., is well-known and some species such as *T. harzianum* are used commercially as sources of these proteins (*e.g.* 'lytic enzyme' preparations L 2773 and L 2265; Catalogue of chemicals; Sigma, MO). Here we review the characteristics, the functions and the diversity of *Trichoderma* chitinolytic enzymes and genes, and how these genes and/or the knowledge about their properties can be used to improve the biocontrol properties of *Trichoderma* spp.

HOW MANY CHITINASE PROTEINS AND GENES DOES *TRICHODERMA* HAVE? It is intriguing that, despite of the interest of many researchers in the application of chitinases, very little is known on the enzymology of chitin breakdown by *Trichoderma* spp. Considering the structure of chitin (a β-1,4-linked insoluble polysaccharide) and in analogy to the cellulose-degrading enzyme system (for which a much more detailed understanding has been achieved; Koivula *et al.,* 1998; Kubicek and Penttilä, 1998), one would expect that endochitinases, exochitinases and chitobiases are required for complete and efficient hydrolysis. Concepts for distinguishing endo- and exo-types of such hydrolase activities have been established (Reese *et al.,* 1968) but not yet directly applied to *Trichoderma* chitinases. In this review, we therefore continue to use the chitinase classification scheme recently proposed by Lorito (1998), which differentiates the chitinases according to the reaction end-products as follows: (*i*) endochitinase (EC 3.2.1.14) which randomly cleaves chitin and chitooligomers and

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releases a mixture of soluble low molecular mass end-products of different sizes, with diacetylchitobiose $(GlcNAc)_2$ being the main end-product; (*ii*) chitin 1,4-β-chitobiosidase (chitobiosidase), which cleaves chitin and chitooligomers [$(GlcNAc)_n$; n>3] progressively from the non-reducing end and releases only $(GlcNAc)_2$; and (*iii*) β-N-acetylhexosaminidase (EC 3.2.1.52), which cleaves chitooligomers and also chitin progressively from the non-reducing end and releases only N-acetylglucosamine monomers (GlcNAc), and thus the only enzyme able to hydrolyze $(GlcNAc)_2$. It should be noted, however, that a firm discrimination between the endochitinase and the chitobiosidase on one hand, and the chitobiosidase and the N-acetyl-β-D-glucosaminidase on the other hand, requires an accurate study on the mechanism of substrate degradation (Reese *et al.,* 1968), which has not been performed yet.

The proteins comprising the chitinolytic system of *Trichoderma* spp. have still not been all identified and/or characterized, although some of the major enzymes have been purified to homogeneity and their biochemistry and biological role studied. Table 1 summarizes the currently available information by identifying each enzyme by its apparent molecular weight (MW) on SDS-PAGE, and shows the diversity of the chitinolytic system of *Trichoderma* spp. It is obvious that homologues of the 42-kDa endochitinase have been most often purified, followed by a frequent isolation of an N-acetyl-β-D-glucosaminidase of 70-73 kDa. This could be explained by the fact that these two enzymes usually represent the major portion of the chitinase proteins in the culture filtrate of *Trichoderma* spp. when the fungus is grown on fungal cell walls as a carbon source. Further, a 37-kDa and a 33-kDa endochitinase (de la Cruz *et al.,* 1992), a 40-kDa chitobiosidase (Harman *et al.,* 1993) and a 28-kDa exochitinase (Deane *et al.*, 1998) have been purified. Yet additional enzymes have already been identified by fluorescent staining of gels (*e.g.* a 102-kDa N-acetyl-β-D-glucosaminidase; Haran *et al.,* 1995), but unfortunately not yet purified.

It should be noted that among the enzymes termed 'endochitinases' listed in Table 1 only the 33-kDa endochitinase of *T. harzianum* releases chitotetraoside as the major end product, along with minor amounts of chitotriose and chitobiose (de la Cruz *et al.,* 1992). This correlates with the fact that this enzyme, in contrast to most of the other endochitinases of *Trichoderma* spp., is practically unable to hydrolyze para-nitrophenol-β-D-chitobioside.

There are major difficulties in the use of the available data to describe the diversity of the chitinase spectrum of *Trichoderma.* First, secreted enzymes like chitinases are likely subjected to proteolytic degradation in the culture filtrate. In fact, this has been a major stumblestone for understanding the enzymology of cellulases from *T. reesei* (Kubicek, 1992), and we have recently reported data suggesting that at least one of the chitinases – the 42-kDa endochitinase – occurs in the culture filtrate of some *Trichoderma* spp. in a proteolytically nicked form (Kullnig *et al.,* 2001). Second, most of the enzyme purifications have been performed on filtrates from different '*T. harzianum*' isolates, which may not actually be all *T. harzianum*. In fact, several authors have recently found a pronounced genetic variability of *T. harzianum* isolates with respect to carbon source utilization patterns (Manczinger and Polner, 1985), secondary metabolite production (Okuda *et al.,* 1982), isoenzyme polymorphism (Stasz *et al.,* 1989; Grondona

Table 1. Chitinase proteins purified from *Trichoderma* spp.

