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Co-expression of two genes, a chitinase (*chit42*) and proteinase (*prb1*), implicated in mycoparasitism by *Trichoderma hamatum*

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Abstract: Mycoparasitism of fungal plant pathogens by *Trichoderma* species is a complex process that involves the production and co-ordinated secretion of cell-wall degrading enzymes. Genes implicated in mycoparasitism by *Trichoderma atroviride* contain motifs in the promoter region, designated MYRE1-MYRE4, that are proposed to act as binding sites for a global inducer of the mycoparasitic response. The aim of our study was to establish whether these motifs also were present in *Trichoderma hamatum* and whether the presence of these motifs could predict co-expression when *T. hamatum* was confronted by a pathogen. Using a combination of targeted, degenerate and inverse PCR, homologues of the mycoparasitism-related genes *ech42* (*chit42*), *prb1* and *lam1.3* (*xbg1.3-110*), which encode an endochitinase, proteinase, and β-1,3-glucanase, respectively, were cloned and sequenced from *T. hamatum.* Alignment of the promoter regions of the three genes revealed identical regions in the *chit42* and *prb1* promoters, which were 6–9 base pairs in length and conserved in position. Specifically, the regulatory motifs MYRE1-MYRE4 were fully conserved, together with a fifth motif, identified by this research. A substrate assay designed to investigate the response of these genes from *T. harzianum* and *T. hamatum* to a simple carbon source (glycerol) showed that, in contrast to *chit42* and *prb1, xbg1.3-110* was not expressed. Further comparison of the expression patterns of these three genes between *T. harzianum* and *T. hamatum* using the glycerol substrate assay showed that no *chit42* or *prb1* expression could be detected in *T. harzianum* when it was grown under the same conditions as *T. hamatum.* This showed that the response of these genes to glycerol was species specific and that a single expression pattern for these genes was not common to all *Trichoderma* species. Confrontation assays were used to investigate the response of the three *T. hamatum* genes to the more complex substrate posed by the fungal pathogen *Sclerotinia sclerotiorum.* Once again gene expression analysis showed that both *chit42* and *prb1* were co-expressed and moderately induced during confrontation against *Sclerotinia sclerotiorum.* Although *xbg1.3-110* previously had been implicated in mycoparasitism by *T. harzianum,* this study detected no *xbg1.3-110* expression during confrontation between *T. hamatum* and *S. sclerotiorum.* These findings show that the MYRE1-MYRE4 together with MYRE5 are present in two species of *Trichoderma, T. atroviride* and *T. hamatum* and that the presence of these motifs could predict co-expression in response to two carbon sources.

Key words: biocontrol, *chit42,* gene regulation, *prb1, xbg1.3-110*

INTRODUCTION

Biocontrol of plant pathogens is an essential component of sustainable agriculture. *Trichoderma* spp. are biocontrol agents of many economically important pathogens, such as species of *Botrytis, Rhizoctonia* and *Sclerotinia.* Control of these diseases by chemical pesticides is losing effectiveness due to enhanced degradation by soil microorganisms and growing resistance within pathogen populations (Jones and Stewart 2000). *Trichoderma* spp. commercially represent one-third of all fungal biocontrol agents of soil-borne fungal phytopathogens (Chernin and Chet 2002), and their prevalence as biocontrol agents has stimulated much research into the mechanisms underlying biocontrol, of which mycoparasitism is considered a major component.

The majority of reported biocontrol agents are isolates of *T. harzianum* or *T. atroviride,* from which 16 genes implicated in mycoparasitism have been sequenced (Benítez et al 1998, Kubicek and Penttilä 1998, Lorito 1998, Cohen-Kupiec et al 1999, Donzelli et al 2001). Transformation studies have demonstrat-

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ed a link between some mycoparasitism-related genes and biocontrol potential. Increased biocontrol activity has been attained by integrating multiple copies of the *prb1* gene into the genome of *T. atroviride* (Flores et al 1997). Introduction of multiple copies of the endochitinase *chit33* in *T. harzianum* also resulted in increased biocontrol ability (Dana et al 2001). In contrast, no difference was observed between *T. atroviride ech42* overexpression mutants, deletion mutants and wild type (Carsolio et al 1999). Although expression of *ech42* and secretion of ECH42 protein during in vitro confrontation with a host pathogen implicated a role for this gene in biocontrol (Carsolio et al 1994), recent diffusion studies have shown *ech42* to be involved in pathogen signalling (Kubicek et al 2001), suggesting a more regulatory role for this enzyme. Considerable attention also has focused on the β-glucanase genes because a large component of the fungal cell wall is composed of β-glucans and, therefore, it is likely that β-glucanases play a role in cell-wall degradation during mycoparasitism. Expression of the β-1,3-glucanase genes *lam1.3* (Cohen-Kupiec et al 1999) and *gluc78* (Donzelli et al 2001) from *T. harzianum* during confrontation have implicated a role for these genes in biocontrol.

