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Trichoderma harzianum genes induced during growth on *Rhizoctonia solani* cell walls

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Trichoderma harzianum **is a biocontrol agent that attacks a range of economically important phytopathogenic fungi. In an attempt to identify genes specifically expressed by *T. harzianum* during growth on cell walls of *Rhizoctonia solani*, we carried out differential screening of an induced cDNA library. In this paper we report the analysis of the sequence and expression of two cDNA clones that encode putative mycoparasitism-related proteins of *T. harzianum*. One of these clones corresponds to a gene, *inda1*, that encodes a protein of 570 amino acids with a predicted molecular mass of 62853 Da. The predicted amino acid sequence of *inda1* showed a high degree of similarity with amino acid permeases from several other organisms. The other cDNA clone corresponds to a gene, *indc11*, that encodes a novel protein of 340 amino acids with a predicted molecular mass of 37010 Da. The use of this methodology should provide specific genetic markers to follow mycoparasitism by *Trichoderma* spp.**

Keywords: *Trichoderma harzianum*, *Rhizoctonia solani* cell walls, mycoparasitism

INTRODUCTION

Modern agriculture is very strongly dependent on fungicides. The repeated use of these pesticides has not only polluted the environment, but many pesticides have also become useless owing to the development of resistance among the target organisms. One possible alternative to synthetic fungicides is the use of biological control agents. Many natural biocontrol agents have been shown to be effective in controlling plant pathogens (see reviews by Chet, 1990; Manocha, 1991). However, biocontrol is not often used in commercial agricultural production because control of plant diseases with microbial agents has been less effective and reliable than with synthetic fungicides. The improvement of biocontrol efficacy requires a better knowledge of the mechanisms involved in the control of plant pathogens.

The fungus *Trichoderma harzianum* has been used as a biocontrol agent and shown to attack a range of economically important aerial and soil-borne fungal plant pathogens. In soil, *T. harzianum* strains have been used as antagonists against several plant-pathogenic fungi, for example *Rhizoctonia solani*, *Botrytis cinerea* and *Sclerotium rolfsii* (Chet, 1987). A great deal of information about the factors involved in the antagonistic properties of *T. harzianum* has been identified. These include volatile and non-volatile antibiotics, and hydrolytic enzymes such as chitinases, glucanases and proteases (Dennis & Webster, 1971a, b; Elad *et al.*, 1982; Ridout *et al.*, 1986, 1988; Claydon *et al.*, 1991; Geremia *et al.*, 1991; Graeme-Cook & Faull, 1991; Ulhoa & Peberdy, 1991; Avent *et al.*, 1992; De La Cruz *et al.*, 1992; Di Pietro *et al.*, 1993; Harman *et al.*, 1993; Lorito *et al.*, 1993). Recently, two mycoparasitism-related genes from *T. harzianum*, encoding an alkaline proteinase (Geremia *et al.*, 1993) and an endochitinase (Hayes *et al.*, 1994), have been isolated and characterized.

When mycoparasitic strains of *Trichoderma* spp. grow on host cell walls, induction and/or derepression of genes involved in mycoparasitism has been found to occur. In addition, glucose represses the activity and/or expression of hydrolytic enzymes important for the degradation of the cell walls (Geremia *et al.*, 1991, 1993). Our approach to identify factors determining mycoparasitic abilities of *T. harzianum* was to isolate genes of this species that show induced or increased expression during growth on *R.*

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The EMBL accession numbers for the *inda1* and *indc11* nucleotide sequences reported in this paper are Z22594 and Z22221, respectively.

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solani cell walls. Identification of the proteins encoded by these genes might help elucidate some of the molecular and cellular events involved in mycoparasite–host interactions.

To isolate the putative mycoparasitism genes, we carried out differential screening of an induced cDNA library. In this paper we report the analysis of the sequence and expression of two cDNA clones from this library. One corresponds to a gene encoding a putative amino acid permease (*inda1*), and the other corresponds to a gene encoding a novel polypeptide (*indc11*).

METHODS

Fungal strains and growth media. *T. harzianum* (IMI 206040) was grown in Erlenmeyer flasks containing 50 ml minimal medium (MM: 0·2% asparagine, 0·2% $NaNO_3$ 0·05% $MgSO_4.7H_2O$, 0·05% KCl, 0·1% KH_2PO_4, pH 6·0) supplemented with 2% (w/v) glucose on a rotatory shaker (150 r.p.m.) at 28 °C for 3 d. The mycelium was then harvested, rinsed with sterile water and transferred to Erlenmeyer flasks with the same medium containing either 2% glucose or 0·1% cell walls of *R. solani* (prepared as described by Goldman *et al.*, 1992a). Mycelium was harvested after 24, 48 and 72 h, lyophilized, and stored at −20 °C.

cDNA library. The cDNA library was constructed using poly$(A)^+$-RNA obtained from *T. harzianum* grown for 24 h in MM containing cell walls of *R. solani* (1 mg ml^{-1}) as sole carbon source (Goldman *et al.*, 1992b). Total RNA and poly$(A)^+$-RNA were isolated from fungal mycelia according to a previously described protocol (Jones *et al.*, 1985), adapted for the large-scale isolation of RNA and using a poly-A tract mRNA isolation kit (Promega).