Species	Strain	Chitinase type	Mr [kDa]	References
T. harzianum	CECT 2413	endochitinase	42 37 33	De la Cruz *et al.,* 1992
T. atroviride	P1	endochitinase chitobiosidase N-acetyl-β-D-glucosaminidase	42 40 73	Harman *et al.,* 1993 Lorito *et al.,* 1994
T. cf. *harzianum*	Nottingham39.1	endochitinase N-acetyl-β-D-glucosaminidase	40 64	Ulhoa and Peberdy, 1992 Ulhoa and Peberdy, 1991
T. cf. *harzianum*	Nottingham39.1	endochitinase	46	Lima *et al.,* 1997
T. cf. *harzianum*	NottinghamT198	exochitinase	28	Deane *et al.,* 1998
T. cf. *harzianum*	AF6-T8	N-acetyl-β-D-glucosaminidase	69	Koga *et al.,* 1991

et al., 1997), RAPD profiles (Fujimori and Okuda 1994; Muthumenakshi *et al.,* 1994; Zimand *et al.,* 1994; Turoczi *et al.,* 1996; Gomez *et al.,* 1997), RFLP patterns (Muthumeenakshi *et al.,* 1994; Bowen *et al.,* 1996), rDNA sequence (Muthumeenakshi *et al.,* 1994; Grondona *et al.,* 1997) and karyotype (Gomez *et al.,* 1997), thus raising the question whether all the isolates identified as *T. harzianum* are actually the same species. In fact, Hermosa *et al*. (2000) have recently showed that a set of 17 biocontrol isolates consisted of at least four different species – *T. harzianum, T. atroviride, T. longibrachiatum* and *T. asperellum*.

We also have investigated the taxonomic identity of eight isolates of '*T. harzianum*' widely used to study the physiology, biochemistry and molecular genetics of biocontrol by *Trichoderma* by sequence analysis of the internally transcribed spacer regions of the rDNA (ITS1 and ITS2), the small subunit of the mitochondrial DNA (mtSSUrDNA), and part of the coding region of the 42-kDa endochitinase encoding gene *ech42* (see below). The gene trees resulting from this study provided a strong statistically-supported evidence that the eight strains belong to three different species: *T. harzianum*, *T. atroviride*, and *T. asperellum*. All eight strains secreted the 42-kDa endochitinase and the 73-kDa N-acetyl-β-D-glucosaminidase into the culture fluid. However, using primers designed to amplify the conserved regions of *chit33,* PCR fragments of this gene were obtained only from *T. harzianum* and closely related species (see below). In agreement with this finding, Schickler *et al.* (1998b) showed that chitinase zymostains can differentiate between different strains of *(bona fide) T. harzianum*, which supports the view that separate species (and eventually different populations) of *Trichoderma* spp. may differ in their chitinase spectrum or chitinase properties.

Cloning of chitinase genes may be a more reliable way to assess chitinase diversity in *Trichoderma*. The chitinase genes cloned up to date are summarized in Table 2. The gene encoding the 42-kDa endochitinase, that has high homology to endochitinases from other fungal species including *Aphanocladium* sp. (Blaiseau and Lafai, 1992), has been the one cloned most often. The respective nucleotide sequence of *ech42* from different *Trichoderma* spp. differs up to 35%, and the amino acid sequence by up to 17%. This difference correlates well with the phylogenetic distance between the investigated species (Kullnig *et al.,* 2001), which indicates *ech42* as a very useful gene for phylogenetic analysis of *Trichoderma* (Lieckfeldt *et al.,* 2000).

The only other endochitinase which has so far been cloned and characterized in detail is *chit33* from *T. harzianum* (Limón *et al.,* 1995), for which no other fungal homologue has so far been found. However, the enzyme has high homology to some plant defense chitinases. Using nucleotide primers corresponding to areas encoding the conserved domains of this protein, we could amplify a corresponding fragment from several other *Trichoderma* spp. with close phylogenetic relationship to *T. harzianum*, but no amplicon was obtained with *T. atroviride, T. viride* or *T. asperellum*. Thus, the presence of this gene may either be specific

Table 2. Chitinase genes cloned from *Trichoderma* spp.

Gene	*Trichoderma* spp.	Strain	Encoded protein	References
Th-En42	*T. atroviride*	P1	42-kDa endochitinase	Hayes *et al.,* 1994
ech42	*T. atroviride*	IMI 206040	42-kDa endochitinase	Carsolio *et al.,* 1994
chit42	*T. harzianum*	CECT2413	42-kDa endochitinase	Garcia *et al.,* 1994
cht42	*T. virens*	Gv29-8	42-kDa endochitinase	Baek *et al.,* 1999
th-ch	*T. hamatum*	Tam-61	42-kDa endochitinase	Fekete *et al.,* 1996
ENC1	*T.* cf. *harzianum*	T25-1	42-kDa endochitinase	Draborg *et al.,* 1996
chit33	*T. harzianum*	CECT2413	33-kDa endochitinase	Limon *et al.,* 1995
nag1	*T. atroviride*	P1	73-kDa N-acetyl-β-D-glucosaminidase	Peterbauer *et al.,* 1996
exc1	*T.* cf. *harzianum*	T25-1	73-kDa N-acetyl-β-D-glucosaminidase	Draborg *et al.,* 1995
exc2	*T.* cf. *harzianum*	T25-1	N-acetyl-β-D-glucosaminidase ??	Draborg *et al.,* 1995

for *T. harzianum* and close relatives, or may be not enough conserved between more distant species so that PCR was unsuccessful.