The endochitinase gene *ech42* and alkaline proteinase gene *prb1* have been characterized extensively from *T. atroviride* (Lorito et al 1996, Cortés et al 1998 [isolate previously referred to as *T. harzianum,* later reclassified as *T. atroviride* {Kullnig et al 2001}]), revealing many insights into the regulation of these genes. Carbon catabolite repression through binding of Cre1 to the promoter (Lorito et al 1996) is considered the major negative regulator of the mycoparasitic response. Nitrogen repression of *prb1* more recently was demonstrated (Olmedo-Monfil et al 2002). Alignment of promoter sequence from *T. atroviride ech42* and *prb1* with known transcription factor motifs has suggested other regulatory pathways, such as light-induced sporulation and stress. Both these stimuli induce *ech42* and *prb1* expression supporting involvement of transcription factor binding at BrlA and STRE motifs. Four putative binding sites, MYRE1-MYRE4 (previously referred to as MYC, Carlos Cortés, pers comm), for an inducer of mycoparasitism have been suggested by alignment of the *ech42* and *prb1* promoters (Cortés et al 1998). This has been supported by knockout studies of MYRE4, in which reduced *ech42* expression was apparent. In addition, a Cre1 motif overlapped MYRE3, from which a proposed mechanism for mycoparasitic induction was formulated, in which Cre1 binding must be relieved to allow binding of the mycoparasitism inducer (Lorito et al 1996).

Mycoparasitism is a process that most *Trichoderma* species use to attack and parasitize other fungi. Therefore, the molecular mechanisms underlying mycoparasitism-based biocontrol are likely to be highly conserved. This hypothesis is in agreement with taxonomic studies on relatedness of *ech42* homologues from multiple *Trichoderma* spp., which have shown this gene to be highly conserved throughout the genus (Lieckfeldt et al 2000). The current study is focused on a *T. hamatum* isolate that has undergone extensive field testing where it has shown a high degree of ability to control *Sclerotinia* diseases of vegetable crops during development for use as a commercial biocontrol agent (BCA) (Rabeendran 2000). In vitro assays have demonstrated that this isolate can mycoparasitize *S. sclerotiorum.* The aim of this study was to isolate and characterize three mycoparasitism-related genes from *T. hamatum.* Subsequent identification of regulatory motifs in the promoter region of these genes and analysis of expression patterns might provide evidence for regulatory pathways that either contrast or are similar to those reported in other *Trichoderma* species. This also might suggest putative molecular targets for manipulating genetic regulation in vivo as a mechanism of optimizing of *T. hamatum* biocontrol activity in the field.

MATERIALS AND METHODS

Fungal isolates and maintenance.—*Trichoderma hamatum* LU593 and *T. harzianum* LU678 were isolated from New Zealand soils and were identified by morphology and ITS1 sequencing. Sequences were compared to species neotypes (*T. hamatum* DAOM 167057, accession number Z48816; *T. harzianum* CBS 226.95, accession number AJ222720). The *Sclerotinia sclerotiorum* isolate G1 used in the direct confrontation assay was from Montana, USA, and has been identified on the basis of morphology. All *Trichoderma* spp. isolates were maintained and stored on potato-dextrose agar (PDA) (Beckton Dickson & Co.) slopes. The *S. sclerotiorum* isolate was maintained on PDA and stored at 4 C as dried sclerotia.

Cloning of the mycoparasitism-related genes.—Genomic DNA was isolated from all *Trichoderma* spp. isolates using the Genomag™ kit (Advanced Biotechnologies Ltd.) as per manufacturer's instructions. *chit42* was amplified from *T. hamatum* LU593 using primers designed to a *T. hamatum chit42* (*ech42* homologue) sequence in GenBank (Tam-61, accession number U88560) (TABLE I). A segment of *prb1* was isolated from *T. hamatum* LU593 and *T. harzianum* LU678 using primers, PRBAtrC-F and PRBAtrC-R (TABLE I), designed to the signal peptide region and exon 2 of *prb1* (accession number M87518) from *T. atroviride* IMI206040. Inverse primers I-PRB-509 (5′-GCTTCTTGTGAGGGCTGC-3′) and I-PRB-1067 (5′-GGTGTTAACATTCTGTCGTC-3′)

TABLE I. Primers used to amplify mycoparasitism-related genes from *T. hamatum* LU593 and *T. harzianum* LU678