Differential screening. This was carried out essentially as described by Gasser *et al.* (1989) and M. H. de S. Goldman *et al.* (1992). Bacterial colonies (3000) were stored in microtitre plates containing Luria–Bertani (LB) medium supplemented with ampicillin. Duplicate nylon replica filters (Hybond-N, Amersham) were used on each of the plates and treated as recommended by the manufacturer. Radioactive plus/minus cDNA probes were synthesized from poly$(A)^+$-RNA of mycelium grown on glucose ('minus') or cell wall ('plus') medium. Hybridization probes were synthesized from 1 µg of poly$(A)^+$-RNA in a reaction similar to the first-strand cDNA synthesis. Modifications introduced were the use of 100 µCi (3·7 MBq) $[^{32}P]$dCTP for the first hour of reaction and the use of random hexanucleotides as primers. Unlabelled dCTP was then added to a final concentration of 0·5 mM and the reaction was continued for 30 min. The labelled cDNA/RNA hybrids were purified on Nensorb columns (Nensorb). In preparation for hybridization, the probes were denatured and the RNA was hydrolysed by adding 60 µl 1·0 M NaOH, followed by 30 min incubation at room temperature. The base was neutralized by adding 60 µl 1·0 M HCl and 60 µl 20× SSPE (1× SSPE: 0·15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7·4). Filters were prehybridized in a solution containing 6× SSC (1× SSC: 0·15 M NaCl, 0·015 M sodium citrate, pH 7·0), 1% (w/v) SDS, 100 µg denatured carrier DNA ml^{-1}, 0·25% milk powder for 1 h at 68 °C. The labelled cDNA was added to the prehybridization solution to a final concentration of 10^6 c.p.m. ml^{-1} and incubated at 68 °C for 36–48 h. The probe-specific activities were adjusted to equality and equivalent amounts of radioactivity per ml of hybridization solution were used. Filters were washed sequentially at 68 °C in 6× SSC, 0·1% SDS for 30 min, and in 2× SSC, 0·1% SDS for 30 min. Filters were exposed to Kodak-X Omat films overnight between intensifying screens at −70 °C.

DNA/RNA manipulations. Restriction enzyme digests and DNA ligation were performed according to the recommendations of the suppliers (Boehringer and Amersham). Plasmid DNA isolation and Southern blotting were all performed using standard procedures (Sambrook *et al.*, 1989). DNA probes were made using a primer system according to the manufacturer (Boehringer). The complete sequence of the *inda1* and *indc11* genes was determined by the dideoxy-chain termination method (Sanger *et al.*, 1977) from both strands using synthetic oligonucleotide primers.

For Northern analysis, *T. harzianum* was grown by inoculating MM supplemented with 2% (w/v) glucose with 3× 10^4 conidiospores per ml medium. The cultures were incubated in a reciprocal shaker at 28 °C for 3 d, harvested by filtration through a Whatman filter (no. 1), and washed thoroughly with TES buffer (50 mM Tris, 50 mM NaCl, 5 mM EDTA, pH 8·0). Equal amounts of mycelium were transferred to MM supplemented with 2% glucose or cell walls (1 mg ml^{-1}) of the phytopathogenic fungus *R. solani*. This last condition was defined throughout this work as cell-wall-grown, i.e. growth in liquid medium having a cell wall preparation of *R. solani* as sole carbon source (Goldman *et al.*, 1992a). Mycelium was collected, disrupted and total RNA extracted. In all Northern analysis experiments, RNA concentration was normalized by hybridization with a β-tubulin gene from *Trichoderma viride* (*tub1*; Goldman *et al.*, 1993).

RESULTS

Isolation of specific *T. harzianum* cDNA clones grown on cell walls

We isolated specific cDNA clones from a *T. harzianum* cell-wall-grown cDNA library by differential screening against probes from *T. harzianum* grown on glucose or on cell walls of *R. solani*, as described by Gasser *et al.* (1989) and M. H. de S. Goldman *et al.* (1992). The initial screening resulted in the identification of 65 clones (from 3000 clones of a non-amplified library) and a secondary screening in 15 cDNA clones that hybridized only to cDNA probes derived from *T. harzianum* grown on cell walls. For further analysis, we decided to choose only the clones which in Northern analysis experiments hybridized with mRNAs isolated from cell-wall-grown *T. harzianum* (see below). These preliminary experiments showed that only eight clones hybridized to mRNA obtained from *T. harzianum* grown on cell walls. Southern analysis experiments indicated that three of these clones cross-hybridized at high stringency (data not shown). Thus, we obtained at the end of our screening six different clones which are expressed specifically during growth on cell walls of *R. solani*. Two of these cDNA clones, INDA1 and INDC11, were chosen for further analysis.

inda1 and *indc11* genes are expressed during growth of *T. harzianum* on cell walls

To clarify the conditions under which the *inda1* and *indc11* genes were expressed, RNA was isolated from *T. harzi-*

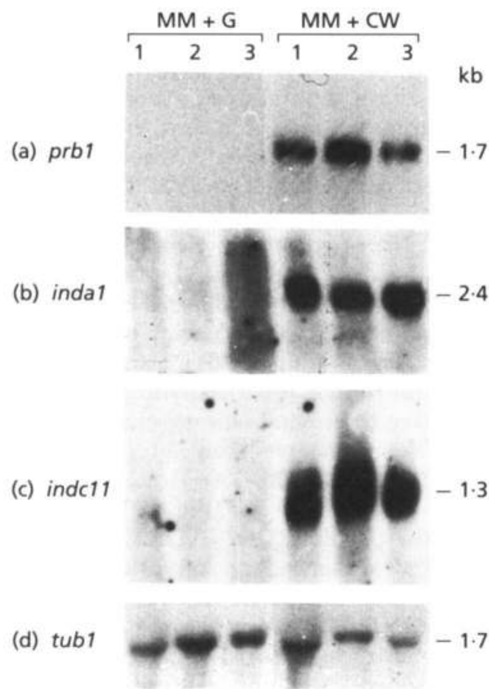

Fig. 1. Northern blot of mRNA obtained from *T. harzianum* grown on MM plus glucose (MM+G) or *R. solani* cell walls (MM+CW). Transcript levels of *prb1* (a), *inda1* (b), *indc11* (c) and *tub1* (d) in mycelia harvested 24 (lane 1), 48 (lane 2) and 72 (lane 3) h after transfer into MM+G or MM+CW.

anum grown on cell walls and Northern analyses were carried out (Fig. 1). One gene expressed during mycoparasitism by *T. harzianum* is the *prb1* gene, which encodes an alkaline proteinase (Geremia *et al.*, 1993). Its expression was repressed in the presence of glucose and increased during cell-wall-grown *T. harzianum* (Fig. 1a). The *inda1* gene specifies a single transcript of about 2·4 kb whereas the *indc11* gene specifies a single transcript of about 1·3 kb (Figs 1b and 1c). *β*-Tubulin mRNA expression was constant during growth on glucose and cell walls (Fig. 1d). Both genes hybridized only with mRNA obtained from *T. harzianum* grown on cell walls. They did not hybridize with mRNA isolated during growth of *T. harzianum* in the presence of glucose. These data indicated that *inda1* and *indc11* genes are specifically expressed in cell-wall-grown *T. harzianum* and repressed during growth on glucose.