Interestingly, none of the chitinases cloned so far from *Trichoderma* spp. has been shown to contain a chitin-binding domain.

Two N-acetyl-β-D-hexosaminidase genes (*exc1* and *exc2*, encoding a 64 and a 68-kDa enzyme – not considering potential glycosylation) have been cloned from *T. harzianum* (Draborg *et al.,* 1995) and called 'exochitinase'. They had 72% aa-homology to each other, and one of them (*exc1*, encoding a 64-kDa enzyme with 7 potential glycosylation sites, which would give rise to an apparent M_r of 73-75 kDa) was similar to the single copy *nag1* gene found in *T. atroviride* (Peterbauer *et al.,* 1996). It is likely that the occurence of the 68-kDa enzyme is unique for the strain used by Draborg *et al.* (1995) (which according to the *ech42* sequence most likely belong to *T. harzianum* or a closely related species), because low stringency hybridization of *T. atroviride* DNA with the *nag1* gene did not reveal any other gene with similarity (C.K. Peterbauer, unpublished results).

In summary, the number of chitinase genes cloned from *Trichoderma* spp. is still much lower than the number of proteins purified, which makes difficult to establish if all of the different purified proteins are actually encoded by different genes. However, there are certainly more chitinase genes present than so far found. Kim *et al.* (1997) reported on the cloning of three chitinase genes from *T. virens*, in addition to the gene encoding the 42-kDa endochitinase. Unfortunately, no full report on this work has so far appeared, leaving open the question if any of these genes is a homologue of *T. harzianum chit33* or *T. atroviride nag1*.

CHITINASES ARE ANTIFUNGAL AGENTS. The antifungal activity of chitinases from bacteria and plants has been known for a long time (Mauch *et al.,* 1988; Gupta *et al.,* 1995; Pleban *et al.,* 1997). The 42-kDa endochitinase, the 40-kDa chitobiosidase and the 73-kDa N-acetyl-β-D-glucosaminidase from *T. atroviride* strain P1 and *T. virens* strain 41 also have a substantial inhibitory effect on the germination of spores and hyphal elongation of several fungal pathogens, including *Botrytis cinerea, Fusarium* spp., *Alternaria* spp., *Ustilago avenae*, *Uncinula necator* and virtually on all fungi containing chitin in their cell-walls (Di Pietro *et al.,* 1993; Lorito *et al.,* 1993, 1994a, b, 1996c; Schirmböck *et al.,* 1994). The endochitinases are usually among the most effective in terms of both antifungal and lytic activities in the comparison with other types of chitinolytic enzymes (de la Cruz *et al.,* 1992; Lorito *et al.,* 1996c). Interestingly, the efficacy as antifungal agents of the *Trichoderma* enzymes was higher than that described for plant, bacterial or other fungal chitinolytic enzymes assayed under the same conditions (Lorito *et al.,* 1996c), which supports the hypothesis that these enzymes are specialized for attacking the cell walls of other fungi. *Trichoderma* enzymes were not only able to lyse the 'soft' structure of the hyphal tip but also the 'hard' chitinous wall of mature hyphae, conidia, chlamydospores and sclerotia (Benhamou and Chet, 1993; Lorito *et al.,* 1993; Benhamou and Chet, 1996; Rousseau *et al.,* 1996).

THE ANTIFUNGAL ACTION OF CHITINASIS REINFORCED BY SYNERGISM WITH ANTIBIOTICS. While the *Trichoderma* chitinases are among the most active antifungal chitinases studied, their LD_{50} values are nevertheless too high to explain mycoparasitism and biocontrol *in vivo* solely on the basis of their action, which has been a major issue raised against the role of chitinases in this process. However, this point was clarified by demonstrating that the action of chitinases is strongly synergistic both with other chitinase components as well as with other components putatively involved in biocontrol, *i.e.* antibiotics (Broglie *et al.,* 1991; Collinge *et al.,* 1993; Lorito *et al,* 1994b; Schirmböck *et al.,* 1994; Haran *et al.,* 1995; Jach *et al.,* 1995; Lorito *et al.,* 1996c). This synergism with other chitinolytic or glucanolytic enzymes results in a dramatic enhancement of the lytic and inhibitory action even in cases where the enzymes have little to no activity when applied alone (de la Cruz *et al.,* 1992; Lorito *et al.,* 1993, 1994a, b, 1996c). Even more important, however, is the ability of chitinases to enhance the antifungal effect of non-enzymatic compounds or other microorganisms (Lorito *et al.,* 1993, 1994b). For instance, chitinolytic enzymes were synergistic with both natural as well as synthetic cell membrane-affecting compounds (MACs) in the inhibition of fungi (Lorito *et al.,* 1996c).