Primer	Gene	Region	Species	Sequence 5′–3′	Anneal Tm. C	Product size (kb)
C42HamP-F	*chit42*	Promoter	*T. hamatum*	atcgatcggtctggcattat	54	1.6
C42HamP-R				ttagtgaagtagacagcgtt		
C42HamC-F		Coding	*T. hamatum*	tgcttgctgcgctgcagg	55	1.3
C42HamC-R				agaccttggtgttgatcaatg		
C42HamC-F			*T. harzianum*	tgcttgctgcgctgcagg	66	1.0
C42HarC-R				tcccagataccggcctcc		
PRBHamP-F	*prb1*	Promoter	*T. hamatum*	gataaggcgccggctacg	60	0.8
PRBHamP-R				gcttcttgtgagggctgc		
PRBAtrC-F		Coding	*T. hamatum*	agctttgctcccggctgtcc	55	1.2
PRBAtrC-R			*T. harzianum*	ccaacaacgtgaggtgtagccatg		
XBGHamP-F	*xbg1.3-110*	Promoter	*T. hamatum*	tggttgacaacagtcatgtacc	64	1.8[a]
XBGHamP-R				tgtactgctgattaccaatggc		
XBGHamC-F		Coding	*T. hamatum*	ggatctgctggattcctcac	65	0.9
XBGHamC-R				gtagccaagttccactcgatg		

[a] Gene fragment extends from 1 kb 5′ to the translational start site to 800 bp of the coding region.

were designed to a region approximately 50 bp inside the LU592 *prb1* DNA sequence. These were used to amplify the *T. hamatum* promoter and 3′ flanking region from a recircularized *Hind*III digest, according to standard inverse PCR protocols (Ochman et al 1990). Specific primers were designed to the extended sequence and used to amplify the promoter region (TABLE I). The *lam1.3* homologue, *xbg1.3-110,* was isolated from *T. hamatum* LU593 using a combination of inverse and degenerate PCR. Degenerate primers were based on regions of identity between the protein sequences of *lam1.3* (accession number AJ002397) from *T. harzianum* T-Y and *exg1* (accession number L48994) from *Cochliobolus carbonum* SB111. The degenerate primers XBGp394 (5′-A/TC/GA/C/TAAGCCA/C/TCAA/GTAC/TGAG-3′) and XBGp535 (5′-G/TCG/TA/G/TGTA/GTGA/GACA/GTCCCA-3′) were used to amplify a 500 bp fragment of *xbg1.3-110* from *T. hamatum* LU593. Inverse primers I-XBG-58 (5′-CCTTGACAAACAATGAATAACAG-3′) and I-XBG-423 (5′-GTCCTCATCGAGTGGAACTTG-3′) were used to amplify flanking regions from a recircularized *Pvu*II digest extending the sequence to ~1.7 kb. Degenerate primer XBGp61 (5′-AACGTC/TAAGGAC/TTACGGA/C/TGCC/TAAG-3′) was designed to a region 5′ to the 1.7 kb *xbg1.3-110* sequence and used with the sequence specific primer XBGext-as (5′-TTATTTCGGTACTGCAACGGC-3′) to amplify a further 550 bp extending the sequence to 2.2 kb. Inverse primers I-XBG4up (5′-TGTTAATTGCTGCCGTGTCGT-3′) and I-XBG2down (5′-CGTGAATAGAACTGGCTCTG-3′) amplified regions flanking the 2.2 kb sequence from a recircularized *Nco*I digest extending the sequence to 3.8 kb. Specific primers were designed and used to amplify the promoter and coding regions (TABLE I).

Using the primers in TABLE I, promoter and coding regions were amplified in an Eppendorf Mastercycler® Gradient PCR machine (Eppendorf-Netherler-Hinz GmbH) for sequence analysis and for use as probes in RNA analysis. Each 25 μL PCR reaction contained 10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP (Roche Molecular Biochemicals Ltd.), 10 pmol of each primer, 10 ng of genomic DNA and 1.25 U *Taq* DNA polymerase (Roche Molecular Biochemicals Ltd.). Amplifications consisted of 5 min at 94 C, followed by 30 cycles of 30 s at 94 C, 30 s at the annealing temperature indicated (TABLE I), and 1 min at 72 C, followed by 7 min at 72 C.

All gene fragments were ligated to pGEM®-T (Promega Corp., Madison, Wisconsin) (Appendix 7.4) as per manufacturer's instructions and transformed into *E. coli* strain INVαF′ (Invitrogen Corp.) using standard techniques (Sambrook et al 1989). Plasmid DNA was prepared using the Perfectprep® Plasmid Mini Kit (Eppendorf-Netheler-Hinz GmbH) as per manufacturer's instructions. Two clones per transformation were sequenced in both directions (University of Auckland). *Trichoderma hamatum* LU593 sequences were deposited in GenBank with these accession numbers: ITS1-AY241456, *chit42*-AY258898, *prb1*-AY258899, *xbg1.3-110*-AY269826.

Promoter sequence analysis.—To identify putative binding sites for regulators, promoter sequences were aligned with homologues from *T. atroviride* IMI206040, with common eukaryotic elements and with fungal specific motifs from other filamentous fungi using DNAman™ (Lynnon Biosoft). In addition, short identical motifs between the three promoters were identified through sequential alignments of 50 bp segments.

