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ORIGINAL ARTICLE

Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four *Trichoderma* species

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Abstract Endophytic isolates of *Trichoderma* species are being considered as biocontrol agents for diseases of *Theobroma cacao* (cacao). Gene expression was studied during the interaction between cacao seedlings and four endophytic *Trichoderma* isolates, *T. ovalisporum*-DIS 70a, *T. hamatum*-DIS 219b, *T. harzianum*-DIS 219f, and *Trichoderma* sp.-DIS 172ai. Isolates DIS 70a, DIS 219b, and DIS 219f were mycoparasitic on the pathogen *Moniliophthora roreri*, and DIS 172ai produced metabolites that inhibited growth of *M. roreri* in culture. ESTs (116) responsive to endophytic colonization of cacao were identified using differential display and their expression analyzed using macroarrays. Nineteen cacao ESTs and 17 *Trichoderma* ESTs were chosen for real-time quantitative PCR analysis. Seven cacao ESTs were induced during colonization by the *Trichoderma* isolates. These included putative genes for ornithine decarboxylase (P1), GST-like proteins (P4), zinc finger protein (P13), wound-induced protein (P26), EF-calcium-binding protein (P29), carbohydrate oxidase (P59), and an unknown protein (U4). Two plant ESTs, extensin-like protein (P12) and major intrinsic protein (P31), were repressed due to colonization. The plant gene expression profile was dependent on the *Trichoderma* isolate colonizing the cacao seedling. The fungal ESTs induced in colonized cacao seedlings also varied with the *Trichoderma* isolate used. The most highly induced fungal ESTs were putative glucosyl hydrolase family 2 (F3), glucosyl hydrolase family 7 (F7), serine protease (F11), and alcohol oxidase (F19). The pattern of altered gene expression suggests a complex system of genetic cross talk occurs between the cacao tree and *Trichoderma* isolates during the establishment of the endophytic association.

Keywords Antibiosis · Endophyte · Mycoparasitisim · *Theobroma cacao* · *Trichoderma*

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Introduction

Theobroma cacao (cacao), the source of chocolate, is grown in many tropical countries (Wood and Lass 2001). Cacao is grown in a range of cropping systems such as full sun, or more traditionally under shade. Cacao diseases cause severe yield losses in many areas where cacao is grown (Bowers et al. 2001; Wood and Lass 2001). The major diseases of cacao include Black Pod (*Phytophthora* species), Witches' Broom (*Crinipellis perniciosa*), and Frosty Pod Rot (*Moniliophthora*

roreri). Although the distributions of the pathogens vary, all three diseases occur in South and Central America (Bowers et al. 2001; Wood and Lass 2001). Fungicides are used to control cacao diseases with varying success and at significant cost to smallholder farmers (Purdy and Schmidt 1996; Adejumo 2005). We are considering biocontrol of cacao diseases as an alternative strategy to fungicide use and as a component of integrated pest management.

In their native habitats, *Theobroma* species, including cacao, are understory forest trees found in tropical regions of Central and South America (Wood and Lass 2001). As such, *Theobroma* species exist in some of the most diverse ecosystems in the world (Wood and Lass 2001). With more extensive study has come the realization that *Theobroma* species carry a portion of this regions diverse microbial community in unique endophytic associations (Arnold et al. 2003; Rubini et al. 2005). Among the endophytic fungi associated with cacao and other forest inhabitants are many species of *Trichoderma* (Samuels et al. 2000; Holmes et al. 2004). These *Trichoderma* species inhabit different plant tissues including roots, trunks, stems, leaves and fruit.

Trichoderma species have recently been described as ''opportunistic, avirulent plant symbionts with potential to control plant diseases'' (Harman et al. 2004). Much of the research concerning *Trichoderma* species/plant interactions centers on the concept of *Trichoderma* species as common soil inhabitants (Harman et al. 2004). Increasing evidence indicates certain *Trichoderma* species have significant potential for biocontrol of plant diseases in the plant canopy where environmental conditions, microbial communities, and plant tissues differ greatly from those encountered in the root zone (Wilson 1997). For example, *T. stromaticum*, an epiphytic mycoparasite, has already proven to be beneficial for control of *C. perniciosa*, the causal agent of witches' broom of cacao (Bastos 1996; Samuels et al. 2000).

We have isolated a collection of endophytes from the above ground portions of cacao trees, related *Theobroma* species, and other plant species found associated with *Theobroma* species in tropical forest. This collection of endophytic fungi represents a potential source of biocontrol agents for cacao diseases. More than 100 isolates from diverse *Trichoderma* species are included in the collection (Evans et al. 2003). The objectives of the research presented here include the characterization of the responses of both the endophytic *Trichoderma* species and the cacao seedlings following establishment of an endophytic relationship. As part of this research we used a seedling assay and differential display, macroarray, and real-time quantitative PCR (Q-PCR) to characterize the interactions between four *Trichoderma* species and cacao at the molecular level.

Materials and methods

Trichoderma isolates and their culture

The isolation of *Trichoderma* endophytes was described by Evans et al. (2003). Four isolates, representing four phylogenetically distinct species of *Trichoderma*, were chosen for study (Holmes et al. 2004). DIS 70a (*T. ovalisporum*) was isolated from high tropical forest along the Pañacocha-Río Yanayacu, Napo River, Sucumbios Province, Ecuador in 1999 (Holmes et al. 2004). The tissue was a witches' broom on a liana, identified by a local Quechua guide as ayahuasca (*Banisteriopsis caapi*, Malpighiaceae). DIS 219b (*T. hamatum*) and DIS 219f (*T. harzianum*) were isolated from a pod of *Theobroma gileri* found in Guadual, Lita, Esmeraldas Province, Ecuador (Evans et al. 2003). DIS 172ai ('Tkon 21' in Holmes et al. 2004) represents an undescribed *Trichoderma* species that is widely distributed in tropical American and African soils. DIS 172ai was isolated from the stem of a 50–60 year old *Theobroma grandiflorum* (cupuaçu) tree located in Brazil (Embrapa, Belem, Para).

Soluble inhibitory metabolite production

Conidia from *Trichoderma* isolates were harvested from 2-week-old cultures grown on 20% PDA at 25°C and subsequently filtered through glass wool to remove mycelia. Three flasks containing 150 ml of minimal salts broth (MIN; Srinivasan et al. 1992) were each inoculated with 1 ml of a 1×10^6 conidial suspension of the endophyte and incubated in an orbital incubator at 25°C and 110 rpm. After 7 days growth, mycelia were collected in cheese cloth and the liquid sterilized by filtration through a 0.22 μm filter (Millipore). Sterile filtrate was placed in a 90°C water bath for 2 h to inactivate enzymes, then added to an equal volume of strengthened agar, 3% MIN [3% agar no. 3], and poured into Petri dishes. A 4-mm-diam. plug of *M. roreri*, from the growing edge of a 7-day-old colony, was placed in the center of the Petri dish. Controls were prepared by replacing the fungal filtrate with uninoculated MIN broth. Three replicate plates were used for each test and all plates were incubated at 25°C. Inhibition of mycelial growth of *M. roreri* was recorded as the difference between mean radial growth in the presence and absence of the fungal filtrate.