The INDA1 cDNA clone corresponds to a gene that encodes a putative amino acid permease

The INDA1 cDNA clone contained an insert of 2·1 kb. The complete sequence of the *inda1* gene of *T. harzianum* is shown in Fig. 2. The cDNA has an ORF of 1710 nucleotides. To determine whether *inda1* is a member of a gene family, we performed DNA blot hybridizations with genomic DNA prepared from *T. harzianum* (Fig. 3a). After digestion of the genomic DNA with appropriate

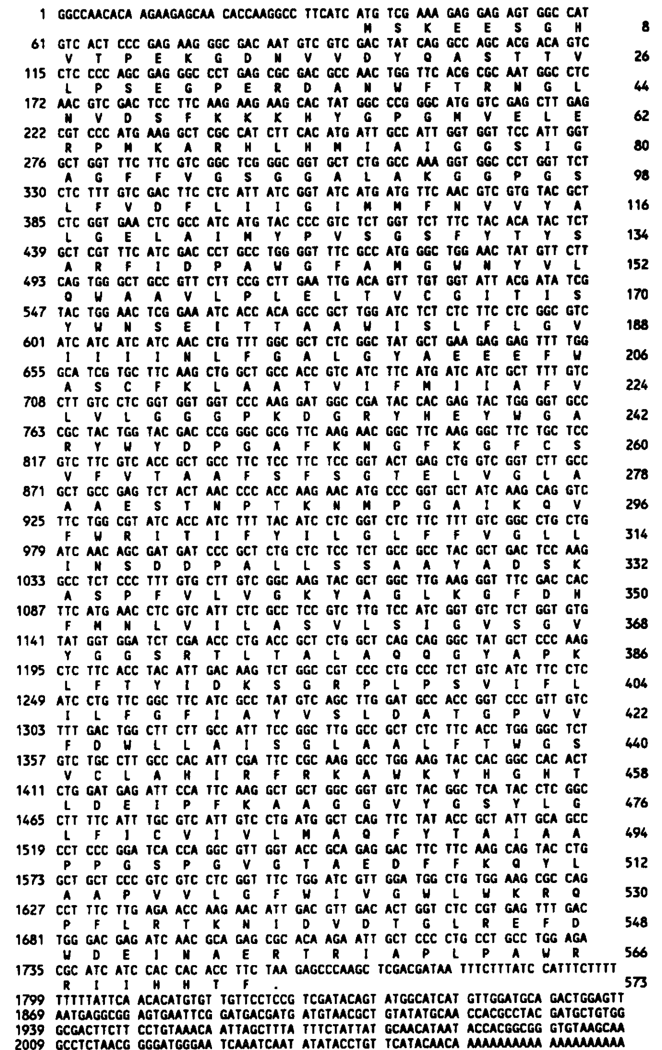

Fig. 2. Nucleotide sequence and predicted amino acid sequence of the *T. harzianum inda1* gene (2·1 kb *SfiI–NotI* fragment).

restriction enzymes, few fragments could be identified in the restriction map of the clone hybridized to the INDA1 cDNA, indicating that *inda1* is probably a single-copy gene. The *inda1* ORF encodes a protein of 574 amino acid residues with a predicted molecular mass of 62 853 Da and a calculated pI of 7·81. The codon usage of the *inda1* gene resembles that of other *Trichoderma* genes (Vanhanen, 1991; Goldman *et al.*, 1992a). Of the 61 codons, 9 are not used at all and 22 are used more than 10 times, coding for about 77 % of the amino acid residues of INDA1 (Table 1). Thus, no codon bias is apparent, a result in agreement with other genes expressed at low levels (Bennetzen & Hall, 1982).

Comparison of the amino acid sequence of INDA1 with those of amino acid permeases from several other organisms revealed a striking identity (Fig. 4): 43·4 % with GAP1 from *Saccharomyces cerevisae*; 35·9 % with HIP1 from *S. cerevisae* (Tanaka & Fink, 1985); 34·1 % with YCC5 from *S. cerevisae* (Tanaka & Fink, 1985); 32·9 %

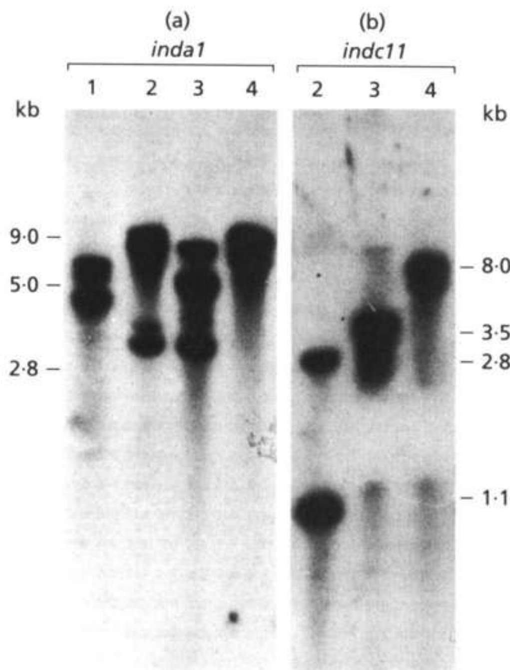

Fig. 3. **Southern blot of *T. harzianum* genomic DNA. DNA was prepared according to Garber & Yoder (1983) and digested with restriction endonucleases *PstI* (lane 1), *HindIII* (lane 2), *EcoRI* (lane 3) or *BamHI* (lane 4). Blots were probed with a ^{32}P-labelled 2·1 kb fragment from an INDA1 cDNA clone (a) or a 1·2 kb fragment from an INDC11 cDNA clone (b).**

