Mol Gen Genet (1998) 260: 218–225

ORIGINAL PAPER

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The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor

Received: 8 June 1998 / Accepted: 28 July 1998

Abstract The mycoparasite *Trichoderma harzianum* has been extensively used in the biocontrol of a wide range of phytopathogenic fungi. Hydrolytic enzymes secreted by the parasite have been directly implicated in the lysis of the host. Dual cultures of *Trichoderma* and a host, with and without contact, were used as means to study the mycoparasitic response in *Trichoderma*. Northern analysis showed high-level expression of genes encoding a proteinase (*prb1*) and an endochitinase (*ech42*) in dual cultures even if contact with the host was prevented by using cellophane membranes. Neither gene was induced during the interaction of *Trichoderma* with lectin-coated nylon fibres, which are known to induce hyphal coiling and appressorium formation. Thus, the signal involved in triggering the production of these hydrolytic enzymes by *T. harzianum* during the parasitic response is independent of the recognition mediated by this lectin-carbohydrate interaction. The results showed that induction of *prb1* and *ech42* is contact-independent, and a diffusible molecule produced by the host is the signal that triggers expression of both genes in vivo. Furthermore, a molecule that is resistant to heat and protease treatment, obtained from *Rhizoctonia solani* cell walls induces expression of both genes. Thus, this molecule is involved in the regulation of the expression of hydrolytic enzymes during mycoparasitism by *T. harzianum*.

Key words Biocontrol · Mycoparasitism · Chitinase · Protease · Cell wall

Communicated by J. Schell

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Introduction

Biological control is a promising tool to help maintain current levels of agricultural production while reducing the release of polluting chemical pesticides to the environment. However, more knowledge of the mechanism of action of the biological agents involved is needed to improve their efficacy. The ability of some species belonging to the genus *Trichoderma* to parasitize other fungi has been exploited for the control of a broad range of phytopathogenic fungi. Some *Trichoderma* species kill their host – often in advance of contact and penetration – and are considered necrotrophic mycoparasites (Papavizas 1985).

Early in the process of parasitization of *Rhizoctonia solani* by *Trichoderma harzianum*, directed hyphal branching by the mycoparasite appears to be induced as a chemotropic reaction to the presence of the host fungus (Chet and Baker 1981). Subsequently, the hyphae of *T. harzianum* coil tightly around those of *R. solani*. It has recently been shown that a purified lectin from *Sclerotium rolfsii* induces coiling of hyphae of *T. harzianum* and the formation of mycoparasitism-related structures (e.g., hooks and appressorium-like bodies) around nylon fibres coated with the lectin, and thus simulates the interaction with the host (Inbar and Chet 1992). Similar lectins have been identified in *R. solani* and other fungal host species (Jeffries and Young 1994). Thus, lectins present on the cell wall of the host are suggested to take part in its recognition. The use of light and scanning electron microscopy has demonstrated the penetration of hyphae of *R. solani* by *T. harzianum* (Elad et al. 1983). A complex set of extracellular enzymes is produced by

mycoparasitic strains of *Trichoderma* when grown on isolated cell walls of *R. solani* (Ridout et al. 1986). Thus, lytic enzymes such as β-1,3-glucanases, chitinases and proteases are probably responsible for hyphal lysis through the digestion of major cell wall components. The chitinolytic system of *T. harzianum* comprises six distinct enzymes, two of which are classified as β-1,4-N-acetylglucosaminidases and the rest as endochitinases (Haran et al. 1995). Three chitinase-encoding genes have been cloned and characterized, namely *chit 33* (*ech33*; Limón et al. 1995), *ech42* (Carsolio et al. 1994; Hayes et al. 1994) and *nag1* (Peterbauer et al. 1996). However, the relevance of the corresponding enzymes to mycoparasitism remains to be proven. The gene *ech42* coding for the endochitinase Ech42 is highly expressed when the fungus is grown on media containing chitin or in dual cultures with a host (direct confrontation assays; Carsolio et al. 1994). Another mycoparasitism-related gene, *prb1*, encoding a 31-kDa basic proteinase is strongly induced during direct confrontation experiments. Strong expression of *prb1* is also observed when *T. harzianum* is grown in media containing chitin as a sole carbon source (Geremia et al. 1993). Furthermore, transgenic *Trichoderma* strains carrying multiple copies of *prb1* showed a much higher level of control of the disease caused by *R. solani* (Flores et al. 1997), suggesting that this gene is indeed directly involved in mycoparasitism.