Mycoparasite screening

The *Trichoderma* isolates were screened for mycoparasitic ability using a pre-colonized plate method as previously described (Evans et al. 2003; Holmes et al. 2004). A strip of inoculum, 2.5×0.5 cm, excised from a freshly sporulating colony of the *Trichoderma* isolate, was placed at one edge of a 9-cm-diam. PDA plate wholly pre-colonized by an isolate of *M. roreri*. Five replicate plates were prepared. The plates were maintained at 25°C in the dark, and on a weekly basis, a total of 15 samples from each replicate were removed with a 5-mm-diam. cork borer starting at the *M. roreri* inoculum. These samples were plated onto 20% PDA and incubated at 25°C under black light (near UV) and observed over 14 days for the growth of the *Trichoderma* sp. or *M. roreri*. The percentage colonization was determined. This was carried out for 5 weeks or until complete colonization by the *Trichoderma* isolate had occurred.

Enzyme assays for *T. ovalisporum* isolate DIS 70a

Isolate DIS 70a was grown 8 days at room temperature and 75 rpm in 250 ml Erlenmeyer flasks containing 25 ml Wiendling's minimal salts (Jones and Hancock 1987) plus 0.2% carboxymethylcellulose, crystalline cellulose, glycerol, pectin, or cacao seedling extract. Cacao seedling extract was prepared by grinding 7-day-old cacao seedlings minus the cotyledons in liquid nitrogen and lyophilizing the resulting powder. The lyophilized powder was used directly in culture medium. The remaining chemicals were reagent grade. DIS 70a was also grown as above in V8 broth. Three replicate cultures of each isolate were prepared from each medium. Culture filtrate was prepared by passing the culture through a sterile 0.2 μ bottle filter unit. Dry weight of mycelia collected on the filter units from each replicate culture was determined. Sterile culture filtrate was stored at –80°C until used.

Culture filtrate was assayed for carboxymethylcellulase (CMC'ase), crystalline cellulase, chitinase, polygalacturonase, pectate lyase, and protease activities. For CMC'ase activity, culture filtrate was mixed with 50 mM MES buffer, pH 5.0, and 0.2% carboxymethylcellulose and incubated at 37°C. For crystalline cellulase activity, culture filtrate was mixed with 100 mM MES buffer, pH 5.0, and 0.2% crystalline cellulose (Sigmacell, Sigma Chemical Company, St. Louis, MO, USA) and incubated at 37°C. For polygalacturonase activity, culture filtrate was mixed with 100 mM succinate, pH 5.0, 10 mM EDTA, and 0.2% polygalacturonic acid and incubated at 37°C. Reducing sugars liberated in these reactions were determined by the method of Nelson (1944) with glucose as standard for carboxymethylcellulase and crystalline cellulase activity and galacturonic acid as standard for polygalacturonase activity. One unit of CMC'ase or crystalline cellulase activity was defined as the amount of enzyme that released 1 μg glucose reducing equivalent per min per μg dry weight mycelium. One unit of polygalacturonase activity was defined as the amount of enzyme that released 1 μg galacturonic acid reducing equivalent per min per μg dry weight mycelium. For pectate lyase activity, culture filtrate was mixed with 50 mM Tris, pH 8.5, 1.5 mM $CaCl_2$, and 0.2% polygalacturonic acid and incubated at 37°C. One unit of pectate lyase activity was the amount of enzyme that increased absorbance at 232 nm one unit per hour per μg dry weight mycelium. Chitinase activity was determined by incubating culture filtrate with 50 mM Tris, pH 7.0 and 200 μg chitin azure (Sigma Chemical Co.) at 37°C. Chitinase activity was also determined in 50 mM Tris, pH 8.0 and in 50 mM succinate buffer, pH 5.0. One unit of chitinase activity was defined as the amount of enzyme that increased absorbance at 575 nm one unit per hour per μg dry weight mycelium. Protease activity was determined by incubating culture filtrate with 50 mM Tris, pH 7.0 and 200 μg azocoll (Sigma Chemical Co.) at 37°C (Chavira et al. 1984). One unit of protease activity was the amount of enzyme that increased absorbance at 520 nm one unit per hour per μg dry weight mycelium. Autoclaved culture filtrates were used as negative controls in determinations of all enzyme activities. Lower limits of detection were 0.198 U for CMC'ase and crystalline cellulase, 0.386 U for polygalacturonase, 0.1 U for pectate lyase, 0.01 U for chitinase, and 0.01 U for protease activities.

Seedling production

Open pollinated seeds of *T. cacao* variety Comum (Lower Amazon Amelonado type) were collected by Alan Pomella from established plantings at the Almirante Cacau, Inc. farm (Itabuna, Bahia, Brazil). The seed coat was removed and the seed surface sterilized by incubation in 14% sodium hypochlorite for 3 min followed by three washes in sterile distilled water. Three sterile seeds were placed side-by-side with the radical tips oriented in the same direction on 1.5% water agar in 10-cm-diam Petri dishes and sealed with parafilm. Seeds were pre-germinated under fluorescent lights at 23°C. After 3 days, two 0.6-cm agar plugs of one of the four *Trichoderma* isolates were placed on the water agar surface below the emerging roots. The *Trichoderma* isolate was allowed to grow out of the

agar plug through the water agar and onto the cacao seedlings. The seedlings were rated for root discoloration after 6 days of colonization using a scale from 0 (no discoloration) to 4 (severe browning).

Seedlings were subsequently processed in one of three ways: (1) Dissection into 1-cm sections of roots, stems, cotyledons, and plumules, surface sterilized as previously described and plated on CDA. (2) Cotyledons were removed and the seedlings frozen in liquid nitrogen and stored until used for RNA isolation. (3) After being colonized by *Trichoderma*, the germinating seeds were planted in sterile soilless mix (80 g of 2:2:1, sand:perlite:promix) in double magenta boxes (20-cm high). Sterile distilled water (20 ml) was added to dry soilless mix after planting and the seedlings grown on fluorescent light benches at 23°C for 2 weeks. The 2-week-old cacao seedlings were dissected into 1-cm sections of roots, stems, cotyledons, plumules, and leaves, surface sterilized as previously described, and the sections incubated on cornmeal agar plates. All plates were incubated at room temperature on the lab bench (23°C) for 5–7 days until the endophytic *Trichoderma* isolates grew out of the cacao tissue sections. At least four replicate plates of three seedlings each were prepared for each *Trichoderma* isolate and the control (uninoculated seedlings) for each of the three seedling processes. Replicate seedling plates were initiated two at a time using seeds from separate shipments. Four seedlings from independent replicate seedling plates were dissected for each *Trichoderma* isolate and the control for seedling processes 1 and 2 in order to verify endophytic colonization of the cacao seedlings.

Statistical analysis

The data were expressed as percent growth inhibition for the antibiosis study and percent colonization for the mycoparasitism and seedling colonization studies. The data were analyzed by analysis of variance using the SAS general linear models procedure (SAS Institute, Cary, NC, USA). The least significant difference ($P < 0.05$) is provided for means comparisons for each tissue in each experiment.

RNA isolation and differential display

Fungal isolates were grown on CDA plates, [1.7% Difco corn meal agar (DIFCO laboratories, Detroit, MI, USA) plus 20% dextrose] in an incubator at 23°C for 5 days before use. Two agar plugs were added to 50 ml of clarified V8 broth in a 250 ml flask, three flasks for each isolate. Mycelia of each isolate were produced by growth at 23°C in stationary cultures for 7 days. Mycelia were collected by filtration onto #2 Whatman paper, frozen in liquid nitrogen, and stored in liquid nitrogen before use in RNA extractions.