Substrate induction assays.—Submerged culture assays were performed as follows. A 100 mL flask containing 25 mL of half-strength potato-dextrose both (PDB) and 2.5×10^8 conidia was incubated 14 h at 22 C in the dark on a rotary shaker (200 rpm). Mycelium was collected by centrifugation at 2000 *g* for 5 min at 4 C, transferred to minimal media (MM) ([0.2 g $MgSO_4 \cdot 7H_2O$, 0.9 g K_2HPO_4, 0.2 g KCl, 1 g NH_4NO_3, 2 mg $FeSO_4 \cdot 7H_2O$, 2 mg $ZnSO_4 \cdot 7H_2O$, 2 mg $MnCl_2 \cdot 7H_2O$ and 2 g asparagine per litre water; pH 5.5] [Carsolio et al 1994]) with 0.4% glycerol and incubated 12

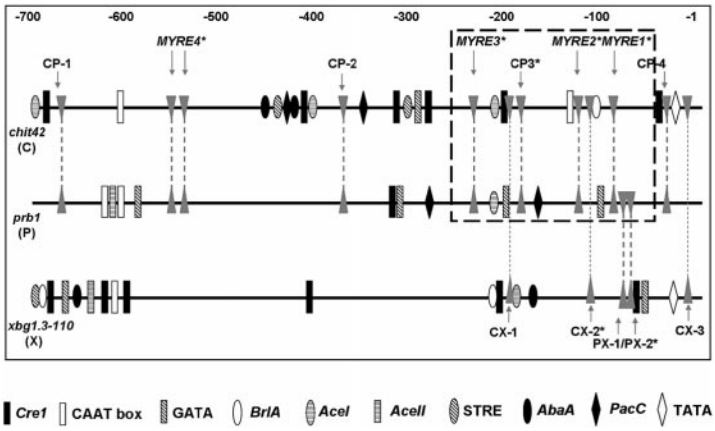

FIG. 1. *T. hamatum* LU593 *chit42, prb1* and *xbg1.3-110* promoter sequences 700 bp 5′ to the transcription start point. *Cre1* (5′SYGGRG3′) binds the catabolite repressor protein (Lorito et al 1996), STRE (CCCCT) is a stress-response motif (Cortés et al 1998), *AbaA* and *BrlA* (5′CATTCY3′, 5′MRAGGGR3′) bind to regulators of light-induced sporulation (Andrianopoulos and Timberlake 1994, Chang and Timberlake 1992), *AceI* and *AceII* (5′AGGCA3′, 5′GGCTAA3′) are involved in induction of cellobiohydrolase genes in reponse to cellulose (Saloheimo et al 2000, Aro et al 2001), GATA (5′HGATAR3′) binds to a regulator of nitrogen repression (Olmedo-Monfil et al 2002), *PacC* (5′GCCARG3′) binds to the ambient pH regulator PacC (Denison2000). MYRE4-MYRE1 are postulated to be involved in mycoparasitism and were identified previously (Cortés et al 1998). CP (*chit42* and *prb1*), CX (*chit42* and *xbg1.3-110*) and PX (*prb1* and *xbg1.3-110*) motifs were identified in this study: CP-1 (5′ATTAGAGCT3′), CP-2 (5′AACGTT3′), CP-3 (5′TTCTAG3′), CP-4 (5′TTGACT3′), CX-1 (5′GGAGAC3′), CX-2 (5′TGGGTT3′), CX-3 (5′TCCTGC3′), PX-1 (5′GAAATCG3′), PX-2 (5′ATTTAAG3′).
* Conserved with *T. atroviride* IMI206040.

h under the previously described conditions. The mycelium again was collected, and the pellet was separated into five portions; one portion was snap frozen (day 0) and the other four were transferred to 2% glycerol MM pH 5.5, or MM without glycerol (control). Mycelium was harvested at 24, 48, 72 and 96 h (days 1–4). The laminarin assay was conducted as above, except that after the second centrifugation step the pellet was separated into two portions; one portion was snap frozen in liquid N_2 (0 h) and the other transferred to 2% glycerol MM pH 5.5 with 0.5% laminarin (SIGMA: L-9634) or 2% glycerol MM only (control). Mycelia were harvested at 48 h and snap frozen. All mycelial samples were stored at −80 C until processed. Each assay was replicated once.

Direct confrontation assay.—Direct confrontation assays were performed on agar plates covered with cellophane using a modification of the method of Carsolio et al (1994). Fungi were grown at 22 C in the dark on minimal media (MM) agar with 0.2% glycerol.