with LYSP from *Escherichia coli* (Steffes *et al.*, 1992); and 27·2% with CAN1 from *S. cerevisiae* (Hoffmann, 1985). The identity to the GAP1 protein from *S. cerevisiae* is the highest value of similarity yet reported for amino acid permeases.

Experiments on the topology of membrane proteins have shown that structures predicted from hydropathy profiles are consistent with experimental methods of determining the structure of permeases (Ahmad & Bussey, 1986; Gött & Boos, 1988; Sophianopoulou & Scazzocchio, 1989). We analysed the hydropathy profile of the INDA1 polypeptide (Fig. 5). The INDA1 putative transporter lacks a hydrophobic N-terminal signal peptide, the whole N-terminal region of 75 amino acids being hydrophilic. The C-terminus of 39 amino acids is also hydrophilic. The remainder of the protein is highly hydrophobic, having 10 distinct putative transmembrane segments of an average hydropathy index greater than 30 (residues 98–114, 151–167, 182–198, 212–228, 300–316, 352–368, 401–417, 421–437, 477–493 and 511–527; Fig. 5). We also examined the hydropathy profile of the INDA1 permease with a number of the sequenced prokaryotic and eukaryotic transport proteins mentioned above. The hydropathy profile of the INDA1 permease was similar to all of the permeases, even though the number of putative transmembrane segments was not identical in all cases (data not shown; Fig. 5). Thus, based on the sequence homology and hydropathy profile of the INDA1, we propose that the *inda1* gene encodes a putative amino acid permease.

The INDC11 cDNA clone corresponds to a gene that encodes a novel polypeptide

The INDC11 cDNA clone contains an insert of approximately 1·2 kb. The cDNA has an ORF of 1020 nucleotides

Table 1. Codon usage of the *T. harzianum inda1* and *indc11* genes

Amino acid	Codon	Occurrence*		Amino acid	Codon	Occurrence*		Amino acid	Codon	Occurrence*		Amino acid	Codon	Occurrence*	
		inda1	*indc11*			*inda1*	*indc11*			*inda1*	*indc11*			*inda1*	*indc11*
Phe	TTT	9	4	Ser	TCT	13	1	Tyr	TAT	8	1	Cys	TGT	1	0
	TTC	36	11		TCC	10	8		TAC	17	14		TGC	4	2
Leu	TTA	0	0		TCA	2	1	Stop	TAA	1	1	Stop	TGA	0	0
	TTG	6	1		TCG	5	2		TAG	0	0	Trp	TGG	18	6
	CTT	12	0	Pro	CCT	6	4	His	CAT	2	2	Arg	CGT	5	0
	CTC	21	15		CCC	16	15		CAC	9	5		CGC	8	9
	CTA	0	1		CCA	2	1	Gln	CAA	0	1		CGA	3	0
	CTG	16	9		CCG	3	5		CAG	8	7		CGG	0	1
Ile	ATT	15	1	Thr	ACT	5	2	Asn	AAT	2	2	Ser	AGT	1	0
	ATC	26	17		ACC	14	9		AAC	13	10		AGC	4	7
	ATA	1	1		ACA	5	1	Lys	AAA	2	3	Arg	AGA	3	1
Met	ATG	12	9		ACG	3	5		AAG	22	17		AGG	0	2
Val	GTT	7	2	Ala	GCT	28	0	Asp	GAT	5	3	Gly	GGT	29	1
	GTC	32	18		GCC	26	2		GAC	16	14		GGC	30	21
	GTA	0	1		GCA	4	2	Glu	GAA	4	4		GGA	3	6
	GTG	3	5		GCG	1	2		GAG	17	11		GGG	0	1

* Total occurrence of this codon in the deduced amino acid sequence of the *inda1* and *indc11* genes.

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GAP1    MSNTS---SYEKNNPDNLKHNGITI------DS-EFLTQEPITIPSNGSAVSIDETGSGSKWQDFK--DSFKRVKPIEVDP-------------------   69
HIP1    MPRNPLKKEYWADVVDGFKPATSPAFENEKEST-TFVTELTSKTDSAFPLSSKDSPGINQTTNDITSSDRFRRNEDTEQED-------------------   80
YCC5    MSSS--KSLY---ELKDLKNSSTEIHATGQDNEIEYFETGSNDRPSSQPHLGYEQHNTSAVRRFF---DSFKRADQGPQDEVEATQMNDLTSAISPSSRQ   92
INDA1   MS---------------KEESGHVTPEKGDNVVDY--------QASTTVLPSEGPERDANW--------FTR-------------NGL---------N   45
CAN1    MTNSK-------------------EDADIEEKHMY-----------------NEPVTTLFHDVEASQTHHRRGSIPLKDEKSKELYPL-RSFPTRVNG   61
LYSP    M---------------------------------------------------------------------------------------------------    1
        *