Total RNA was isolated from cacao seedlings and treated with DNase I as previously described (Bailey et al. 2005a). Total RNA of fungal mycelia was extracted using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendation, with an extra DNase I treatment. Using the GenHunter Corporation (Nashville, TN, USA) RNAspectra Red Fluorescent mRNA Differential Display System, the RNA was reverse-transcribed with three unlabeled anchor primers following manufacturers instructions. Subsequent PCR reactions used all 8 random primers included in RNAspectra^{TM} Red Kit 1, with the incorporation of the fluorescent label using labeled anchor primers. The samples were electrophoresed on a 4-mm-thick denaturating polyacrylamide (6% acrylamide, 8 M urea) sequencing gel. The GenHunter procedure was followed for loading and electrophoresis. Gel images were captured using an Amersham Biosciences Typhoon 8600 Variable Mode Imager (GE Healthcare, Piscataway, NJ, USA) with a 532 green laser, and Cy3, 555, BP20 emission filter. Bands of interest were selected, excised and eluted from the gel, and reamplified following the procedure described in the GenHunter protocol. The reamplified cDNA fragments were cloned using the PCR-TRAP Cloning System (GenHunter). Plasmids containing DNA from bands of interest from the differential display were isolated using a Wizard Plus SV Minipreps DNA kit (Promega, Madison, WI, USA) following kit directions. The mini-prepped plasmids were sequenced and then identified using BLAST.

Macroarray procedure

Plasmid DNA (2 μg) was diluted with sterile distilled water and transferred into each well of a 96-well microtiter plate to give 16 μl. Denaturing solution (40 ml 2 M NaOH plus 2 ml 1% bromophenyl blue) was added at a rate of 4 μl per well. Empty wells were loaded with 16 μl water plus 4 μl NaOH/dye. The plates were incubated at room temperature for 20–30 min before printing.

Two different plates were set up for each printing. Using a 9-hole grid template, each plate was stamped on each membrane (Zeta-Probe GT, BioRad, Hercules, CA, USA) four times in either a 4-corner pattern or a compass-point (diamond) pattern, leaving the center of the 9-hole grid empty, giving four identical replications per blot. This process resulted in the

transfer of approximately 25 ng of plasmid DNA for each of four spots for each plasmid. Immediately after printing, the membranes were crosslinked using a Stratagene (La Jolla, CA, USA) UV Stratalinker 1800, air-dried and then stored at –20°C.

Radioactive probes were made from 5 μg RNA using Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) following the instructions supplied with the kit, except volumes were doubled. dCTP-αP^{32} was supplied by Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). Unincorporated nucleotides were removed with an Edge Biosystems (Gaithersburg, MD, USA) Performa DTR-RT gel filtration cartridge spin column. Three independent biological samples were used to create probes for each *Trichoderma* isolate/*T. cacao* combination and controls.

Membranes were pre-wetted in 1× SSC and incubated twice in BD Biosciences-Clontech (Palo Alto, CA, USA) ExpressHyb buffer for 30 min at 65°C. Fresh, pre-warmed ExpressHyb buffer was placed in each bottle and the cleaned probe added to the buffer and gently mixed before returning the bottle to the incubation oven. Membranes were hybridized 2 h at 65°C with constant rotation. Blots were washed following the ExpressHyb protocol, exposed to a Molecular Dynamics (Amersham Biosciences, part of GE Healthcare) Storage Phosphor screen, and visualized using an Amersham Biosciences Typhoon 8600 Variable Mode Imager.

Real-time quantitative PCR

Four micrograms of each RNA sample isolated as described above were used to generate first strand cDNA using SuperScript III RNase H^- Reverse Transcriptase (Invitrogen) with Oligo $(dT)_{20}$ primer. The synthesized first strand cDNA was diluted tenfold and used as template for Q-PCR. The Q-PCR analysis of gene expression was performed using Mx3005P® Q-PCR System and Brilliant® SYBR® Green Q-PCR Master Mix (Stratagene, La Jolla, CA, USA). Primers (Supplementary File S1), 23–27 oligomers, for selected genes were designed to generate a product of 200–250 bp, and to have a T_m (melting temperature) of 60±3°C using the Primer 3 program of Biology Workbench (http://www.workbench.sdsc.edu/). A dissociation (melting) curve was run for each sample at the end of the amplification reaction to verify a single product was amplified in the PCR reaction (95°C for 1 min, 55°C for 30 s and 95°C).

Reactions contained 12.5 μl of 2× Brilliant SYBR® Green Q-PCR Master Mix, 5 μl of tenfold diluted cDNA, 2,500 nM of each gene-specific primer and diluted reference dye (final concentration = 300 nM) in a final volume of 25 μl. A master mix of cDNA, 2× SYBR® Green QPCR Master Mix, and reference dye was prepared to reduce pipetting errors and to ensure the same amount of reagent in each well. A threshold of 0.1 was manually defined to obtain a threshold cycle (C_T) value, which is the cycle number that is required for the SYBR® Green fluorescent signal (ΔRn) to cross the threshold value. Averages and standard errors (SEs) for C_T values were calculated for each gene of interest based on three replications with three different biological samples. The following default thermal profile was used: 95°C for 10 min, 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s.

The constitutively expressed cacao *ACTIN* gene (P55) and *Trichoderma ACTIN* gene, (AY376676) were used as expression references. PCR efficiencies (E) of all primers were calculated using dilution curves with five dilution points, threefold dilution, and the equation $E = [10^{(-1/\text{slope})}] - 1$ as described previously by Pfaffl (2001). To compare data from different PCR reactions and cDNA templates, C_T values for all genes of interest (GOI) (C_{TGOI}) were normalized to the C_T values of ACT (C_{TACT}) for each replication. The relative expression ratios of the target plant genes (treatment/control) were computed using the following equation: $\left[(E_{GOI})^{\Delta C_{TGOI}}/(E_{ACT})^{\Delta C_{TACT}}\right]$, where $\Delta C_T = C_{TC} - C_{TT}$, C_{TT} is the C_T for treatment, and C_{TC} is the C_T for the corresponding control. The data are presented as Log_{10} values of the fold induction/repression ± standard error. The data for plant genes is expressed as Log_{10} of the induction/repression ± standard error for each *Trichoderma* isolate/cacao seedling combination. Mean fold induction was calculated after determination of individual replication fold induction.

Since some target genes failed to amplify in specific *Trichoderma* isolate/cacao seedling combinations or in fungal mycelia controls, the data for fungal ESTs are presented as Log_{10} of expression levels (EXP_{GOI}) normalized against *Trichoderma ACTIN* (Funact) using the equation $EXP_{GOI} = E_{GOI}^{\Delta C}$ where $\Delta C = C_{TACT} - C_{TGOI}$, C_{TACT} is the C_T for Funact in the sample, and C_{TGOI} is the C_T for the corresponding gene of interest in the sample. The ratios of Fungal EST expression in treatment/Fungal EST expression in mycelia were calculated as described above for fungal ESTs where appropriate controls were available.