RNA dot-blot analysis.—Total RNA was extracted (TRIzol, Invitrogen) and 20 μg transferred to Hybond N+ nylon membrane (Amersham) with a Bio-Dot Microfiltration Apparatus (Bio-Rad). Coding region fragments of *chit42* and *prb1* from *T. hamatum* and *T. harzianum* and *xbg1.3-110* from *T. hamatum* were used as probes in northern hybridizations. Chemiluminescent detection was performed using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham).

RESULTS

Upstream regulatory sequence analysis of three mycoparasitism-related genes.—A common approach to sequence analysis has been comparison of gene homologues in related species or coregulated genes in the same species. Both approaches were taken in this study. Three mycoparasitism-related genes were sequenced from a *T. hamatum* biocontrol agent. Transcription start points (TSP) and exon/intron boundaries were assigned on the basis of conservation with related sequences. The endochitinase *chit42* was compared to the *T. hamatum* (Tam-61) sequence deposited on GenBank (accession number U88560), sharing 99.84% nucleotide and 99.41% amino acid identity. *Trichoderma hamatum prb1* shared 88.75% nucleotide identity across the coding region and 96.4% amino acid identity with *T. atroviride prb1* (accession number M87518) and *xbg1.3-110* shared 83.55% identity in the coding region and 90.18% amino acid identity with *T. harzianum lam1.3 (xbg1.3-110)* (AJ002397).

Sequence analysis of the promoter regions identified numerous putative regulatory motifs previously reported in other fungal genes, including *Trichoderma* mycoparasitism-related genes (FIG. 1). Regions were aligned with available promoter sequences from the homologous *T. atroviride ech42* (843 bp, accession number Z803580) and *prb1* (1274 bp, Alfredo

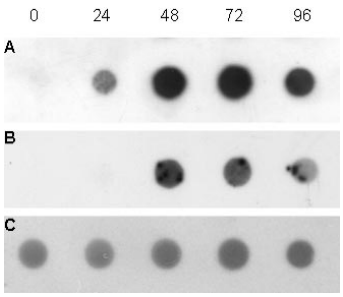

FIG. 2. Expression of *T. hamatum* LU593 *chit42* and *prb1* in MM + 2% glycerol submerged cultures. Sampling times in hours are shown above the figure. A, B, C RNA dot-blot analysis of 20 μg total RNA. A *chit42*. B *prb1*. C ITS1 rRNA.

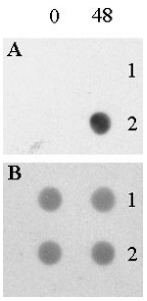

FIG. 3. Expression of *T. hamatum* LU593 *xbg1.3-110* in laminarin submerged cultures. Sampling times in hours are shown above the figure. A, B, RNA dot-blot analysis of 20 μg total RNA from LU593 grown in MM + 2% glycerol (1) or MM + 2% glycerol + 0.5% laminarin (2). A *xbg1.3-110*. B ITS1 rRNA.

Herrera-Estrella, pers comm) to determine which motifs were conserved in both biocontrol species. Only 58 bp 5′ to the TSP from *T. harzianum lam1.3* (xbg1.3-110) (accession number AJ002397) was available for alignment with *xbg1.3-110*. Approximately half the motifs identified in *chit42* and *prb1* were conserved with their *T. atroviride* homologues. Comparative alignments of the promoter regions between the three *T. hamatum* genes revealed that *chit42* and *prb1* shared more sequence identity than either with *xbg1.3-110*. In addition, while the putative mycoparasitic motifs MYRE4-MYRE1 (Cortés et al 1998) were conserved in *chit42* and *prb1,* none were present in *xbg1.3-110*.

Differential expression of chit42, prb1 *and* xbg1.3-110.—Strong sequence identity between the regulatory regions of *chit42* and *prb1* led to the prediction that they shared common regulatory pathways. To test this hypothesis, gene expression patterns of *chit42, prb1* and *xbg1.3-110* were analyzed in the presence of the simple carbon source glycerol. *chit42* and *prb1* were induced strongly at 48 h, decreasing at 96 h, in *T. hamatum* grown in minimal media broth with 2% glycerol (FIG. 2), whereas in *T. harzianum* no expression of *chit42* or *prb1* was observed. In MM without glycerol conidia germinated and died (data not shown) whereas in 2% glycerol, cultures increased in size throughout the experiment, indicating metabolism of the available carbon. No expression of *xbg1.3-110* was detected, however strong expression was observed in 2% glycerol MM with 0.5% laminarin, a common substrate for β-1,3-glucanases, demonstrating the gene to be active (FIG. 3).

Expression patterns of *T. hamatum chit42, prb1* and *xbg1.3-110* further were analyzed during confrontation with the plant pathogen *Sclerotinia sclerotiorum* (FIG. 4). Both *chit42* and *prb1* were expressed moderately upon physical contact with *S. sclerotiorum,* however no *xbg1.3-110* expression was detected at any sampling interval. Low expression of *chit42* and *prb1* also was observed in the *T. hamatum* versus *T. hamatum* treatment.