GAP1    --NLSEAEKVAIITAQT-----PLKHHLKNRHLQMIAIGGAIGTGLLVGSGTALRTGGP-SLLIGWGSTGTMIYAMVMALGELAVIFP-ISGGFTTYATR  160
HIP1    -------------INNT-----NLSGDLSVRHLLTLAVGGAIGTGLYVNTGAALSTGGPASLVIDWVIISTCLFTVINSLGELSAAFP-VVGGFNVYSMR  161
YCC5    AQELEKNESSDNIGANTGHKSDSLKKTIQPRHVLMIALGTGIGTGLLVGNGTALVHAGPAGLLIGYAIMGSILYCIIQACGEMALVYSNLTGGYNAYPVS  192
INDA1   VDSFKKKHYGPGMVE--------LERPMKARHLHMIAIGGSIGAGFFVGSGGALAKGGPGSLFVDFLIIGIMMFNVVYALGELAIMYP-VSGSFYTYSAR  136
CAN1    EDTFSMEDGIGDEDEGEVQNAE-VKRELKQRHIGMIALGGTIGTGLFIGLSTPLTNAGPVGALISYLFMGSLAYSVTQSLGEMAT-FIPVTSSFTVFSQR  159
LYSP    ------------VSETKTTEAPGLRRELKARHLTMIAIGGSIGTGLFVASGATISQAGPGGALLSYMLIGLMVYFLMTSLGELA-RYMPVSGSFATYGQN   88
                   .      .    .   **.  .*.*..**.*. .. ....  .** .   ...    .    .   .  . **..   .   . ... ..  .

GAP1    FIDESFGYANNFNYMLQWLVVLPLEIVAASITVNFWGTDPKYRDGFVALFWLAIVIINMFGVKGYGEAEFVFSFIKVITVVGFIILGIILNCGGGPTGG-  259
HIP1    FIEPSFAFAVNLNYLAQWLVLLPLELVAASITIKYWNDKIN-SDAWVAIFYATIALANMLDVKSFGETEFVLSMIKILSIIGFTILGIVLSCGGGPHGG-  259
YCC5    LWMMVFGFAVAWVYCLQWLCVCPLELVTASMTIKYWTTSVN-PDVFVIIFYVLVITINIFGARGYAEAEFFFNCCKILMMTGFFILGIIIDVGGAGNDG-  290
INDA1   FIDPAWGFAMGWNYVLQWAAVLPLELTVCGITISYWNSEIT-TAAWISLFLGVIIIINLFGALGYAEEEFWASCFKLAATVIFMIIAFVLVLGGGPKDGR  235
CAN1    FLSPAFGAANGYMYWFSWAITFALELSVVGQVIQFWTYKVPLA-AWISIFWVIITIMNLFPVKYYGEFEFWVASIKVLAIIGFLIYCFCMVCGAGVTGP-  257
LYSP    YVEEGFGFALGWNYWYNWAVTIAVDLVAAQLVMSWWFPDTP-GWIWSALFLGVIFLLNYISVRGFGEAEYWFSLIKVTTVIVFIIVGVLMIIGI------  181
          .. *  .  *    *     .... .    .. .*  .     . .*   .   *  .  . .* *.  .  *.   . ● ●    .  *

GAP1    ---YIGGKYWHDPGA-----FAGDTPGAKFKGVCSVFVTAAFSFAGSELVGLAASESVEPRKSVPKAAKQVFWRITLFYILSLLMVGLLVPYNDKSLI-G  350
HIP1    ---YIGGKYWHDPGA-----FVGHSSGTQFKGLCSVFVTAAFTYSGIEMTAVSAAESKNPRETIPKAAKRTFWLITASYVTILTLIGCLVPSNDPRLLNG  351
YCC5    ---FIGGKYWHDPGA-----FNGKHAIDRFKGVVATLVTAAFAFGGSEFIAITTAEQSNPRKAIPGAAKQMIYRILFLFLATIILLGFLVPYNSDQLL-G  381
INDA1   YHEYWGARYWYDPGA-----FKNG-----FKGFCSVFVTAAFSFSGTELVGLAAAESTNPTKNMPGAIKQVFWRITIFYILGLFFVGLLINSDDPALLSS  325
CAN1    ----VGFRYWRNPGAWGPGIISKDKNEGRFLGWVSSLINAAFTFQGTELVGITAGEAANPRKSVPRAIKKVVFRILTFYIGSLLFIGLLVPYNDPKLTQS  353
LYSP    ---FKGA----QPAGWSNWTIGEAPFAGGFAAMIGVAMIVGFSFQGTELIGIAAGESEDPAKNIPRAVRQVFWRILLFYVFAILIISLIIPYTDPSLLRN  274
            ●     .*..    .         ● .  .  . ..*.. * *.......*  .* ...* *..  . *   ..  . ... ..  .. *  .

GAP1    ASSVDAAASPFVIAIKTHGIKGLPSVVNVVILIAVLSVGNSAIYACSRTMVALAEQRFLPEIFSYVDRKGRPLVGIAVTSAFGLIAFVAASKKEGEVFNW  450
HIP1    SSSVDAASSPLVIAIENGGIKGLPSLMNAIILIAVVSVANSAVYACSRCMVAMAHIGNLPKFLNRVDKRGRPMNAILLTLFFGLLSFVAASDKQAEVFTW  451
YCC5    STGGGTKASPYVIAVASHGVRVVPHFINAVILLSVLSMANSSFYSSARLFLTLSEQGYAPKVFSYIDRAGRPLIAMGVSALFAVIAFCAASPKEEQVFTW  481
INDA1   AAYADSKASPFVLVGKYAGLKGFDHFMNLVILASVLSIGVSGVYGGSRTLTALAQQGYAPKLFTYIDKSGRPLPSVIFLILFGFIAYVSLDATGPVVFDW  425
CAN1    TSYVST--SPFIIAIENSGTKVLPHIFNAVILTTIISAANSNIYVGSRILFGLSKNKLAPKFLSRTTKGGVPYIAVFVTAAFGALAYMETSTGGDKVFEW  451
LYSP    DV-KDISVSPFTLVFQHAGLLSAAAVMNAVILTAVLSAGNSGMYASTRMLYTLACDGKAPRIFAKLSRGGVPRKAAVCTTVIAGLCFLTSMFGNQTVYLW  373
        .   .   ** ...    ●          * .** ...* . *. *   .* . ...       ●  ..  ..● *  .      .. . . .     *. ●