The mechanism of this enzyme-antibiotic synergism has been investigated in some detail, and shown to be due to a concerted effect of enzyme and MAC activity on the integrity of the cell wall, as evidenced in the case of peptaibol antibiotics (Lorito *et al.,* 1996b). These are linear oligopeptides of 12-22 amino acids, which are rich in α-aminoisobutyric acid, N-acetylated at the N-terminus and containing an amino alcohol (Pheol or Trpol) at the C-terminus (Rebuffat *et al.,* 1989). They are known to form voltage-gated ion channels in black lipid membranes and modify the membrane permeability of liposomes in the absence of applied voltage (El Hajji *et al.,* 1989). Hence, while the chitinases reduce the rigidity (the barrier effect of) of the cell-wall, peptaibol antibiotics inhibit the membrane bound synthases of

cell-wall components and thereby impair the ability of the hyphae to repair the lytic effect of the enzymes on the cell walls (Fig. 1). This view is also supported by the findings of permeability changes in the host during mycoparasitic interaction with *Trichoderma* (Lewis and Papavizas, 1987). However, this may not be the only explanation for the observed synergism between chitinases and antibiotics, as the mode of action of other components (*e.g.* g-pentyl pyrone) also found to be important for antagonism *in vivo* (Claydon *et al.,* 1987; Serrano-Carreon *et al.,* 1993; Howell, 1998), has not yet been elucidated. For a detailed survey of the current knowledge, see Lorito (1998).

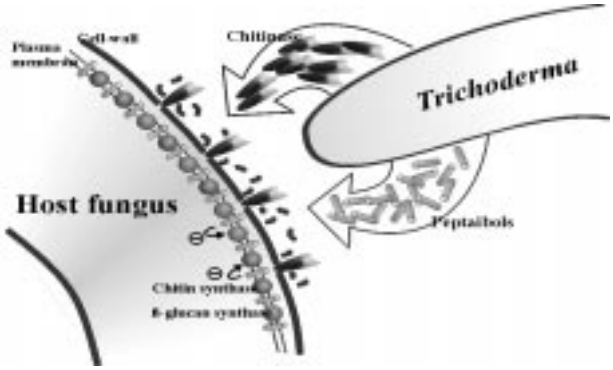

Fig. 1. Hypothetical model of the synergism of *Trichoderma* cell-wall hydrolases and membrane affecting compounds such as peptaibols.

CHITINASE GENE EXPRESSION IN *TRICHODERMA* IN RELATION TO BIOCONTROL. High-level induction of extracellular chitinolytic enzymes is usually obtained by growing *Trichoderma* on purified chitin, fungal cell walls or mycelia as sole carbon sources. No or much less induction is normally attained when related compounds such as chitosan, cellulose, unpurified chitin or laminarin are used, indicating a rather specific induction *in vitro* (Ulhoa and Peberdy, 1991a; de la Cruz *et al.,* 1993; Harman *et al.,* 1993; Lorito *et al.,* 1994a). The production of chitinolytic enzymes could be induced by soluble chitooligomers (Smith and Grula, 1983; St Leger *et al.,* 1986; Reyes *et al.,* 1989), as seems to be the case with the 73-kDa N-acetyl-β-D-glucosaminidase and the 42-kDa endochitinase of *T. harzianum* (de la Cruz *et al.,* 1993; Ulhoa and Peberdy, 1991b, 1993; Schickler *et al.,* 1998a). However, this does not mean that the different chitinases have the same mechanism of induction. For example, during mycoparasitic action of *T. harzianum* on agar plates, the formation of different chitinases, particularly the 102-kDa and 73-kDa N-acetyl-β-D-glucosaminidases and the 42-kDa endochitinase, was affected differently if the fungus was confronted with different hosts (Inbar and Chet, 1995; Haran *et al.,* 1996).

In addition, formation of most chitinolytic enzymes does not occur or is even inhibited by glucose, sucrose and chitinolytic end-products (Ulhoa and Peberdy, 1991b; de la Cruz *et al.,* 1993; Carsolio *et al.,* 1994; Garcia *et al.,* 1994; Margolles-Clark *et al.,* 1996; Peterbauer *et al.,* 1996), suggesting that direct induction and/or catabolic repression are major regulatory parameters for chitinase formation. Several studies reported glucose repression of *chit42/ech42* gene expression (de la Cruz *et al.,* 1993; Carsolio *et al.,* 1994; Garcia *et al.,* 1994; Margolles-Clark *et al.,* 1996). However, some researchers have found that at least trace quantities of the 102-kDa N-acetyl-β-D-glucosaminidase, the 42-kDa endochitinase and the 33-kDa endochitinase are produced constitutively (Ulhoa and Peberdy, 1991a; Garcia *et al.,* 1994; Haran *et al.,* 1995; Inbar and Chet, 1995; Margolles-Clark *et al.,* 1996; Carsolio *et al.,* 1999). Additional enzymes may also be formed under starvation or stress conditions. In summary several enzymes may collaborate in the release of chitin oligomers from hosts present in the environment and so induce the full chitinolytic system.