It may be expected that the production of hydrolytic enzymes takes place simultaneously with the formation of parasitic structures and that both events might be activated by a common mechanism. However, the recognition events at the molecular level are not yet clear. Oligosaccharides containing N-acetyl-D-glucosamine (GlcNAc), including chitin oligomers, which are components of the potential host, might act as elicitors, enabling the invader to trigger the so-called mycoparasitic reaction (Elad et al. 1983; Benhamou and Chet 1993; Lora et al. 1994). Inbar and Chet (1994, 1995) have shown an increase in the activity of an intracellular 102-kDa glucosaminidase using a biomimetic system based on the binding of a purified lectin from *S. rolfsii* to nylon fibres, suggesting that the recognition event mediated by lectins serves as a signal that triggers a general antifungal response in *Trichoderma*. However not a single mycoparasitism-related gene has been directly associated with the response to the lectin-carbohydrate interaction. The mycoparasitic relationship between *Trichoderma* and its potential host might involve biochemical and physiological reactions that precede the microscopically visible phenomena of hyphal coiling, appressorium formation, penetration and cytoplasmic degradation.

In the present study, the possible involvement of lectins in the induction of two hydrolytic enzymes in *T. harzianum* during mycoparasitism was investigated. A novel system was used to analyze the parasitic response in terms of its dependence on contact with the host.

Materials and methods

Strains and plasmids

T. harzianum strain IMI206040 was used throughout this work. *Sclerotium rolfsii* type A (ATCC 26325) was used for the preparation of the lectins used in the biomimetic assays. *Rhizoctonia solani* AG-1 and *S. rolfsii* were used in direct confrontation assays. Plasmid pPrBg78 is a derivative of pT3T7Lac carrying a 5.5-kb fragment that includes the basic proteinase gene *prb1* (Flores et al. 1997). Plasmid quiAc38 is a derivative of pSPORT (Gibco-BRL, Madison, Wis.) carrying a full-length cDNA derived from the endochitinase gene *ech42* (Carsolio et al. 1994).

DNA and RNA manipulations

Total RNA was isolated essentially according to the protocol previously described (Jones et al. 1985). Northern analysis were performed by standard procedures (Sambrook et al. 1989). DNA probes were labeled with a random primer kit (Dupont, Boston, Mass.).

Biomimetic system

Lectins from *S. rolfsii* were purified and bound to nylon fibers as previously described (Inbar and Chet 1994). Briefly, *T. harzianum* was grown on a cellophane membrane placed on a Petri dish containing synthetic medium (SM) (Okon et al. 1973) supplemented with 0.2% (w/v) glucose, in the presence of lectin-coated nylon fibers or nylon fibers without lectin. After 48 h of incubation, mycelium was collected, frozen in liquid nitrogen and used for RNA extraction.

Direct confrontation assays

T. harzianum confrontations with *R. solani, S. rolfsii* or *T. harzianum* itself were carried out on plates of solid minimal medium (MM) supplemented with 0.2% glucose covered with a cellophane sheet basically as described (Carsolio et al. 1994). Confrontation assays without contact were carried out essentially in the same way except that the *R. solani* colony was covered with two cellophane sheets before placing the *T. harzianum* agar plug on top. After 36, 48, 60 or 72 h, when overgrowth is observed, mycelia from the area of interaction was recovered. A schematic representation of this assay is shown in Fig. 2a. Control plates, covered with cellophane, were inoculated with *T. harzianum* alone. The ability of the double cellophane barrier to prevent contact was checked by light microscopy in all experiments. All fungi were grown and manipulated in the dark.