DISCUSSION

Various environmental inducers of *Trichoderma* mycoparasitism-related genes have been postulated on

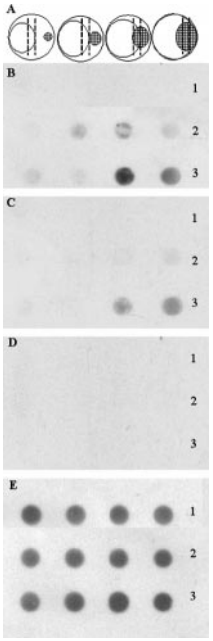

FIG. 4. Expression of *T. hamatum* LU593 *chit42* and *prb1* during confrontation with *S. sclerotiorum* G1. A. Diagrammatic representation of assay and sampling zones. Outer circle represents the plate, the inner circles represent *T. hamatum* (open circle) and *S. sclerotiorum* (checkered circle) and the dashed lines the sampling zones. B, C, D, E. RNA dot-blot analysis of 20 μg total RNA from LU593 alone (1), LU593 v LU593 (2) and LU593 v *S. sclerotiorum* G1 (3) at the sampling intervals above. B. *chit42*. C. *prb1*. D. *xbg1.3-110*. E. ITS1 rRNA.

the basis of sequence, northern and protein analysis in *T. atroviride* and *T. harzianum*. In this study, three mycoparasitism-related gene homologues from *T. hamatum,* a lesser known biocontrol species, were analyzed with reference to sequence structure of their promoter regions and their expression in response to a simple carbon source (glycerol) and complex carbon source in the form of the plant pathogen *Sclerotinia sclerotiorum*.

Identification of regulatory motifs in the promoter region might provide evidence for common regulatory pathways. A detailed summary of the motifs identified in the *T. hamatum* promoter region are shown in FIG. 1. Of the three genes, *chit42* and *prb1* shared the most sequence identity in the promoter/enhancer region. Strong conservation of the four putative binding sites (MYRE1-MYRE4) for a global inducer of mycoparasitism-related genes that was identified in *T. atroviride chit42 (ech42)* and *prb1* (Cortés et al 1998) is evident. These exact motifs were conserved in the *T. hamatum* homologues yet absent in *T. hamatum xbg1.3-110*. In *T. hamatum* MYRE3, CP3, MYRE2 and MYRE1 formed a highly conserved cluster proximal to the transcription start site that spanned exactly 142 bp in both *chit42* and *prb1*. The 6 bp identity CP3 also was conserved in *T. atroviride,* however, it had not been identified by Cortés et al (1998). In *T. hamatum,* each motif within the cluster lay exactly the same distance apart in both *chit42* and *prb1*. The conservation of the regulatory elements MYRE1-MYRE3 and CP3 in the *T. hamatum* genes *chit42* and *prb1* suggested that these genes more likely are to be co-expressed during mycoparasitism.

In its simplest form mycoparasitism is the acquisition of carbon from another fungus. In this study glycerol was used as the simple carbon source in a substrate assay. Strong gene expression of *Trichoderma* mycoparasitism-related genes in minimal media supplemented with elevated glycerol has not been reported before. No expression of *ech42* and only weak expression of *prb1* from *T. atroviride* has been observed previously under these conditions (Cortés et al 1998), and no *chit42* or *prb1* expression was detected from *T. harzianum* grown under the same conditions (this study). The results presented here suggest that in *T. hamatum chit42* and *prb1* might be coregulated by a metabolic pathway that responds to elevated glycerol. Because all three genes have been implicated in cellular differentiation and growth, it also is possible that up-regulation of LU593 *chit42* and *prb1* was in response to growth-stage regulation of expression not elevated glycerol. However, the very closely related *T. harzianum* showed no gene expression of homologues at any time, despite undergoing parallel growth and differentiation (data not shown). For this reason it is unlikely that growth-stage-specific regulation was the cause of the increased *chit42* and *prb1* expression. Mycoparasitism and glycerol utilization are examples of alternative carbon source metabolic pathways. Other nonglucose carbon sources, such as V8 juice, directly enhance chitinase protein activity in both *T. atroviride* (P1) and *T. virens* (Tronsmo and Harman 1992). In the presence of both chitin and V8 juice, chitinase production followed similar patterns in both isolates. However, *T. virens* produced substantially lower yields of extracellular chitinases to *T. atroviride,* suggesting differential regulation. The *T. hamatum* response in minimal media amended with high glycerol may represent an alternative carbon induction pathway absent or not active in other *Trichoderma* species. These data support the results of the sequence analysis, where the *chit42* and *prb1* promoter regions both included the MYRE4-MRYE1 elements and showed greater similarity to each other than to *xbg1.3-110,* in which MYRE4-MYRE1 were absent.