GAP1    LLALSGLSSLFTWGGICICHIRFRKALAAQGRGLDELSFKSPTGVWGSYWGLFMVIIMFIAQFYVALF--PVGDSP-SAEGFFEAYLSFPLVMVMYIGHK  547
HIP1    LSALSGLSTIFCWMAINLSHIRFRQAMKVQERSLDELPFISQTGVKGSWYGFIVLFLVLIASFW-----------------------TFSVPI------R  522
YCC5    LLAISGLSQLFTWTAICLSHLRFRRAMKVQGRSLGELGFKSQTGVWGSAYACIMMILILIAQFWVAIA--PIGEGKLDAQAFFENYLAMPILIALYVGYK  579
INDA1   LLAISGLAALFTWGSVCLAHIRFRKAWKYHGHTLDEIPFKAAGGVYGSYLGLFICVIVLMAQFYTAIAAPPGSPGVGTAEDFFKQYLAAPVVLGFWIVGW  525
CAN1    LLNITGVAGFFAWLFISISHIRFMQALKYRGISRDELPFKAKLMPGLAYYAATFMTIIIIIQGFTAF------APKFNGVSFAAAYISVFLFLAVWILFQ  545
LYSP    LLNTSGMTGFIAWLGIAISHYRFRRGYVLQGHDINDLPYRSGFFPLGPIFAFILCLIITLGQNYEAFLKDTIDWGGVAA-----TYIGIPLFLIIWFGYK  468
        *  . .*.. .. ●  . . * **...   .. . ... . .        .   .     .. .  .                         .  .  .

GAP1    -IYKRNWKLFIPAEKMDIDTGRREVDLDLLKQEIAEEKAIMATKPRWYRIWNF-WC  601
HIP1    -RFRSQRRIIL---------------------------------------------  532
YCC5    -VWHKDWKLFIRADKIDLDSHRQIFDEELIKQEDEEYRERLRNGPYWKRVVAF-WC  633
INDA1   -LWKRQ--PFLRTKNIDVDTGLREFDWD----EINAERTRIAPLPAWRRIIHHTFX  574
CAN1    CIFRC--RFIWKIGDVDIDSDRRDIE-AIVWEDHE-------PKTFWDKFWNVV-A  590
LYSP    LIKGTH---FVRYSEMKFPQNDKK-------------------------------  489
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Fig. 4. Comparison of the amino acid sequence deduced for the *T. harzianum* INDA1 protein with amino acid permeases from *S. cerevisiae* (GAP1, HIP1, YCC5 and CAN1), and *E. coli* (LYSP). See Results for source of data. Asterisks indicate identical residues, dots indicate similar residues and hyphens indicate gaps inserted into the sequences to maximize alignment.

(Fig. 6) which encodes a protein of 340 amino acid residues with a predicted molecular mass of 37010 Da and a calculated pI value of 8·25. The *indc11* nucleotide and amino acid sequences do not display a high degree of homology with any sequence deposited in the databanks. A typical N-terminal sequence resembling signal sequences for excreted proteins was not found (Intelligenetics), suggesting that the INDC11 is not a secreted protein. The DNA sequence surrounding the translation initiation site ATG is CCACAATGGC, similar to the consensus sequence CCACCATGGC (Kozak, 1984). The consensus sequence AATAAA, which is thought to be involved in the 3′-end formation and polyadenylation of the precursor mRNA (see review by Ballance, 1991), was found in the *indc11* gene at nucleotide position 1351, 19 nucleotides upstream from the apparent poly(A) addition site. No other significant sequence similarities were identified in computer-assisted searches of the databanks. Codon usage data for the *T. harzianum indc11* gene are presented in Table 1. The coding region of the *indc11* gene of *T. harzianum* contains approximately 55 mol% G + C. Of the 61 codons, 6 are not used at all and 12 are used more than 10 times, coding for about 56% of the amino acid residues of INDC11. Thus codon bias is apparent, a result in agreement with other genes expressed at high levels (Bennetzen & Hall, 1982; Ballance, 1991). The bases G and C are generally preferred at the third position of the codons. The codon usage of the *T. harzianum indc11* gene resembles that of other *Trichoderma* genes (Vanhanen, 1991; Goldman *et al.*, 1992a).

To determine whether *indc11* is a member of a gene family, we performed DNA blot hybridizations with genomic DNA prepared from *T. harzianum* (Fig. 3b). After