Expression of the mycoparasitism-related genes in submerged cultures

Cell wall induction. *T. harzianum* was inoculated (2.5×10^8 conidia) in 125-ml flasks containing 25 ml of half-strength Potato Dextrose Broth (PDB; Difco, Detroit, Mich.), and grown on a rotary shaker for 14 h at 28° C. Mycelia were collected by centrifugation at 3000 rpm for 15 min, transferred to MM at pH 5.5, containing 0.4% glycerol, and incubated for 22 h. After a second centrifugation mycelia were transferred to MM without any carbon source and incubated 2 h at 28° C. Mycelia were collected, washed, divided into five portions and transferred to 25 ml of MM supplemented with *R. solani* cell walls, treated cell walls or a soluble fraction of cell walls obtained by heat treatment.

Chitin induction. *T. harzianum* was grown essentially as described above except that, after the first centrifugation step, it was transferred to MM pH 5.5 and incubated for 12 h. After the second

centrifugation, mycelia were transferred to MM with 2% glycerol or a combination of 2% glycerol and 0.75% chitin.

In both cases, mycelia were allowed to grow for the indicated time under these conditions, collected by filtration, frozen in liquid nitrogen and kept at −70° C until use.

Cell wall treatment

R. solani cell walls were allowed to stand in 2 M NaCl for 2 h, boiled in 2% SDS for 5 min and extensively washed with distilled water (NS), or extracted with chloroform-methanol (2:1) (CM).

Preparation of the soluble fraction and determination of the size of the inducer

R. solani cell walls (0.2% in water) were autoclaved and filtered through Whatman paper No. 5 and the filtrate used as inducer. To determine the size of the inducer, 20 ml of the filtrate was then subjected to ultrafiltration using a YM30 membrane in an Amicon system. Where indicated the total soluble fraction was incubated for 15 min at 37° C with 100 µg/ml of Pronase (Lakeside) and Proteinase K (GIBCO-BRL).

Results

Expression of *prb1* and *ech42* in the biomimetic system

To test whether the purified lectin from *S. rolfsii*, previously implicated in recognition, is also involved in the induction of the mycoparasitism-related genes *prb1* and *ech42*, RNA from *T. harzianum* grown in the presence of either lectins bound to nylon fibres or fibres alone was extracted and subjected to Northern analysis (Fig. 1). Hybridization with *prb1* and *ech42* probes showed that there was no expression of either of the two genes in the biomimetic system (lanes 1 and 2), even though a formation of mycoparasitism-related structures was clearly observed (data not shown). In contrast, a significant induction of *prb1* and *ech42* was detected during direct confrontation assays using both *S. rolfsii* and *R. solani* as hosts (lanes 4 and 6). These results suggest that lectins are not the signal that triggers the induction of at least two genes involved in the mycoparasitic response of *T. harzianum*.

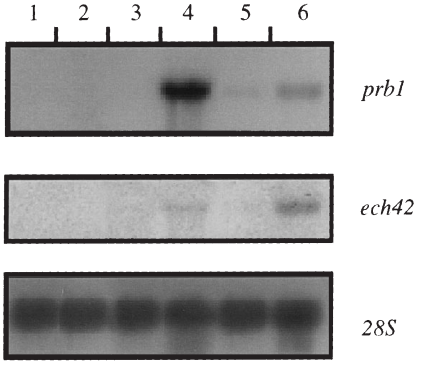

Fig. 1 Expression of *prb1* and *ech42* in direct confrontation assays and in the biomimetic system. Twenty micrograms of total RNA from each sample were subjected to Northern analysis, with the following probes: a *Pst*I fragment of the *prb1* gene, the *ech42* cDNA clone, and a human 28S ribosomal RNA clone, as indicated. The RNAs were obtained from the following sources: *T. harzianum*: grown in the presence of nylon fibers (lane 1); grown in the presence of lectin-coated nylon fibers (2); recovered from the area of interaction with *Rhizoctonia* at 60 h (4); recovered from the area of interaction with *S. rolfsii* at 72 h (6); lanes 3 and 5 contained RNA from a control culture of *T. harzianum* grown in the absence of the host for 60 and 72 h, respectively

Induction of *prb1* and *ech42* in *T. harzianum* is contact independent

In order to investigate the dependence of gene expression on host contact in mycoparasitism by *T. harzianum*, we developed a system in which confrontation between *Trichoderma* and *R. solani* occurs but contact is avoided (Fig. 2A). Mycelia of *Trichoderma* were collected after 36 or 48 h of interaction. In both cases *T. harzianum* was overgrowing the colony of *Rhizoctonia*. Northern analysis of total RNA from these samples was carried out