Results

Soluble inhibitory metabolite production and mycoparasitism

The *Trichoderma* isolates varied in their abilities to produce metabolites that inhibited growth of *M. roreri*, the Frosty Pod pathogen (Table 1). Culture filtrates from DIS 172ai grown in MIN medium completely inhibited growth of *M. roreri* when incorporated into MIN agar while the DIS 219f filtrate inhibited growth of *M. roreri* 46%. Culture filtrates from the other two isolates were not inhibitory. The isolates studied also varied in their ability to colonize *M. roreri* (Table 1). DIS 70a, DIS 219b, and DIS 219f completely colonized the psuedostroma of *M. roreri* on precolonized plates after 5 weeks. DIS 172ai was a poor mycoparasite of *M. roreri* eliminating the pathogen on only 12% of the agar plugs from precolonized plates after 5 weeks.

Enzyme assays for *T. ovalisporum* isolate DIS 70a

β-Glucanase, cellulase, polygalacturonase, and protease activities were detected in filtrates of DIS 70a liquid cultures grown on cacao extract or V8 Juice as substrate (Table 2). The β-glucanase, cellulase, polygalacturonase, and protease activities were also detected when carboxymethyl cellulose, cellulose, polygalacturonic acid, and gelatin were used as substrates, respectively. The β-glucanase, cellulase, and protease enzyme activities detected showed a dependence on the substrate used with cacao extract and V8 juice serving as complex substrates inducing multiple enzyme activities. Polygalacturonase activity was particularly high among the enzymes assayed.

Colonization of cacao seedlings

DIS 219b and DIS 219f caused intense discoloration of the root surface of cacao seedlings within 6 days of

Table 1 Colonization of cacao seedlings and antibiosis and mycoparasitism of *Moniliophthora roreri* by endophytic *Trichoderma* isolates

	Control	*Trichoderma* isolate				
		DIS 70a	DIS 219b	DIS 219f	DIS 172ai	$LSD_{0.05}$
Antibiosis[a]		–2	11	46	100	36
Mycoparasitism[b]		100	100	100	12	4
Discoloration rating[c]	0.1	1.7	3.9	3.0	2.8	0.9
Seedling colonization (plate culture)[d]						
Root	0	75	87	100	100	26
Stem	0	100	100	87	63	36
Cotyledon	0	87	100	100	100	17
Plumule	0	50	100	50	50	67
Seedling colonization (magenta box culture)[e]						
Root	0	100	100	100	100	0
Stem	0	100	100	100	100	0
Bark	0	100	100	100	100	0
Xylem	0	75	100	100	67	37
Cotyledon	0	100	100	100	100	0
Plumule	0	80	100	100	100	26
Leaf	0	60	100	100	87	35

Colonization studies included pregerminated cacao seeds inoculated with agar plugs of the *Trichoderma* isolate. The *Trichoderma* isolates were allowed to colonize the seedlings. Ratings for root discoloration were made and seedling colonization determined. Alternatively, the seedlings were planted in sterile soil and grown 2 weeks prior to determining seedling colonization. Studies of antibiosis and mycoparasitism were carried out as described in the Materials and methods

[a]Means represent percent inhibition of growth of *M. roreri* on plates including filtrates from *Trichoderma* isolates grown on MIN medium

[b]Means represent percentage of plate precolonized with *M. roreri* where *Trichoderma* isolate was re-isolated and *M. roreri* eliminated

[c]Rating for discoloration of the root zone was determined using a scale of 0 (no discoloration) to 4 (dark brown discoloration) prior to harvest

[d]Seven days after inoculation, seedlings were harvested, cut into sections, surface sterilized, and plated on cornmeal agar. Data represents mean percentage of tissue pieces colonized by the test *Trichoderma* isolate

[e]Seedlings were planted in sterile soil in magenta box culture. Magenta-box-culture seedlings were sampled similar to plate culture seedlings. Data represents mean percentage of tissue pieces colonized by the test *Trichoderma* isolate

Table 2 Enzyme assays for *T. ovalisporum* isolate DIS 70a

Substrate	Mycelium[a] g dry wt	β-Glucanase[b]	Cellulase[b]	Pgase[c]	Protease[d]
Glycerol	0.010(0.001)[e]	BDT	BDT	8.10(0.71)	0.005(0.002)
CMC	0.007(0.001)	2.58(0.03)	–	–	–
Cellulose	0.042(0.001)	–	0.08(0.04)	–	–
PGA	0.011(0.001)	–	–	–	0.026(0.004)
Cacao	0.026(0.004)	1.01(0)	0.64(0.03)	3.89(0.13)	0.013(0.003)
V8 Juice	0.110(0.040)	0.21(0.01)	0.10(0.02)	0.27(0.06)	0.009(0.003)

Isolate DIS 70a was grown 8 days in Wiendling's minimal salts plus 0.2% carboxymethylcellulose, crystalline cellulose, glycerol, pectin, or powdered lyophilized cacao seedling. DIS 70a was also grown in V8 broth. Three replicate cultures of each isolate were prepared from each medium. The culture filtrates were assayed for β-glucanase, cellulase, chitinase, polygalacturonase, pectate lyase, and protease activities. Autoclaved filtrate was used as negative controls in determinations of all enzyme activities. Pectate lyase and chitinase activities were below the level of detection

BDT Below the level of detection, – not done

[a]Dry weight of mycelium in 25 ml culture filtrate

[b]One unit of β-glucanase or cellulase activity is the amount of enzyme that released 1 μg glucose reducing equivalent per minute per g dry weight mycelium

[c]One unit of polygalacturonase (Pgase) activity is the amount of enzyme that released 1 μg galacturonic acid reducing equivalent per min per g dry weight mycelium

[d]One unit of protease activity was the amount of enzyme that increased absorbance at 520 nm one unit per hour per g dry weight mycelium

[e]Numbers in parenthesis represent standard errors

inoculation while DIS 172ai and DIS 70a caused less intense root responses but did discolor cacao roots (Table 1). The roots of uninoculated seedlings remained white. Using 10× to 50× magnification with a stereoscopic microscope, all four fungi were observed growing on the surface of inoculated cacao seedlings. When seedling tissues were surface sterilized and plated on cornmeal dextrose agar (CDA) plates, the *Trichoderma* isolates were observed growing out of all cacao tissues. All four isolates could be re-isolated from all parts of colonized seedlings (Table 1). When considered across the *Trichoderma* isolates tested, plumules were least colonized at 62.5% and cotyledons were most heavily colonized at 96.8%. DIS 219b tended to be the most aggressive in colonizing cacao seedlings under the conditions used. When colonized, germinating seeds were planted in sterile soil in magenta box culture, the seedlings emerged and grew normally. The *Trichoderma* isolates were observed growing out of surface sterilized cacao tissue isolated from magenta box grown seedlings when plated on CM-dextrose agar (Table 1). Under the conditions used, *Trichoderma* isolates maintaining the colony morphologies for the isolates used in the inoculations were the only microbes observed growing out of the cacao tissues. DIS 219b and DIS 219f completely colonized all tissues sampled and, roots, stems, bark, and cotyledons were completely colonized by all four isolates. Among the additional tissues sampled from emerged seedlings in the magenta box culture, leaf and xylem tended to be less colonized although all four isolates were isolated from these tissues more than once.