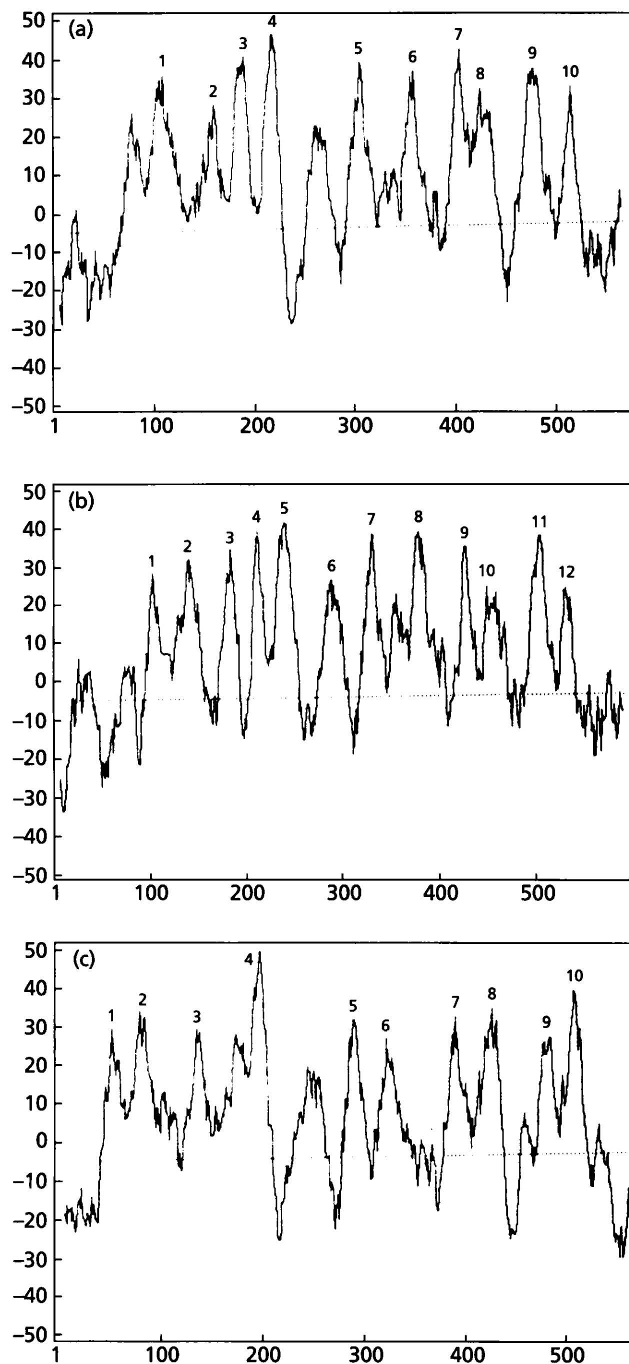

Fig. 5. **Hydropathy profiles of the predicted amino acid sequence of the *T. harzianum* INDA1 amino acid transporter (a), *S. cerevisiae* GAP1 general amino acid permease (b), and *A. nidulans* PRNB proline transport protein (c). The profiles of the amino acid transporters are plotted according to Kyte & Doolittle (1982) from the N-terminus to the C-terminus by averaging hydropathy values over a window of 15 residues.**

digestion of the genomic DNA with appropriate restriction enzymes, few fragments that could be identified in the restriction map of the clone hybridized to the INDC11 cDNA, suggesting that *indc11* is a single-copy gene.

DISCUSSION

We have described the isolation and characterization of two cDNA clones that correspond to genes encoding novel putative mycoparasitism-related proteins of *T. harzianum*. These two clones were obtained by differential screening of an induced library and correspond to genes that are expressed during growth on *R. solani* cell walls. One of them corresponds to a gene, *inda1*, that encodes a putative amino acid permease whereas the other corresponds to a gene, *indc11*, which is expressed at high levels during mycoparasitism. The *indc11* gene encodes a novel polypeptide that does not show any similarity with other proteins deposited in the databanks.

Although the *inda1* gene encodes a presumptive amino acid transporter that shows high similarity to a general amino acid permease of *S. cerevisiae* (see Results), the specificity of the permease activity of INDA1 remains to be elucidated. The hydropathy profile of the INDA1 transport protein is similar to many different permeases (see Results). There is no classical cleavage leader sequence in the INDA1 amino acid permease. All fungal and many bacterial and mammalian transporters lack an N-terminal hydrophobic signal peptide. Blobel (1980) suggested that one or more of the hydrophobic segments of these proteins might function as an 'internal signal peptide'. Further investigation is required to determine the actual role of INDA1 in mycoparasitic interactions, and how its regulation can be affected by a diversity of environmental factors.

It has been proposed that the mycoparasitic activity of *Trichoderma* proceeds in three major steps (Chet, 1987). Initially, the mycoparasite hyphae grow towards the host hyphae (Chet *et al.*, 1981). Then the parasite attaches to the target hyphae, presumably using a host lectin (Elad *et al.*, 1983a, b; Barak *et al.*, 1985), and appressoria-like structures coil around the attacked cells (Chet *et al.*, 1981; Elad *et al.*, 1983b). Concurrently, degradation of 1,3-β-glucans and chitin from the host cell wall is observed (Elad *et al.*, 1983b). Finally, the mycoparasite penetrates and/or lyses the host hyphae (Chet *et al.*, 1981), releasing the cellular contents and providing nutrients to sustain its growth. Both mechanical pressure and cell-wall-degradation by hydrolytic enzymes are probably involved in the process of penetration. Extracellular enzymes that hydrolyse the main chemical constituents of the fungal cell wall, i.e. chitin, glucans and proteins, were detected when *T. harzianum* was grown on *R. solani* mycelia or cell walls as sole carbon source (Ridout *et al.*, 1988; Geremia *et al.*, 1991). The enzymes appeared sequentially: first an alkaline proteinase was produced followed by glucanases and chitinases (Geremia *et al.*, 1993; G. H. Goldman & D. Jacobs, unpublished results). We have recently purified the alkaline proteinase specifically induced by *R. solani* cell walls and chitin (Geremia *et al.*, 1991) and cloned the corresponding gene (*prb1*; Geremia *et al.*, 1993). We suggested that PRB1 was involved in the degradation of the phytopathogen cell walls, membranes and possibly proteins released after the lysis of the host, which could then be used as nutrients by the mycoparasite. Thus, the