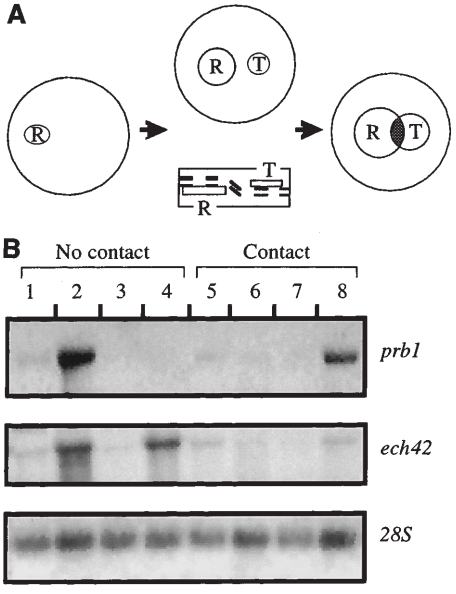

Fig. 2 **A** Schematic representation of the direct confrontation assay without contact. *T. harzianum* vs *R. solani*. The *outer circle* represents the plate. *Inner circles* represent colonies of the fungi of interest: *R. solani* (R) and *T. harzianum* (T). The *rectangle* represents a transverse view of the confrontation, *broken lines* represent two cellophane sheets placed between the colonies. Mycelia were collected from *shaded areas* (interaction zone). **B** Expression of *prb1* and *ech42* in direct confrontation assays and on confrontation without contact. The Northern blot was prepared and hybridized with the probes indicated in the legend to Fig.1. RNAs loaded in the different lanes were obtained from: control cultures of *T. harzianum* grown in the absence of *R. solani* at 36 and 48 h, respectively (lanes 1 and 3, and 5 and 7); the area of interaction between *T. harzianum* and *R. solani* – without contact – at 36 and 48 h, respectively (2 and 4); the area of interaction between *T. harzianum* and *R. solani* – with contact – at 36 and 48 h, respectively (6 and 8)

using *prb1* and *ech42* as probes. It was clear that even where contact was avoided, *Trichoderma* strongly expressed *prb1* mRNA at 36 h (Fig 2B, lane 2), expression drops to a basal level after 48 h (lane 4). Induction of *ech42* showed a similar pattern, but in this case, expression was sustained for up to 48 h (Fig 2B, lane 4). On the other hand, during direct confrontation assays (with contact) expression of these two genes increased with time, reaching a maximum at 48 h (lane 8), as previously shown (Carsolio et al. 1994; Flores et al. 1997). Surprisingly, induction of *prb1* and *ech42* during interaction without contact was stronger than during direct confrontation assays. These data show that the induction of the genes encoding cell wall-degrading enzymes is contact independent. Furthermore, these experiments suggest that a diffusible molecule from the host is required to act as an elicitor that triggers the expression of both *ech42* and *prb1*.

Direct confrontation assays were carried out in which *Trichoderma* was inoculated on opposite sides of a petri dish and grown until contact was made to verify that the expression of *ech42* and *prb1* observed during interaction with *R. solani* with and without contact was a parasitism-specific response and not due simply to starvation provoked by the presence of a competing colony. A similar experiment in which contact was prevented was also carried out to compare the responses. Cultures were incubated in the dark and mycelia were collected after 36 and 48 h. Total RNA was extracted, and a Northern assay was carried out on these samples. No induction of *prb1* (Fig. 3, lanes 1, 2, 5, and 6) was observed in either case, as compared with the control grown alone (lanes 3 and 4) and *ech42* was expressed only at nearly undetectable levels (data not shown), indicating that the response observed in experiments with *R. solani* was specific, and not simply due to the decrease in the availability of nutrients.