Some of these findings have also been supported by the analysis of gene expression. The expression of *T. atroviride nag1* is triggered by fungal (*B. cinerea*) cell walls and the commercially available chitin monomer N-acetyl-glucosamine, and the oligomers di-N-acetylchitobiose and tri-N-acetylchitotriose (Mach *et al.,* 1999). In contrast, *ech42* expression in *T. atroviride* was also observed during growth on fungal cell walls, but could not be triggered by those chitin degradation products (Margolles-Clark *et al.,* 1996; Carsolio *et al.,* 1999; Mach *et al.,* 1999), whereas in *T. harzianum* it is induced by N-acetyl-β-D-glucosamine (Garcia *et al.,* 1994; Schickler *et al.,* 1998a). In contrast, *ech42* from *T. atroviride* was remarkably expressed after prolonged carbon starvation (Margolles-Clark *et al.,* 1996; Mache *et al.,* 1999). The latter effect occured with glucose as well as with glycerol as a carbon source, and is thus probably not due to a relieve from carbon catabolite repression. In addition, *ech42* gene transcription was triggered by some conditions of physiological stress (4°C, high osmotic pressure, addition of ethanol; Mach *et al.,* 1999), as well as by light- induced sporulation (Carsolio *et al.,* 1994). Interestingly, *T. harzianum chit33* expression, while being inducible by N-acetyl-β-D-glucosamine, was also triggered by carbon starvation, nitrogen starvation and physiological stress (de la Mercedes *et al.,* 2000), suggesting that stress-mediated

regulation may be a general phenomenon involved in chitinase gene expression of *Trichoderma* spp.

Some studies have so far been performed towards understanding how and in which order the chitinases are induced during mycoparasitic interaction. In their pioneering studies, Inbar and Chet (1992, 1995) demonstrated that formation of chitin-degrading enzymes in *T. harzianum* is elicited by a lectin-based physical interaction with the host, which was suggested to be the earliest event of interaction, and precede induction by possible chitooligomers. A support for this model was the finding that nylon threads, covered with *Sclerotinia sclerotiorum* lectin nicely mimicked the presence of the host. In a subsequent study, Inbar and Chet (1995) also showed that the 102-kDa chitinase is specifically induced by contact with the host lectin, whereas formation of all the other chitinases requires the presence of the living host. They concluded that the 102-kDa enzyme may be responsible for the first attack and induction for the other chitinases. However, the induction cascade *in vivo* appears today much more complex. Zeilinger *et al.* (1998) studied the expression of *ech42* and *nag1* during mycoparasitic interaction of *T. atroviride* with *R. solani in vivo* by using the *Aequorea victoria* green fluorescent protein as a non disruptive reporter system. They showed that *ech42*, but not *nag1*, was formed before any detectable contact of *Trichoderma* with its host. Similar studies with *chit33:GFP* in *T. harzianum* (de la Mercedes *et al.,* 2000) showed that this pre-contact gene expression did not occur with the 33-kDa endochitinase-encoding gene *chit33*, and therefore may be specific for *ech42*. Interestingly, *ech42* gene expression was prevented when a dialysis membrane was placed between the two fungi (Zeilinger *et al.,* 1999), but still occur when a cellophane membrane was used for this purpose (Cortes *et al.,* 1998). This led to contradicting conclusions regarding the nature of the molecule triggering *ech42* gene expression (Cortes *et al.,* 1998; Zeilinger *et al.,* 1999). This issue could be solved by showing that the cellophane – but not the dialysis – membrane, was partially permeable to proteins of relatively large size (up to 100 kDa) (Kullnig *et al.,* 2000). Thus the data from both studies (Cortes *et al.,* 1998; Zeilinger *et al.,* 1999) were in perfect agreement and showed that *ech42* is expressed before contact of *Trichoderma* with its host, probably representing one of the earliest event in mycoparasitism and biocontrol. By using two types of membranes (one permeable and one not permeable to proteins), which allowed the removal of either *Trichoderma* or *Rhizoctonia* colony from the plate and thus the performing of subsequent cultivations, Kullnig *et al.* (2000) also showed that a chitinase activity, secreted constitutively by *Trichoderma,* is essential for the triggering of *ech42* gene expression. The nature of this enzyme is still unknown, but could very well be the 42-kDa endochitinase or the 102-kDa protein indicated by Inbar and Chet (1995). Interestingly, this activity is also present in a commercial Novozyme 234 enzyme preparation (Kullnig *et al.,* 2000) which can thus be used to purify it in significant amounts.