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   1 GGCCAACCTTCGAAACAAGCACAGAGAGCAATAGCACGCATCAATTCGAAGTGTGCTGTTGATTGATTGCTTCTTGATAGAGACTCAATCACAATCTCCACCAATTGCTTC
 112 GAGGATCTCTCCAACAAGGCCACTACAGATCGGATCCTGCTTCAACACCATCACC ACA ATG GCC ATC CCC AAC CGC AAA GTC GTC ATC ACC GCC TAC
                                                              M   A   I   P   N   R   K   V   V   I   T   A   Y   13
 209 GGC CCC CCC TCC ACC GCC CTC CAG TTC GTC ACC GAG GAT CTG CCC CCG CCG CCC AAG GAC CAC GTC CAG GTC AAG ATC CTC TAC
      G   P   P   S   T   A   L   Q   F   V   T   E   D   L   P   P   P   P   K   D   H   V   Q   V   K   I   L   Y   41
 293 GCA GGC TTC GCC GGC GCC GAC GTC AAC ATG CGC CTC GGC GTC TAC CCC ATG CAG AGC GCC CCT CCC TTC ACG CCG GGC TAC TGC
      A   G   F   A   G   A   D   V   N   M   R   L   G   V   Y   P   M   Q   S   A   P   P   F   T   P   G   Y   C   69
 377 TTC GCC GGC CGC GTC TCC GTC AAC GGG CCC GGC AGC GGC AAG TTC GAG CCC GGC ACC CTC GTC ACG GCG CTG ACA AAG TAC GAC
      F   A   G   R   V   S   V   N   G   P   G   S   G   K   F   E   P   G   T   L   V   T   A   L   T   K   Y   D   97
 461 TCC GAC GCC GAG TAC ATC AAC ATC CCC GAA AAG TAC CTC CTG GCC ATC CCC GAC GGC GTC GAC CCC AAG GTG GCC GCT GCC CTG
      S   D   A   E   Y   I   N   I   P   E   K   Y   L   L   A   I   P   D   G   V   D   P   K   V   A   A   A   L   125
 545 CCC GTC GAC TGG TCC ACC GCC TAC GGC ATG GTG CAC CGC GCT GCC AAA GTG TCC GAG GGC CAG CGC GTC TTC ATC CAC GGC ATC
      P   V   D   W   S   T   A   Y   G   M   V   H   R   A   A   K   V   S   E   G   Q   R   V   F   I   H   G   I   153
 629 AGC GGA GCA GTT GGC CAG GCC GTC ATG TAT CTC TCG CTC CTC CAG GGC GCC ACC GTC TAC GGC ACG GCC TCT GAG AGG AAC CAC
      S   G   A   V   G   Q   A   V   M   Y   L   S   L   L   Q   G   A   T   V   Y   G   T   A   S   E   R   N   H   181
 713 GCC GCC CTC AAG GAA GCA GGC GCC CAT CCG TAC CTC TAC ACC AAC AAG GAC TGG ATC GCG GCC ATG AAG GAC CTC GGC GGC GTG
      A   A   L   K   E   A   G   A   H   P   Y   L   Y   T   N   K   D   W   I   A   A   M   K   D   L   G   G   V   209
 797 CAC GCT GTG TTT GAC GCC CTG GGC TTT GAA AGC TTC GAC GAG TCC TAC TCC ATC TTG ACT CCC AAG GAG AGA AGC GTA GTC GTC
      H   A   V   F   D   A   L   G   F   E   S   F   D   E   S   Y   S   I   L   T   P   K   E   R   S   V   V   V   237
 881 GCC TAC GGA AAC AAC CTC AGC AAT CTC ACT GGT GCG AAG CGG CGC AGC CCT TGG ATC CCC ATG GCC AAG CTG CTA TTC AAG AAT
      A   Y   G   N   N   L   S   N   L   T   G   A   K   R   R   S   P   W   I   P   M   A   K   L   L   F   K   N   265
 964 CTC TAT TTC TGG TCC AAC AAG GGA GCT ATA TTC TAC TTC ATC ACG CGC GAC CAA AAG ACG TTT GAG CCT GAA CTG CAG CTG CTG
      L   Y   F   W   S   N   K   G   A   I   F   Y   F   I   T   R   D   Q   K   T   F   E   P   E   L   Q   L   L   293
1049 CTC AAC ATG ACC AGG GAT GGA ATC ATT ACC CCT CCG ATC AAG GCC GTC TGG GAG TTT GAC GAT ATC AAA GAG GCC CAT GAG GCT
      L   N   M   T   R   D   G   I   I   T   P   P   I   K   A   V   W   E   F   D   D   I   K   E   A   H   E   A   321
1133 TGG TGC AAG GGC TCA GGA ATG GGA TCG CCA GTT ATC CGC ATC GCC CGC GAC GCT TAA AGTGGAAATCGTGCTCTTGTCTCTCTTTGATACCT
      W   C   K   G   S   G   M   G   S   P   V   I   R   I   A   R   D   A   •                                       340
1225 CGTTTAATAGTGTCTTGATTTGGTTAAAGATTGTTGTCGGATCTGCAGCGGTTTTCTGTTATTGTCTCCTCCCCAATTTAATTCCCAATTATTTAGTTCAGCAACTTGAAA
1336 CATTCACTAATTAATAATAAAATCCATGTACTTTGTCTGCAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 6. Nucleotide sequence and predicted amino acid sequence of the *T. harzianum indc11* gene (1·2 kb *Sfi*I–*Not*I fragment).

mycoparasite could utilize amino acids derived from the enzymic hydrolysis of these proteins as carbon and/or nitrogen sources. Consequently, the uptake of these amino acids will be of importance for the nutrition of the mycoparasite. The coordinated transcription of the *prb1* and *inda1* genes fits this model, but formal proof of this hypothesis must await additional studies.

The use of this methodology for identifying and characterizing mycoparasitism-related genes should provide more biochemical information about mycoparasitism and provide specific genetic markers to follow the biological interaction. Further steps in this direction would be specific knock-out of the *inda1* and *indc11* genes in order to prove their role in mycoparasitism. Additionally, investigations will be carried out into the development of better biocontrol strains of *Trichoderma* spp. In this regard, the fact that *inda1* and *indc11* genes are expressed during growth on R. *solani* cell walls suggests that their promoters could be useful for many types of genetic engineering. The fusion of genes coding for products that are inhibitory to plant pests could, under the control of these promoters, have great potential for producing transgenic *Trichoderma* strains with enhanced biocontrol activity. Enzymes that degrade fungal cell walls, especially chitinases or glucanases, may be attractive candidates for such products.

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