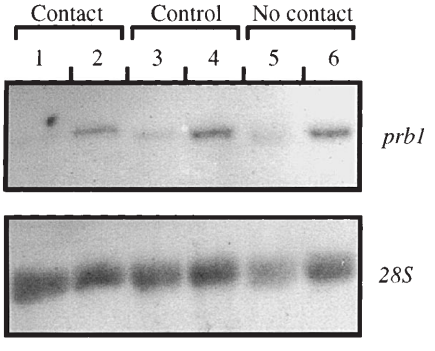

Fig. 3 Northern analysis of the expression of *prb1* on confrontation of *T. harzianum* with *T. harzianum* with and without contact. The Northern blot was prepared and hybridized with the probes indicated as described in the legend to Fig.1. RNAs were obtained from: the area of *Trichoderma-Trichoderma* interaction – with contact – at 36 and 48 h, respectively (lanes 1 and 2); *T. harzianum* grown alone for 36 and 48 h, respectively (3 and 4); and the area of *Trichoderma-Trichoderma* interaction without contact at 36 and 48 h, respectively (5 and 6)

A soluble fraction obtained from the *R. solani* cell wall induces expression of *prb1* and *ech42*

In order to test if the postulated diffusible molecule might be derived from the cell wall of the host, mycelia from *Trichoderma* cultures were grown in liquid medium containing *R. solani* cell walls or a soluble fraction obtained from them. Cultures grown in minimal medium were used as a control. RNA was extracted from the samples and subjected to Northern analysis, using *prb1* and *ech42* as probes. When *Trichoderma* was grown in the presence of cell walls or the soluble fraction, the *prb1* and *ech42* mRNAs were both strongly expressed at 24 h (Fig. 4a, lanes 4 and 6). Very low expression was detected in the control experiment (Fig. 4a, lanes 1 and 2). These experiments demonstrated that a soluble component released from the cell wall of *R. solani* is sufficient to induce the expression of genes associated with mycoparasitism. In addition, cell walls treated with either NaCl/SDS (Fig. 4a, lane 8) or chloroform/methanol (Fig. 4a, lane 9) also induced expression of *prb1* and *ech42* mRNAs at the same level as untreated cell walls (Fig. 4a, lane 4). To learn something of the size and nature of the inducer(s), the soluble fraction of *R. solani* cell walls was subjected to ultrafiltration through a membrane with a 30 kDa cut off. Both the filtrate and the retained fraction were used in induction experiments. The filtrate strongly induced both genes, whereas the retained fraction induced expression of the genes only poorly (data not shown). Furthermore, after treatment with proteases, the soluble fraction still induced the expression of both *prb1* and *ech42* (Fig. 4b, lane 2).

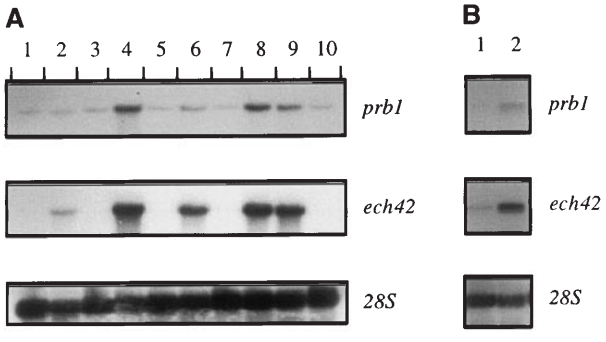

Fig. 4A,B Analysis of the expression of *prb1* and *ech42* of *T. harzianum* in submerged cultures grown in the presence of different cell wall fractions. The Northern blot was prepared and hybridized with the probes indicated as described in the legend to Fig 1. RNAs were obtained from *T. harzianum* grown in MM with (**A**) no carbon source (lanes 1 and 2); *R. solani* cell walls (3 and 4); *R. solani* cell wall, soluble fraction (5 and 6); NS-treated cell walls (7 and 8); CM-treated cell walls (9 and10). Odd- and even-numbered lanes contained samples collected 6 and 24 h after induction, respectively. **B** *T. harzianum* was grown in the absence of a carbon source (lane 1) or in the presence of the protease-treated, soluble cell wall fraction (2)