Differential display analysis

Differential display was carried out using total RNA from cacao seedlings individually colonized by DIS 70a, DIS 219b, DIS 219f, and DIS 172ai plus several *Trichoderma* isolates not further studied. 164 EST clones were sequenced and analyzed for putative protein function. A total of 116 independent ESTs were identified and a putative function or conserved domain identified for 59 ESTs (Supplementary File S2). Thirty-nine ESTs were most closely aligned with plant genes and 16 ESTs were most closely aligned with fungal genes. The majority (11 of 16) of ESTs aligning with fungal genes originated from DIS 219b-colonized seedlings.

Macroarray analysis of cacao gene expression in response to endophytic colonization by *Trichoderma* isolates

An additional 21 ESTs (Supplementary File S2) identified from previous studies of cacao stress responses were included in the macroarray (Bailey et al. 2005a, b). Fifty-nine ESTs of cacao origin and 57 ESTs of uncertain origin (*Trichoderma* species or cacao) were included in the macroarray analysis (Supplementary File S2). The fungal ESTs were included on the macroarray but were generally not detectable by macroarray

analysis and these results are not further discussed (Supplementary File S2). Sixty ESTs could be detected on the macroarrays in at least one of the treatment combinations, 32 ESTs of plant origin related to published sequences and 28 ESTs of uncertain origin (*Trichoderma* species or cacao) unrelated to reported sequences. Many of the EST clones included on the macroarray were not detected using probes derived from total RNA isolated from either fungal-colonized or control cacao tissues suggesting their expression levels were low *in planta*. Data on a subset of the ESTs carried forward to QPCR are presented in Fig. 1. Cacao-derived ESTs P3, P4, P13, P26, P59, and U4 were induced due to colonization by at least three of the four isolates studied with the greatest induction being 18.5-fold for EST P59 when cacao seedlings were colonized by DIS 219b. Expression of EST P12 was repressed in cacao seedling due to colonization by isolates DIS 172ai and DIS 219b. Colonization by *Trichoderma* isolates DIS 219b and DIS 172ai resulted in very similar patterns of both induction and repression for the cacao-derived ESTs being studied.

Real-time quantitative-PCR analysis

A subset of 19 cacao-derived ESTs (Table 3) was selected for further study using Q-PCR based partially on the macro-array results (induced or repressed) and in some cases on the tentative ID of the gene itself.

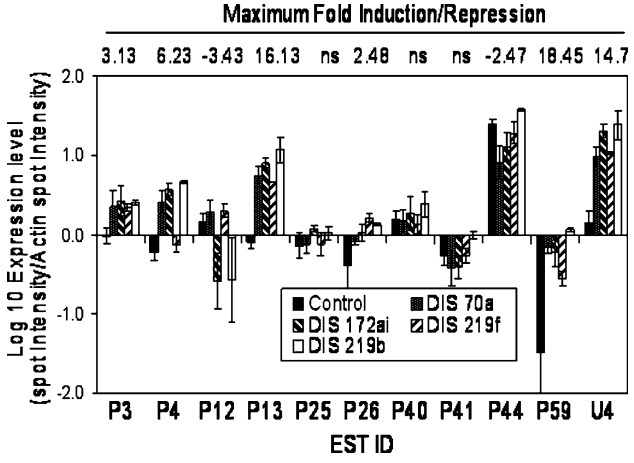

Fig. 1 Expression levels and fold induction/repression values for 11 cacao ESTs on macroarrays probed with cDNA derived from cacao seedlings colonized with four *Trichoderma* isolates. Six days were allowed for colonization of cacao seedlings on water agar before harvest. Sterile cacao seedlings were maintained as controls. The data are presented as the Log_{10} value of the gene of interest (*GOI*) expression level normalized against cacao *ACTIN* (P55). The fold induction/repression in response to colonization is presented numerically over expression level bars. Fold changes in expression less than ±2 are indicated by ns (nonsignificant)

This allowed analysis of some genes that were not detected on the macro-array but were of particular interest based on their putative function. In addition, 17 ESTs (Table 3) characterized as fungal in origin based on sequence analysis were used for Q-PCR analysis. For the ESTs showing similarity to plant gene sequences, all were detectible in the non-colonized cacao seedling at some level. In some cases, depending upon the isolate being studied, primer sets for fungal genes failed to amplify the target gene transcript in colonized plant tissue or in the control fungal biomass. In eight fungal EST/fungal isolate combinations, seven of which occurred with DIS 70a, the primer sets for fungal genes amplified target gene transcript in colonized plant tissue but not in the control fungal biomass. In all cases the targeted gene could be amplified using fungal genomic DNA as a template. In three fungal EST/fungal isolate combinations, the target transcript could be amplified in the control fungal biomass and not in the colonized plant.

T. ovalisporum-isolate DIS 70a interaction with cacao

Based on real time Q-PCR analysis of colonization of cacao seedlings, isolate DIS 70a induced accumulation of transcripts for five plant ESTs more than twofold (P1, P4, P13, P59, and U4) (Fig. 2a). Transcript levels for ESTs P1, P4, P59, and U4 accumulated in DIS 70a-colonized tissues to levels fivefold more than these found in the controls. EST P59 was most highly induced, accumulating in DIS 70a-colonized tissues to levels 60-fold more than levels found in non-colonized seedlings. Transcripts for plant ESTs P12, P31, and P41 decreased in DIS 70a-colonized tissues more than 50% with P12 being decreased more than fourfold.

Transcripts for 12 fungal ESTs including FunTef1 and FunAct (Fig. 2b) were detected in cacao seedlings colonized by DIS 70a. FunTef1 was not induced during colonization, showing a constitutive expression level *in planta* and in pure fungal mycelia relative to FunAct. ESTs F3 and F9 were most strongly induced at 6.4-fold and 2.4-fold, respectively, of the expression levels observed in liquid-culture-grown DIS 70a mycelia. Transcripts of seven fungal ESTs (F2, F5, F7, F11, F12, F14, and F19) were detected in colonized seedlings but were not detected in liquid-culture-grown mycelia.

T. hamatum isolate DIS 219b interaction with cacao

Transcript levels for eight plant derived ESTs (P1, P4, P13, P26, P29, P57, P59, and U4) accumulated in seedlings colonized by DIS 219b to more than

Table 3 Summary of ESTs studied in cacao seedlings colonized by *Trichoderma* species using macro-array and Q-PCR analysis