It is likely that several other events play a role in the induction of mycoparasitism including recognition, chemical secretion by the pathogen and defence or evasion responses. To further study the response of the mycoparasitism genes from *T. hamatum* in a more realistic situation, confrontation against plant pathogens in an agar plate assay was used. The *T. hamatum* BCA used in this study had demonstrated previously high biocontrol activity toward *Sclerotinia* diseases in the field (Rabeendran 2000) and mycoparasitism of *S. sclerotiorum* by LU593 has been observed in vitro (data not shown). For these reasons the plant pathogen *S. sclerotiorum* was used in all confrontation assays. Expression analysis of *chit42, prb1* and *xbg1.3-110* in response to co-inoculation with the plant pathogen *S. sclerotiorum* confirmed predictions made on co-expression by promoter sequence analysis and the glycerol assay. As predicted by this sequence analysis *chit42* and *prb1* shared expression patterns under all test conditions. The lack of *xbg1.3-110* expression during confrontation with *S. sclerotiorum* and in response to glycerol demonstrated that it did not share the same expression patterns as *chit42* and *prb1* and did not play a role in initial mycoparasitism of *S. sclerotiorum*. These data support the results of the sequence analysis, where the *chit42* and *prb1* promoter regions both included the MYRE1-MRYE4 and CP3 elements and showed greater similarity to each other than to *xbg1.3-110,* in which these elements were absent. This suggests that similarity between promoter regions of different genes can predict co-expression.

Weak expression of *chit42* and *prb1* was observed in the *T. hamatum-T. hamatum* contact and is prob-

ably the result of a reduced nutrient availability. In the *T. hamatum* alone controls expression of both *chit42* and *prb1* was undetectable. In *T. atroviride* there was no increase in *prb1* relative to *T. atroviride* alone controls and *ech42* expression barely was detectable, which suggested expression during confrontation with the pathogen *R. solani* was parasitism specific (Cortés et al 1998). In *T. hamatum* expression of *chit42* and *prb1* during confrontation with *S. sclerotiorum* is due likely in part to a nutrient-regulated factor, however, the fungal biomass of the confrontation was less than that of the self-self controls. Therefore, the increase in expression during confrontation is likely to be primarily the result of mycoparasitism rather than increased nutrient depletion.

These studies also demonstrated that expression of different mycoparasitism-related genes in *Trichoderma* species is highly variable. Extracellular secretion of variable enzymes profiles between biocontrol strains of *T. harzianum* have been observed in response to polysaccharides and host cell walls (Elad et al 1982, Mach et al 1999). This study found interspecies variation in expression of three mycoparasitism-related genes. In addition, it has been suggested during confrontation *T. atroviride* Prb1 might release an inducer from the host that induces *ech42* expression (Cortés et al 1998). This is unlikely to be true for *T. hamatum* because *chit42* is expressed earlier and stronger than *prb1*. The regulation of genes encoding enzymes that have been implicated in mycoparasitism is clearly complex and, in many instances, species-specific pathways occur. However, this work has shown that conservation of regulatory motifs can predict coregulation and provide information on metabolic pathways likely to be conserved among *Trichoderma* species. It is interesting to note that all MYRE elements identified in *T. atroviride* were conserved in *T. hamatum,* whereas only half the putative transcription factor motifs identified in this study were conserved between both species.

Mycoparasitism is simply an alternate means of carbon assimilation, characterized by catabolite repression and induction during carbon starvation. When carbon deprived, alternative pathways are turned on in response to environmental stimulators, such as presence of another fungus or easily metabolizable carbon source. Genes implicated in mycoparasitism are regulated differentially among *Trichoderma* species in a carbon-source-dependent fashion. Knowledge of carbon sources that up-regulate mycoparasitism-related genes is likely to have implications for the design of *Trichoderma* inoculants and might improve reliability of commercial biocontrol agents.

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LITERATURE CITED

Andrianopoulos A, Timberlake WE. 1994. The *Aspergillus nidulans* abaA gene encodes a transcriptional activator that acts as a genetic switch to control development. Mol Cell Biol 14:2503–2515.

Aro N, Saloheimo A, Ilmén M, Penttilä M. 2001. ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. J Biol Chem 276:24309–24314.

Benítez T, Limón C, Delgado-Jarana J, Rey M. 1998. Glucanolytic and other enzymes and their genes. In: Harman GE, Kubicek CP, eds. *Trichoderma* and *Gliocladium,* vol 2: enzymes, biological control and commercial application. London: Taylor & Francis. p 101–128.

Carsolio C, Gutiérrez A, Jiménez B, van Montagu M, Herrera-Estrella A. 1994. Characterization of *ech-42,* a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. Proc Natl Acad Sci USA 91: 10903–10907.

———, Benhamou N, Haran S, Cortés C, Gutiérrez A, Chet I, Herrera-Estrella A. 1999. Role of *Trichoderma harzianum* endochitinase gene, *ech42,* in mycoparasitism. Appl Environ Microbiol 65:929–935.