Induction of *prb1* and *ech42* by chitin

Cell wall-degrading enzymes, including Ech42 and Prb1, are highly expressed and secreted during growth of *T. harzianum* in liquid medium containing chitin as sole carbon source. In contrast, when grown on glucose as sole carbon source both enzymatic activity and mRNA levels are very low (Geremia et al. 1993; Carsolio et al. 1994). However, true induction by chitin has not been proven. To study in more detail the possibility of coordinate induction of *ech42* and *prb1* genes by this homopolymer, RNAs from mycelia grown under inducing conditions were compared by Northern analysis with the corresponding probes. Figure 5 shows the results for cultures grown with agitation in media containing glycerol-chitin or glycerol. The levels of *prb1* RNA in cultures grown in the glycerol-chitin media were clearly higher than the levels in glycerol alone, reaching a peak at 72 h and decreasing by 110 h under both conditions. On the other hand, levels of *ech42* RNA gradually increase in chitin-induced cultures, reaching a maximum at 110 h. No signal was detected at any time with this probe when glycerol was used as sole carbon source in the culture (Fig. 5, glycerol).

Discussion

Trichoderma species display strong antagonistic activity against many soil and root-dwelling fungi. The success of *Trichoderma* as a biocontrol agent is believed to involve various modes of action, including antibiotic production, secretion of lytic enzymes and direct penetration of the host hyphae. A given *Trichoderma*-host interaction may involve any of these mechanisms individually or encompass more than one of them acting simultaneously. The hydrolytic enzymes produced by *Trichoderma* may play a key role in its ability to penetrate and kill a host fungus. Genes coding for an endochitinase (*ech42*) and a proteinase (*prb1*) from *T. harzianum* have been cloned, characterized and directly implicated in its mycoparasitic response (Geremia et al. 1993; Carsolio et al. 1994). Common substrates are implicated in the induction of both genes and they show similar glucose repression patterns. Thus, it has been speculated that a common induction pathway involving a specific inducer activates all hydrolytic enzymes produced by *Trichoderma* during mycoparasitism. The possibility that the whole cascade of antagonistic events during parasitism by *T. harzianum* might be regulated by a common mechanism has previously been discussed (Schirmböck et al. 1994). Lora and coworkers (Lora et al. 1994) proposed that oligosaccharides containing GlcNAc could act as elicitors which might trigger a general antifungal response in *Trichoderma*, including the production of hydrolytic enzymes.

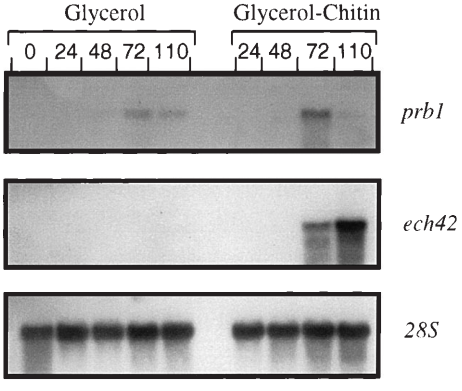

Fig. 5 Expression of *prb1* and *ech42* of *T. harzianum* in submerged cultures grown in the presence of glycerol and chitin. The Northern blot was prepared and hybridized with the probes indicated as described in the legend to Fig.1. RNAs were obtained from *T. harzianum* grown: in glycerol for 0, 24, 48, 72 and 110 h (lanes 1–5); or in the presence of glycerol and chitin for 24, 48, 72 and 110 h (6–9)

In the biomimetic system developed by Inbar and Chet (1992) morphological changes including coiling and appressorium formation have been observed. The same authors demonstrated that coiling of *Trichoderma* around the host hyphae is an early event mediated by lectin-carbohydrate interactions. To address the question of whether hydrolytic enzymes can be induced by the purified lectin of *S. rolfsii,* the expression of *ech42* and *prb1* was tested in *T. harzianum* that was subjected to the biomimetic system. We clearly show here that no induction could be detected when *Trichoderma* was grown on nylon fibres coated with the lectin, even though coils and hooks were observed by light microscopy (data not shown). It therefore appears that the morphological changes associated with the lectin-carbohydrate interactions are independent of the induction of the secreted cell wall-hydrolyzing enzymes.