Clone no.	Accession no.	Size (bp)	Source[a]	Clone ID	Putative ID	Identity(%)/ expected ratios
P1	DW246134	>389	DIS 219b	AF029349	*Lycopersicon esculentum*/ornithine decarboxylase	69/1E-56
P3	DW246135	646	DIS 219b	CNS0A0ZN	*Arabidopsis thaliana*/unknown protein	81/4E-95
P4	DW246136	321	DIS 219b	AB087837	*Pisum sativum* /glutathione S-transferase	65/6E-9
				NM_120357	*Arabidopsis thaliana* /In2-1 protein, putative	63/6.0E-8
P12	DW246137	407	DIS 70a	AM117766	*Theobroma cacao*/unknown protein/extensin-like protein Protease inhibitor/seed storage/LTP family	97/5E-80
P13	DW246138	215	DIS 219b	CA992708	*Gossypium hirsutum* /EST-Zinc-Finger protein, putative Cys2-His2 type	87/7.0E-12
P18	DW246140	415	DIS 219b	NM_148885	*Arabidopsis thaliana*/Zinc-Finger protein	74/2E-25
P20	DW246141	354	DIS 219b	AAM62748	*Arabidopsis thaliana*/B-cell receptor-associated protein	67/4E-9
P25	DW246142	313	DIS 219b	NM_122210	*Arabidopsis thaliana*/expressed protein	86/7E-55
P26	DW246143	190	DIS 219b	CA798633	*Theobroma cacao*/Putative wound protein	100/4.0 E-30
P29	DW246144	440	DIS 70a	AF531362	*Gossypium barbadense*/EF-hand, calcium binding motif	92/6E-49
P30	DW246145	363	DIS 70a	CK144295	*Theobroma cacao*/ORFX/fw2.2-like	99/3E-78
P31	DW246146	662	DIS 219f	BT012976	*Lycopersicon esculentum*/tonoplast intrinsic protein	89/2E-75
P40		572	Published	CK144296	*Theobroma cacao*/Apoplastic quiacol peroxidase-like	
P41		303	Published	CK144297	*Theobroma cacao*/Photosystem I 24 kDa protein-like	
P44		557	Published	CF973685	*Theobroma cacao*/Chitinase-TcChiB	
P55		477	Published	CA797197	*Theobroma cacao*/Actin	
P57		423	Published	CF974274	*Theobroma cacao*/chloroplast elongation factor	
P59	DW246148	517	DIS 219b	AF503442	*Nicotiana* spp./Nectrin 5-glucose oxidase activity	73/3E-28
U4	DW246147	356	DIS 219b		No homology	
F2	DW246118	273	DIS 219b	XP_369811	*Magnaporthe grisea*/Vacuolar ATP synthase subunit	89/4.0 E-07
F3	DW246119	621	DIS 219b	XM_654907	*Aspergillus nidulans* /Glycosyl hydrolases family 2	69/4.0 E-73
F4	DW246120	461	DIS 219b	AY850350	*Magnaporthe grisea* /eukaryotic trans. initiation factor 3	70/4.0 E-10
F5	DW246121	377	DIS 219b	XM_385607	*Gibberella zeae*/Phenylalanyl-tRNA synthetase	86/8.0 E-23
F6	DW246122	300	DIS 219b	XM_369855	*Magnaporthe grisea*/ATP dependent DNA ligase	93/2.0 E-10
F7	DW246123	243	DIS 219b	XM_745951	*Aspergillus fumigatus* /Glycosyl hydrolase family 7	78/4.0 E-15
F9	DW246124	216	DIS 219b	XM_327884	*Neurospora crassa*/Uracil phosphoribosyltransferase	72/4.0 E-18
F11	DW246125	247	DIS 219b	AY258899	*Trichoderma hamatum*/alkaline proteinase	100/1.0 E-53
F12	DW246126	536	DIS 219b	XM_322385	*Neurospora crassa*/Enoyl-CoA hydratase/isomerase	71/6.0 E-55
F13	DW246127	518	DIS 219b	XM_390361	*Gibberella zeae*/DnaJ molecular chaperone	62/2.0 E-55
F14	DW246128	263	DIS 219b	CF872154	*Trichoderma reesei*/nuclear pore membrane glycoprotein	64/2.0 E-04
F15	DW246129	514	DIS 70a	XM_386421	*Gibberella zeae*/actin depolyem. factor/cofilin-like	73/5.0 E-28
F19	DW246131	695	DIS 219f	AF232903	*Cochliobolus victoriae*/alcohol oxidase	76/1.0 E-103
F20	DW246132	363	DIS 219f	XM_389986	*Gibberella zeae*/scavenger mRNA decapping enzyme	81/8.0 E-44
F21	DW246133	264	DIS 219f	CK434057	*Trichoderma harzianum*/unknown	65/2.0 E-28
FunTef1		230	Published	AF348101	*Trichoderma harzianum*/TEF1	
Funact		283	Published	AY376676	*Trichoderma* sp./actin	

[a]Source for P#, U#, and F# ESTs represents the cacao/*Trichoderma* isolate combination from which the EST was isolated using Differential Display

threefold the levels observed in non-colonized seedlings (Fig. 3a). Transcript levels for plant ESTs P1, P4, P59, and U4 were induced more than tenfold in seedlings colonized by DIS 219b. Transcript levels for plant EST P44 decreased fourfold and transcripts for plant ESTs P12 and P31 decreased more than 100-fold in these same seedlings.

Fifteen fungal genes were detected in cacao seedlings colonized by DIS 219b including FunTef1 and FunAct (Fig. 3b). FunTef1 transcript was induced 4.5-fold in the DIS 219b-colonized cacao seedlings compared to transcript levels observed in liquid-culture-grown DIS 219b mycelia. Transcript levels for six fungal ESTs (F4, F6, F12, F13, and F15) were induced less than FunTef1. Of the remaining fungal ESTs whose transcripts were detected in DIS 219b-colonized cacao seedlings, five were induced more than 20-fold with F3, F7, and F11 being most highly induced at 125-fold, 2514-fold, and 116,653-fold, respectively. Transcripts for EST F21 were detected in liquid-culture-grown mycelia but not in DIS 219b-colonized cacao seedlings.

DIS 219f-*T. harzianum* interaction with cacao

Plant ESTs P1, P4, P13, and P57 were induced in DIS 219f-colonized seedlings by just over twofold compared to non-colonized, seedlings, respectively. Transcripts

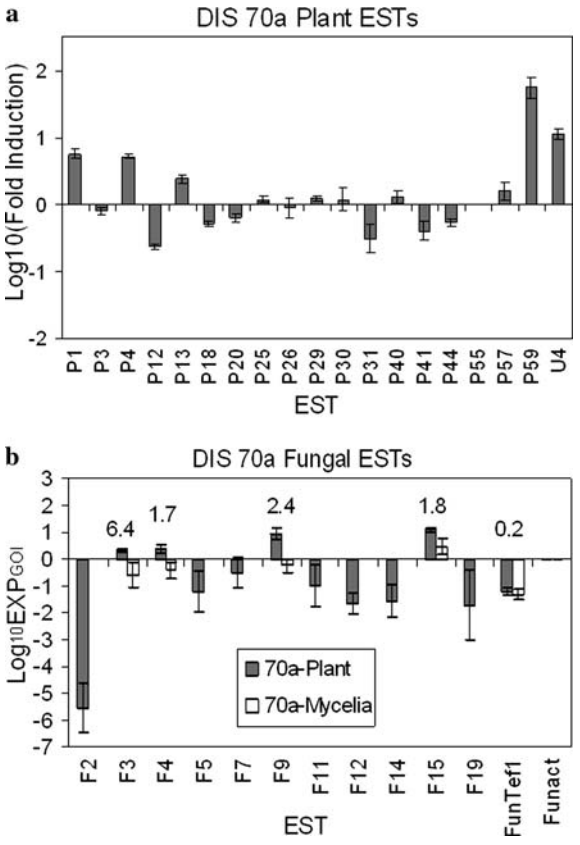

Fig. 2 Fold induction and expression levels for cacao and fungal ESTs in cacao seedlings colonized by *Trichoderma ovalisporum* DIS 70a. **a** Data for plant ESTs is presented as Log_{10} of the fold induction/repression. **b** Data for fungal ESTs is presented as Log_{10} EXP_{GOI} for expression level of each EST relative to Fungal *ACTIN* (*Funact*). *Bars* represent Fungal EST expression in colonized plant tissue (*Plant*) or pure fungal mycelia (*Mycelia*). The fold induction/repression in response to colonization is presented numerically over expression level bars where ESTs were amplified in both treatment and control samples