TOWARDS UNDERSTANDING THE NATURE OF THE HOST-DERIVED MOLECULES THAT INDUCE BIOCONTROL GENES. The availability of mutants in which the promoters of the biocontrol genes control the expression of vital reporter genes, such as GFP or detectable extracellular enzymes, have permitted studies at a molecular level of the mechanism involved in the activation of the antagonistic behaviour in *Trichoderma* biocontrol strains, and the occurrence of host-derived molecules that induce the biocontrol gene expression cascade (Mach *et al.,* 1999; Zeilinger *et al.,* 1999). It was found that digestion of the host cell walls with specific combinations of purified *Trichoderma*-secreted chitinases and glucanases (both endo- and exo-acting) releases products that are strong elicitors of *ech42*/*nag1* gene expression and consequent mycoparasitic activity. Interestingly, the same strain used different combinations of cell wall degrading enzymes to detect and respond to different fungal hosts, which suggests that the high redundancy of the *Trichoderma* chitinolytic and glucanolytic enzyme system serves to increase the host range. We have recently purified by HPLC and analyzed by electrospray mass spectrometry the low-molecular weight, biocontrol-inducing molecules released from the host cell walls. These compounds were much more active in vitro than purified chitin or glucan monomers or oligomers, and could be considered as the first identified biocontrol inducers for fungi (Lorito *et al.,* manuscript in preparation). They are being tested in different conditions to determine their activity *in vivo* and the direct effect on biocontrol performance of selected *Trichoderma* strains. Experiments are underway to determine if the purified elicitors activate mycoparasitic gene expression in situ (*i.e.* in strains applied on plant leaves), stimulate colony growth and enzyme production (as already observed in preliminary experiments), improve biocontrol/mycoparasitism performance *in vitro* and *in vivo* by increasing the 'aggressiveness' of the parasite or perhaps by reducing the efficacy of the host response. In addition, it would be interesting to determine the level of specificity of the mycoparasitism-inducing molecules, by determining, for instance, their efficacy on various *Trichoderma* strains or species. In fact, we are testing if the *Trichoderma* endochitinase-generat-

ed elicitors from *Rhizoctonia solani* cell walls also act as plant elicitors of PR protein expression if injected in leaves or other tissues (Lorito *et al.,* 1998). Obviously, the potential applications of host-derived molecules that have the ability to activate biocontrol and/or enzyme production are very appealing, and concern agriculture (*i.e.* they can be used to stimulate antagonistic activity of native strains reducing the need of a typical inundative applications) as well as the *Trichoderma*-mediated industrial production of extracellular enzymes (*i.e.* they can help to increase the yield and/or reduce the fermentation time).

GENETIC ENGINEERING OF *TRICHODERMA* BIOCONTROL. The obvious antifungal activity of *Trichoderma* chitinases has consequently lead to attempts to improve or alter biocontrol properties of strains by chitinase gene manipulations. Somewhat conflicting data have been reported on the effect of overexpression and/or deletion of selected chitinase genes of *Trichoderma*. Using *T. atroviride* IMI 206040, Carsolio *et al.* (1999) found no difference between an *ech42*-disrupted strain and its parent in the biocontrol activity in glasshouse tests against *Sclerotium rolfsii* and *R. solani* on cotton. They thus concluded that *ech42* is not essential for biocontrol activity. In contrast, Woo *et al.* (1999), investigating the effect of *ech42* gene disruption in *T. atroviride* P1, and Baek *et al.* (1999), performing similar investigations with *T. virens*, noted pronounced effects on the biocontrol efficacy of the fungus. The latter authors reported an increased and decreased biocontrol activity against *R. solani* on cotton in strains of *T. virens* containing two *ech42* copies and a disrupted *ech42* gene copy, respectively. In the study of Woo *et al.* (1999), *in vitro* assays of *B. cinerea* spore germination and hyphal elongation showed a significant reduction in antifungal activity for the *ech42* disrupted strain. The inhibitory effect of the culture filtrate could be fully recovered by adding 10 µg of purified 42-kDa endochitinase. Interestingly, no obvious differences between the disrupted strain and the parent were observed in the ability to overgrow either *B. cinerea* or *R. solani* in plate confrontation assays. However, *in vivo* tests against *B. cinerea* by leaf inoculations of bean plants revealed a significant reduction of biocontrol ability of the disrupted strain. In contrast, a significant increase in the biocontrol efficacy of soils heavily infested with *R. solani* was noted for the *ech42*-disrupted strain. Macro- and microscopic examinations of the attached seed coats after plant emergence indicated an apparent increase of *T. atroviride* growth on the surface, suggesting that the lack of the 42-kDa endochitinase may have stimulated the colonization of the spermo- and rhizosphere. This study therefore shows that manipulation of *ech42* can alter the biocontrol mechanism both in a positive as well as in a negative way. We are now constructing *T. atroviride* strains manipulated in other chitinase and also other hydrolase genes to learn how these enzymes affect biocontrol activity.

The only other chitinase, whose effect on biocontrol has been studied, is *chit33* from *T. harzianum* (Limon *et al.,* 1999). Using a constitutively expressed *pki1::chit33* fusion, recombinant strains with higher antagonizing activity against *R. solani* on agar plates were obtained. However, results from experiments with this mutants performed in glasshouse or soil have not been reported.