It is generally believed that mycoparasitism in *Trichoderma* is controlled by an ordered series of events that might be regulated by common signals. The fact that *prb1* and *ech42* are not induced in the biomimetic assays could be explained by the lack of a triggering factor. Thus, recognition through lectins is not sufficient to trigger the complete response observed during the *Trichoderma*-host interaction. Direct confrontation assays have been shown to be a powerful tool with which to study in detail the phenomenon of mycoparasitism by *Trichoderma* (Carsolio et al. 1994; Flores et al. 1997). The use of cellophane membranes placed between the mycoparasite and the host helped us to define the relevance of hyphal contact during mycoparasitism. When contact was prevented induction of *prb1* and *ech42* was even stronger than in the assay with contact. The growth of *R. solani* was clearly inhibited during interactions without contact, suggesting that the cellophane membranes permit free diffusion of molecules from *T. harzianum* to *R. solani* and vice versa. That *prb1* and *ech42* expression occurs during confrontation assays without contact indicates that diffusible molecules produced by the host trigger the induction of both genes. However, whether the presence of the mycoparasite is required to

induce the release of such molecules is still not clear. Studies on *Trichoderma-Trichoderma* interactions demonstrated that induction of *prb1* and *ech42* is a response to the presence of the host and not only to nutrient limitation provoked by the competing colony, as previously speculated. No induction of *prb1* or *ech42* was detected during *T. harzianum vs T. harzianum* confrontation assays. Interestingly, no overgrowth was observed when *Trichoderma* reached the opposite colony even when contact was prevented by the double cellophane membrane. This observation suggests that self recognition is also mediated by a diffusible molecule and is contact independent. Thus, the signals involved in sensing a potential host and those implicated in self-recognition might share common pathways.

The demonstration of the existence of a molecule capable of inducing the *prb1* and *ech42* genes was carried out using soluble fractions of *R. solani* cell walls. When *T. harzianum* was grown in the soluble fraction a clear induction of *prb1* and *ech42* was detected. The inducing capacity of this fraction implies that a molecule solubilized from the cell walls can act as an inducer. To study further the general characteristics of such an inducing molecule, the soluble fraction was subjected to ultrafiltration and both fractions were used in induction assays. The filtrate fraction clearly induced *prb1* and *ech42*, whereas the retained fraction produced a very poor response (data not shown). These data suggest that the molecule involved in the induction of both genes has a molecular mass of less than 30 kDa. Although the nature of the inducer is still unclear it is likely that polysaccharides or heat resistant protein-carbohydrate molecules are involved. The results obtained with the protease-treated molecule(s), and the fact that cell walls treated to remove non-covalently linked proteins (N/S) and lipids (C/M) induce to the same levels as untreated cell walls strongly supports the idea that the molecule(s) is polysaccharide in nature. Interestingly, induction of *prb1* and *ech42* with C/M-treated cell walls is more rapid, suggesting that lipids might interfere with the release of the inducer from the cell walls.

Induction of *prb1* and *ech42* by chitin was demonstrated in a medium containing 2% glycerol. This carbon source was previously used to study cellulase regulation in *T. reesei* without significantly affecting the growth of the fungus (Ilmen et al. 1997). Our results show that proteinase and chitinase mRNAs accumulate in fungi grown on glycerol in the presence of the inducer (chitin), reaching a maximum at 72 h or 110 h, respectively. This is the first evidence that chitin is a true inducer of both *prb1* and *ech42* expression under conditions where starvation conditions were avoided. Low levels of expression of *prb1* mRNA were observed during cultivation with glycerol alone. In contrast, no chitinase mRNA could be detected under control conditions. The difference observed in the time of induction between both genes might reflect a coordinated regulation where proteinase is first required to release the inducing molecule that also triggers chitinase expression.

Comparison of the 5′-flanking DNA sequences of co-regulated genes may be useful to identify conserved regulatory sequences (Gwynne et al. 1987). Such regions could constitute binding sites for either general or specific regulatory proteins. Thus, this strategy was used to identify promoter elements that might be involved in the coordinated expression of *prb1* and *ech42*. Promoter alignments revealed the presence of four identical sequences, named MYC1, MYC2, MYC3 and MYC4 in both promoters (Fig. 6), localized at similar positions relative to the corresponding CAAT box. The MYC2 box is located exactly 83 bp upstream of the CAAT box in both promoters. Recent results show that transgenic *Trichoderma* strains obtained using an *ech42* clone lacking MYC4 have much lower chitinase activity than those carrying the complete promoter (manuscript in preparation). These data suggest that this motif is necessary to obtain full expression of the chitinase gene.