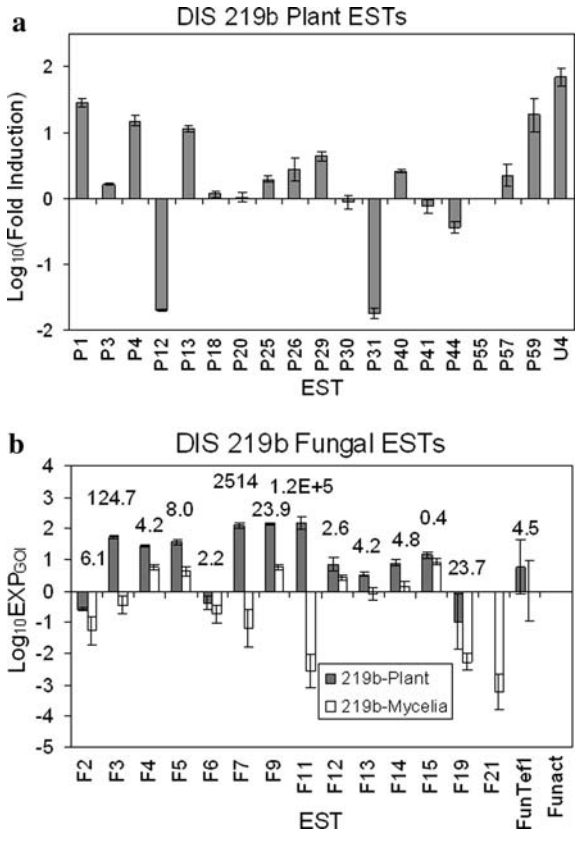

Fig. 3 Fold induction and expression levels for cacao and fungal ESTs in cacao seedlings colonized by *Trichoderma hamatum* DIS 219b. **a** Data for plant ESTs is presented as Log_{10} of the fold induction/repression. **b** Data for fungal ESTs is presented as Log_{10} EXP_{GOI} for expression of each EST relative to Fungal *ACTIN* (*Funact*). *Bars* represent Fungal EST expression in colonized plant tissue (*Plant*) or pure fungal mycelia (*Mycelia*). The fold induction/repression in response to colonization is presented numerically over expression level bars where ESTs were amplified in both treatment and control samples

for plant ESTs P26, P59, and U4 increased 5.4-fold, 19.2-fold, and 8.5-fold, in seedlings colonized by DIS 219f as compared to non-colonized seedlings (Fig. 4a). Transcript levels for plant EST P12 decreased by more than twofold in DIS 219f-colonized seedlings when compared to non-colonized seedlings.

Transcripts for five fungal ESTs were detected in cacao seedlings colonized by DIS 219f including FunTef1 and FunAct (Fig. 4b). FunTef1 was not induced during colonization, showing a consistent expression level in seedlings colonized by DIS 219f and in pure fungal mycelia when compared to FunAct expression levels. ESTs F9 and F21 accumulated in cacao seedlings colonized by DIS 219f to levels 99.5 and 6.7-fold levels detected in mycelia grown in liquid culture, and EST F19 was induced more than 1,000,000-fold in DIS 219f-colonized seedlings. Transcripts for ESTs F4, F15 and F20 were detected in liquid-culture-grown DIS 219f mycelia but not in DIS 219f-colonized seedlings.

DIS 172ai-*T. species* interaction with cacao

Transcript levels for 7 of 20 plant ESTs (P1, P4, P13, P26, P29, P59, and U4) accumulated in seedlings colonized by DIS 172ai to more than threefold the levels observed in non-colonized seedlings (Fig. 5a). Transcripts for plant ESTs P1, P4, P59, and U4 accumulated in seedlings colonized by DIS 172ai to levels that were tenfold higher than the levels observed in non-colonized seedlings. Transcripts for plant ESTs P12 and P31 decreased by more than tenfold in these same colonized seedlings when compared to transcript levels observed in non-colonized seedlings.

Transcripts for eight fungal ESTs were detected in the DIS 172ai-colonized cacao seedlings including

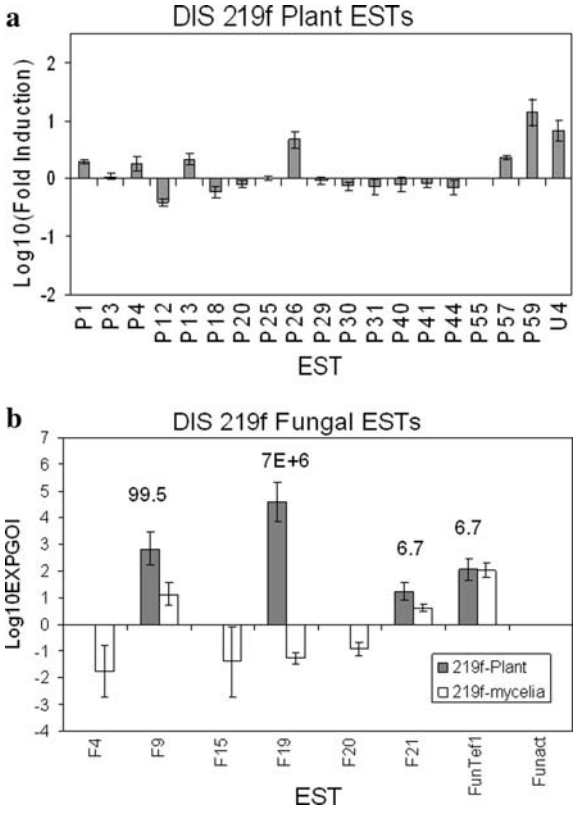

Fig. 4 Fold induction and expression levels for cacao and fungal ESTs in cacao seedlings colonized by *Trichoderma harzianum* DIS 219f. **a** Data for plant ESTs is presented as Log_{10} of the fold induction/repression. **b** Data for fungal ESTs is presented as Log_{10} EXP_{GOI} for expression of each EST relative to Fungal *ACTIN* (*Funact*). Bars represent Fungal EST expression in colonized plant tissue (*Plant*) or pure fungal mycelia (*Mycelia*). The fold induction/repression in response to colonization is presented numerically over expression level bars where ESTs were amplified in both treatment and control samples

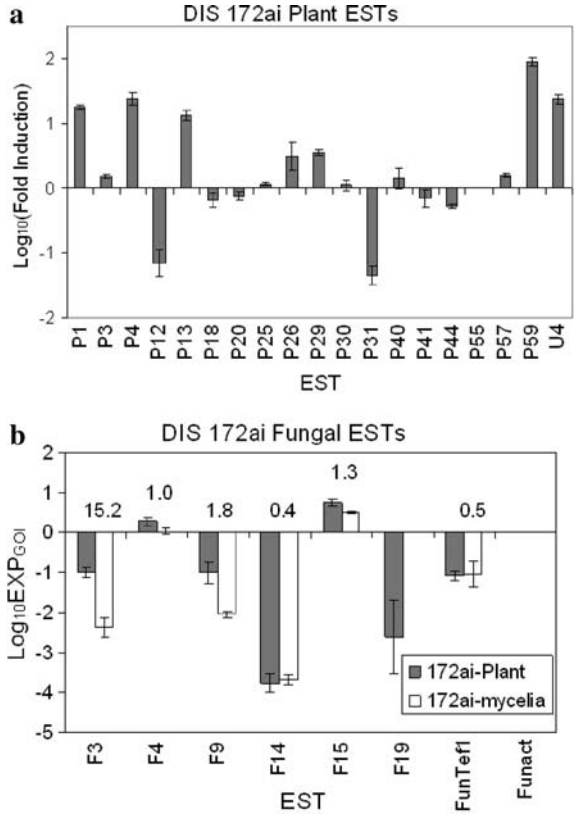

Fig. 5 Fold induction and expression levels for cacao and fungal ESTs in cacao seedlings colonized by *Trichoderma* species DIS 172ai. **a** Data for plant ESTs is presented as Log_{10} of the fold induction/repression. **b** Data for fungal ESTs is presented as Log_{10} EXP_{GOI} for expression of each EST relative to Fungal *ACTIN* (*Funact*). *Bars* represent Fungal EST expression in colonized plant tissue (*Plant*) or pure fungal mycelia (*Mycelia*). The fold induction/repression in response to colonization is presented numerically over expression level bars where ESTs were amplified in both treatment and control samples