Chang YC, Timberlake WE. 1992. Identification of Aspergillus *brlA* response elements (BREs) by genetic selection in yeast. Genet 133:29–38.

Chernin L, Chet I. 2002. Microbial enzymes in the biocontrol of plant pathogens and pests. In: Burns RG, Dick RP, eds. Enzymes in the Environment: Activity, Ecology and Applications. New York: Marcel Dekker. p 171–226.

Cohen-Kupiec R, Broglie KE, Friesem D, Broglie RM, Chet I. 1999. Molecular characterization of a novel β-1,3-exoglucanase related to mycoparasitism of *Trichoderma harzianum*. Gene 226:147–154.

Cortés C, Gutierrez A, Olmedo V, Inbar J, Chet I, Herrera-Estrella A. 1998. The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. Mol Gen Genet 260:218–225.

De las Mercedes Dana M, Limón MC, Mejías R, Mach RL, Benítez T, Pintor-Toro JA, Kubicek CP. 2001. Regulation of chitinase 33 (*chit33*) gene expression in *Trichoderma harzianum*. Curr Genet 38:335–342.

Denison SH. 2000. pH regulation of gene expression in fungi. Fungal Genet Biol 29:61–71.

Donzelli BGG, Lorito M, Scala F, Harman GE. 2001. Cloning, sequence and structure of a gene encoding an antifungal glucan 1,3-β-glucosidase from *Trichoderma atroviride* (*T. harzianum*). Gene 277:199–208.

Elad Y, Chet I, Henis Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Can J Microbiol 28:719–725.

Flores A, Chet I, Herrera-Estrella A. 1997. Improved bio-

control activity of the proteinase-encoding gene *prb1*. Curr Genet 31:30–37.

Jones EE, Stewart A. 2000. Selection of mycoparasitise of sclerotia of *Sclerotinia sclerotiorum* isolated from New Zealand soils. NZ J Crop Hort Sci 28:105–114.

Kubicek CP, Penttilä ME. 1998. Regulation of production of plant polysaccharide degrading enzymes by *Trichoderma*. In: Harman GE, Kubicek CP, eds. *Trichoderma* and *Gliocladium,* vol 2: enzymes, biological control and commercial application. London: Taylor & Francis. p 49–72.

———, Mach RL, Peterbauer CK, Lorito M. 2001. *Trichoderma*: from genes to biocontrol. J Plant Pathol 83:11–23.

Kullnig CM, Krupica T, Woo SL, Mach RL, Rey M, Benítez T, Lorito M, Kubicek CP. 2001. Confusion abounds over identities of *Trichoderma* biocontrol isolates. Mycol Res 105:770–772.

Lieckfeldt E, Cavignac Y, Fekete C, Börner T. 2000. Endochitinase gene-based phylogenetic analysis of *Trichoderma*. Microbiol Res 155:7–15.

Lorito M, Mach RL, Sposato P, Strauss J, Peterbauer CK, Kubicek CP. 1996. Mycoparasitic interaction relieves binding of the Cre1 carbon catabolite repressor protein to promoter sequences of the *ech42* (endochitinase-encoding) gene in *Trichoderma harzianum*. Proc Natl Acad Sci USA 93:14868–14872.

———. 1998. Chitinolytic enzymes and their genes. In: Harman GE, Kubicek CP, eds. *Trichoderma* and *Gliocladium,* vol 2: enzymes, biological control and commercial application. London: Taylor & Francis. p 73–100.

Mach RL, Peterbauer CK, Payer K, Jaksits S, Woo SL, Zeilinger S, Kullnig CM, Lorito M, Kubicek CP. 1999. Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. Appl Environ Microbiol 65:1858–1863.

Ochman H, Medhora MM, Garza D, Hartl DL. 1990. Amplification of flanking sequences by inverse PCR. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego: Academic Press. p 219–227.

Olmedo-Monfil V, Mendoza-Mendoza A, Gómez I, Cortés C, Herrera-Estrella A. 2002. Multiple environmental signals determine the transcriptional activation of the mycoparasitism related gene *prb1* in *Trichoderma atroviride*. Mol Genet Genomics 267:703–712.

Rabeendran N. 2000. Biological control of *Sclerotinia* diseases of vegetables. [Doctoral thesis]. Lincoln University, Canterbury, New Zealand.

Saloheimo A, Aro N, Ilmén M, Penttilä M. 2000. Isolation of the *ace1* gene encoding a Cys2-His2 transcription factor involved in regulation of activity of the cellulase promoter *cbh1* of *Trichoderma reesei*. J Biol Chem 275: 5817–5825.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Tronsmo A, Harman GE. 1992. Coproduction of chitinolytic enzymes and biomass for biological control by *Trichoderma harzianum* on media containing chitin. Biol Control 2:272–277.