In addition, *Trichoderma* chitinase genes may be used to improve the resistance and defense mechanisms of plants. Towards this goal, Lorito *et al.* (1998) have transferred the *T. atroviride ech42* gene into tobacco and potato. This resulted in a nearly complete resistance to *R. solani*, *Alternaria solani, A. alternata*, and *B. cinerea*, which exceeded the levels of resistance previously achieved by transgenic expression of bacterial or plant chitinases. This strategy, confirmed also by subsequent studies (Bolar *et al.,* 2000), is now used in many laboratories to improve disease resistance of tobacco, potato, cabbage, broccoli, apple, tomato, grapes, lemon, wheat, rice, forest trees and several other plants (Lorito and Scala, 1999).

***CIS* AND *TRANS*-ACTING GENETIC FACTORS RELEVANT TO THE EXPRESSION OF BIOCONTROL GENES.** Despite the above described success, the possibility of manipulation of the *Trichoderma* chitinase genes by gene disruption/gene amplification strategies may be limited by the concentration of the *cis*- and *trans*-acting factors involved in their regulation. Therefore, we have recently initiated an in depth investigation on the factors regulating chitinase gene expression in this fungus.

As a first approach, we used the plant pathogen *B. cinerea* as the model host, confronted it on agar plates with *T. atroviride* P1, prepared crude protein extracts from mycelia harvested at different phases during mycoparasitism, and used them in electrophoretic mobility shift assays (EMSAs) with two promoter fragments of the *ech-42* gene of *T. atroviride* (Lorito *et al.,* 1996a). High-molecular weight protein-DNA complexes were obtained with all cell-free extracts, but the complexes obtained with extracts from mycelia during mycoparasitic attack exhibited a smaller size. Competition experiments, using oligonucleotides containing functional and non-functional consensus sites for binding of the carbon catabolite repressor Cre1 (Strauss *et al.,* 1995; Ilmen *et al.,* 1996), provided evidence that the complex from non-mycoparasitic mycelia involves the binding of

Cre1 to both fragments of the *ech-42* promoter. These findings are consistent with the presence of two and three consensus sites, respectively, for binding of Cre1 in the two *ech-42* promoter fragments used. In contrast, the protein-DNA complex from mycoparasitic mycelia does not involve Cre1, as its formation is unaffected by the addition of the competing oligonucleotides. Addition of equal amounts of protein of cell-free extracts from non-mycoparasitic mycelia converted the mycoparasitic DNA-protein-complex into the non-mycoparasitic complex. The addition of a purified Cre1-glutathion-S-transferase fusion protein to mycoparasitic cell-free extracts produced the same effect. These findings allowed us to present a preliminary model for regulation of *ech-42* expression in *T. harzianum* which involves at least (*i*) binding of Cre1 to two single sites in the *ech-42* promoter; (*ii*) binding of a 'mycoparasitic' protein/protein complex to the *ech-42* promoter in vicinity of the Cre1 binding sites; and (*iii*) functional inactivation of Cre1 upon mycoparasitic interaction to enable the formation of the 'mycoparasitic' protein-DNA complex (Lorito *et al.,* 1996a).

Some of our recent results, previously discussed in this review, have for the first time provided evidence for a role of stress-response reactions in the triggering of *ech42* gene transcription, although it is notable that only some forms of stress (osmotic pressure, ethanol, low temperature) were effective (Mach *et al.,* 1999). A regulation of *ech42* gene transcription by stress would also be consistent with the findings of four stress-response elements (AGGGG) on its promoter, which in *Saccharomyces cerevisiae* bind the zinc finger transcription factors Msn2p and Msn4p and are capable of mediating a number of different stress responses, including nutrient deprivation and others applied in our experiments with *Trichoderma* (Marchler *et al.,* 1993; Treger *et al.,* 1998). Corresponding AGGGG-elements are present also in two other *Trichoderma* chitinase (*nag1*, *chit33*) promoters (Mach *et al.,* 1999; de la Mercedes *et al.,* 2000). Thus, we have recently cloned the *T. atroviride* homologue of *MSN2/MSN4.* It encodes a DNA-binding protein whose zinc finger domain has a high aa-similarity to that of Msn2 and Msn4, but lacks any similarity to the other domains of these yeast proteins (Fig. 2). A fragment of the *Trichoderma* protein expressed in *E. coli*, indeed bound specifically to the AGGGG-box (C.K. Peterbauer, R.L. Mach and C.P. Kubicek, unpublished data). A gene disruption study is now under way to indentify the eventual role of this regulator gene in chitinase gene expression and mycoparasitism in *T. atroviride*.

In addition to the AGGGG-binding protein, which may also regulate *ech42* gene expression in response to starvation (Marchler *et al.,* 1993), we have recently identified another element that may contribute to this regulatory mechanism. The *ech42* promoter sequence contains two short nucleotide sequences which resemble the consensus for binding of the *Aspergillus nidulans brlA* (*bristle*) regulator (5'-MRAGGGR-3'; Chang and Timberlake, 1992). The encoded BrlA protein is a general regulator of conidial development, which itself responds to carbon starvation (Skromne *et al.,* 1995). Cell-free extracts of *T. atroviride*, prepared from mycelia subjected to carbon starvation, form a specific, consensus-dependent complex with BrlA site-containing oligonucleotide fragments of the *ech42* promoter (K. Brunner, C.K. Peterbauer and C.P. Kubicek, unpublished data). This provides a preliminary support to the idea of an involvement of BrlA in transcription of biocontrol genes.