The *prb1* promoter displayed a single consensus site (5′ SYGGRG 3′) for binding of the carbon catabolite repressor CreA (Sophianopoulou et al. 1992), located 963 bp upstream of the CAAT box. A total of seven GATA sites are present in the *prb1* promoter. The core GATA sequences are recognized by the major positively acting regulatory elements *Are A* in *Aspergillus nidulans*, *Nit-2* in *Neurospora crassa* and *GLN3* in *Saccharomyces cerevisiae* (Fu and Marzluf 1987; Kudla et al. 1990; Minehart and Magasanik 1991), which mediate global nitrogen repression and derepression. The role of these sequences in the regulation of *prb1* requires further investigation.

The presence of binding sites for the *A. nidulans* carbon-response regulator CreA in the *ech42* gene has previously been reported (Carsolio et al. 1994; Lorito et al. 1996). Four consensus target sites for binding of

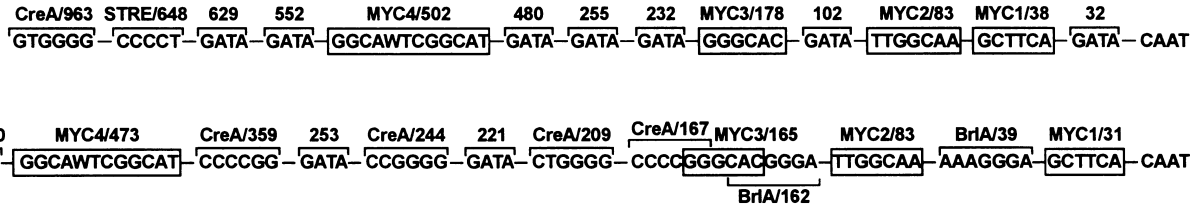

Fig. 6 Analysis of *prb1* and *ech42* promoter sequences. Discontinuous nucleotide sequence of the 5′ upstream regions of the *T. harzianum prb1* and *ech42* genes. The *boxed sequences* correspond to putative binding sites for regulatory proteins involved in mycoparasitism (MYC). Sequences in *brackets* correspond to consensus binding sites for previously described transcriptional regulators. The numbers indicate the position relative to the CAAT box. Sequence comparisons were made using the program DNASIS

this transcriptional regulator were identified in the 5′ upstream sequence of the *ech42* gene (see Fig. 6). Additionally two GATA sequences were located 221 and 253 bp upstream of the CAAT box. The MYC3 box (-GGGCAC-) overlaps one of the CreA binding sites by 2 bp (-GG-). This kind of arrangement is compatible with the model proposed by Lorito et al. (1996), in which target sequences for Cre1 and mycoparasitism-specific complexes overlap or are close together. Thus, it is likely that MYC3 is the target sequence for a my coparasitism regulator in *T. harzianum*.

The non-palindromic pentanucleotide CCCCT (C_4 T) is an upstream element implicated in the regulation of several yeast genes in response to stress (Kobayashi and McEntee 1993). This C_4 T sequence is present in the *prb1* and *ech42* promoters 648 and 820 bp upstream of the CAAT box, respectively. The presence of this sequence could explain the fast induction (4 h) of *prb1* observed when *Trichoderma* is grown in a media containing cell walls as sole carbon source, which combines stress conditions and the presence of an inducer (Flores et al. 1997). In contrast, our results showed that induction of the proteinase gene is much delayed, occuring at 48 h, in a medium containing the inducer and an alternative carbon source. Whether the proposed regulatory boxes are functional remains to be investigated.

Our data strongly support the hypothesis that an inducing factor is required for the expression of mycoparasitism-related genes. However, nutrient availability and/or other types of stress might still play an important role in the control of this set of genes, as suggested by the presence in their promoters of the different cis-acting regulatory elements described above.

Acknowledgements We wish to thank Drs. June Simpson, Luis Herrera-Estrella and Rafael Rivera for critical reading of the manuscript. V. O. and C. C. are indebted to CONACYT for doctoral fellowships.

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