Since the expression of *nag1* (encoding a 73-kDa N-acetyl-β-D-glucosaminidase) can be induced within a very short time (2 h) by the addition of purified low molecular chitooligosaccharides (Mach *et al.,* 1999), we have used this gene to study the involvement of various factors in the induction of chitinase gene expression by

Fig. 2. Zinc finger region of yeast Msn2 and Msn4 and the putative stress regulator isolated from *T. atroviride*. EST fragments of the *A. nidulans* and *F. sporotrichioides* data bank with amino acid sequence similarity to the stress regulator (http://www.genome.ou.edu/fungal.html) are included for comparison.

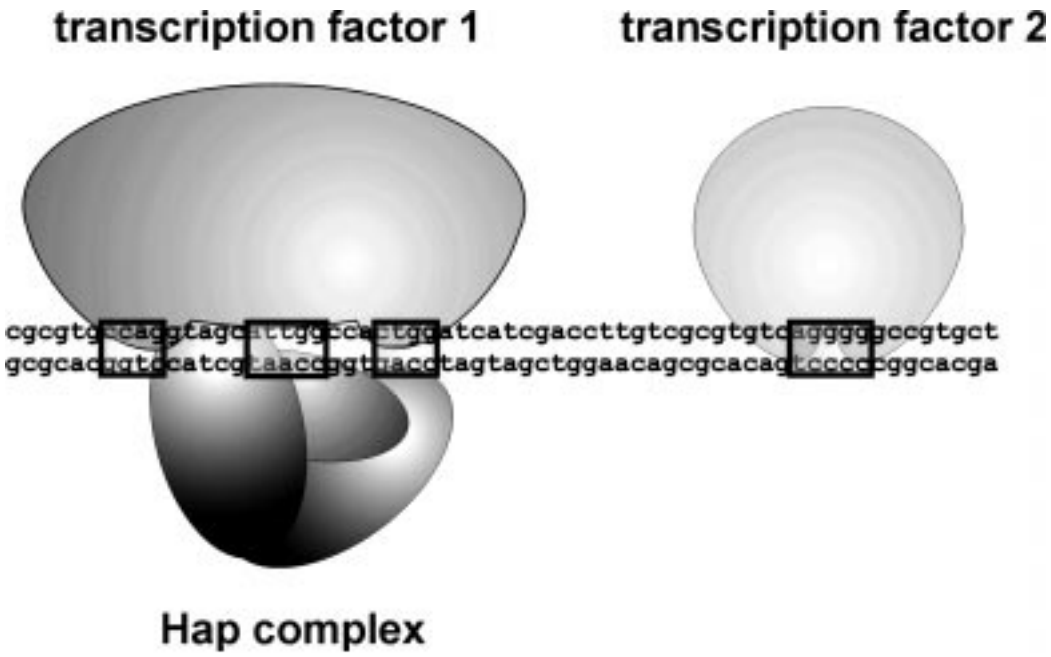

Fig. 3. Hypothetical model of regulation of *nag1* gene expression in *T. atroviride*.

chitin oligomers. Using a combination of promoter deletion, *in vivo* footprinting, and EMSA experiments, we have investigated the regulation of the *nag1* gene of *T. atroviride* P1 by N-acetyl-glucosamine. The results revealed protein binding to the AGGGG-element at –240, to a $CCAGN_{13}CTGG$ motif at –284 and to a CCAAT-box found in the spacer of the latter motif (Fig. 3). The organization of the binding site at –284 would be compatible with the binding of a Zn(II)2Cys6-type zinc finger protein (Todd *et al.,* 1997), whereas the CCAAT-box binds a protein complex consisting of at least three proteins Hap2, Hap3 and Hap5, which were originally described in *S. cerevisiae* and more recently characterized from the filamentous fungi *A. nidulans* (Steidl *et al.,* 1999) and *T. reesei* (Zeilinger *et al.,* 2001). Interestingly, Narendja *et al.* (1999) have recently demonstrated that the main functions of the Hap-proteins is the establishment of an open chromatin structure.

CONCLUSIONS

The current evidence, as summarized in this review, documents that some chitinases such as the 42-kDa endochitinase can have an enormous impact on the biocontrol ability of *Trichoderma* spp., thereby affecting biocontrol both positively and negatively. As most of the data available have been reported only with one species of *Trichoderma*, *i.e. T. atroviride*, it cannot be ruled out that other chitinases may prove similarly critical for biocontrol by other species such as *T. harzianum*. A comparative investigation of the role of chitinases in different biocontrol species therefore seems to be highly warranted. In addition, the identification of *cis*- and *trans*-acting factors which control chitinase gene expression – besides increasing our understanding on how some fungi recognize others – will enable us to exogenously control the biocontrol activity of *Trichoderma in planta* and in the field, and to design promoters for time-specific expression of other antifungal genes, such as those involved in the production of secondary metabolites.

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