ESTs FunTef1 and FunAct (Fig. 5b). EST F3 was most strongly induced *in planta* at 15.2-fold the level found in liquid-culture-grown mycelia. Expression levels for the remaining seven ESTs were not altered during colonization. Transcript for F19 was not detected in liquid-culture-grown DIS 172ai mycelia but was detected in colonized cacao seedlings.

Discussion

Trichoderma isolates with biocontrol potential are endophytic on cacao

Trichoderma ovalisporum DIS 70a has been more intensively studied than the other three isolates tested here (Holmes et al. 2004). In studies by Holmes et al. (2004), and verified here, DIS 70a completely colonized the pseudostroma of the Frosty Pod pathogen *M. roreri* in plate culture screens. DIS 70a established an endophytic association with cacao seedlings in greenhouse studies and could be re-isolated from the apical meristem and the younger tissues (Holmes et al. 2004). DIS 70a was also observed overgrowing the pseudostroma of *M. roreri* on pods in the field and could be re-isolated from surface-sterilized pods 10 weeks after the inoculation (Holmes et al. 2004). Less is known about the biocontrol abilities of the other three isolates tested. However, as shown here (Table 1), *T. harzianum* isolate DIS 219f and *T. hamatum* isolate DIS 219b actively colonized colonies of *M. roreri* grown on 20% PDA. *Trichoderma* sp. Isolate DIS 172ai was a poor mycoparasite of *M. roreri* but produced metabolites capable of completely inhibiting growth of *M. roreri* in culture. The culture filtrates of the other three isolates were significantly

less inhibitory toward *M. roreri*. Antibiosis, the production of antimicrobial compounds, and mycoparasitism, the feeding on a fungus by another organism, are major mechanisms whereby *Trichoderma* species provide protection to plants against plant pathogens (Chet et al. 1998; Howell 2003; Harman et al. 2004).

Data presented here (Table 1) verify that all four isolates studied are capable of establishing endophytic associations with cacao. They differ in their abilities to cause root discoloration but all four isolates were able to colonize all plant parts. Root discoloration was superficial and cacao seedlings grew normally when planted. The root discoloration may be related to the synthesis of antimicrobial compounds. The induction of terpenoid biosynthesis was closely correlated with the ability of *Trichoderma virens* isolates to protect cotton seedlings against *Rhizoctonia solani* (Howell et al. 2000). Our results indicate DIS 219f and DIS 219b are slightly more aggressive in colonizing cacao seedlings than DIS 70a and DIS 172ai, based on the root discoloration and colonization data.

Additional data on enzyme production in response to growth on cacao extract indicates *T. ovalisporum* isolate DIS 70a has many of the enzyme activities required to breakdown plant cell wall components including β-glucanase, cellulase, polygalacturonase, and protease activities (Table 2). Particularly, polygalacturonase activity, required for degradation of the middle lamella, may be essential for movement of *Trichoderma* isolates between cacao cells. β-Glucanase, cellulase, and protease activities have also been associated with the ability of *Trichoderma* isolates to parasitize plant pathogens (Chet et al. 1998).

Gene expression in cacao shows endophyte-isolate-specific induction patterns

All the plant ESTs studied that showed altered expression responded to colonization by more than one *Trichoderma* isolate suggesting some similarity of response across isolates (Table 4). The expression profiles of cacao ESTs were not identical when comparisons were made between *Trichoderma* isolate/cacao interactions ESTs indicating *Trichoderma* isolate dependent differential regulation.

Careful study of the plant responses to the four endophytes suggests these four groupings for the plant genes showing altered gene expression (Table 4): ESTs P1, P4, P12, P13, and P31; EST P26; EST P29; and ESTs P59 and U4. Where grouped with other ESTs, the ESTs showed coordinated expression patterns being either induced or repressed together. Based on the QPCR results, cacao ESTs P1, P4, and P13 were induced in response to colonization by all four *Trichoderma* isolates but only twofold by DIS 219f. EST P12 was repressed in cacao seedlings by colonization with all four *Trichoderma* isolates, only 60% with DIS 219f and, EST P31 was repressed in cacao seedlings colonized by DIS 70a, DIS 219b, and DIS 172ai. EST P26 was induced in response to colonization with DIS 219f, DIS 219b, and DIS 172ai. EST P29 was induced in

Table 4 Summary table for Q-PCR results indicating induction/repression of ESTs identified in *Trichoderma* spp./*Theobroma cacao* interactions

Clone	*Trichoderma* isolate			
	DIS 70a	DIS 219b	DIS 219f	DIS 172ai
P1	+[a]	2+	+	2+
P3	d[b]	d	d	d
P4	+	2+	+	2+
P12	–[c]	2–	–	2–
P13	+	2+	+	+
P18	–	d	–	d
P20	d	d	d	d
P25	d	d	d	d
P26	d	+	+	+
P29	d	+	d	+
P30	d	d	d	d
P31	–	2–	d	2–
P40	d	d	d	d
P41	–	d	d	d
P44	d	d	d	–
P55	d	d	d	d
P57	d	+	+	d
P59	2+	2+	2+	2+
U4	2+	2+	+	2+
F2	∞[d]	+	nd	nd
F3	+	3+	nd	++
F4	d	+	nd	d
F5	∞	+	nd	nd
F6	nd[e]	+	nd	nd
F7	∞	4+	nd	nd
F9	+	2+	2+	d
F11	∞	6+	nd	nd
F12	∞	+	nd	nd
F13	nd	+	nd	nd
F14	∞	+	nd	–
F15	d	–	nd	d
F19	∞	2+	7+	∞
F20	nd	nd	nd	nd
F21	nd	nd	+	nd
FunTef1	–	+	+	–
FunAct	d	d	d	d

[a] +, 2+, 3+, 4+, 5+, 6+, and 7+ represent 2, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6-fold induction of gene expression

[b] d, detected but not induced

[c] – and 2– represent 2 and 10^1-fold repression of gene expression

[d] ∞, combinations were fungal gene expression was detected *in planta* but not in the control mycelia. At least a 16-fold induction is predicted for fungal transcripts detected *in planta* but not in control mycelia

[e] nd, expression was not detected *in planta*

cacao seedlings by DIS 219b and DIS 172ai. ESTs P59 and U4 were induced more than eightfold in cacao seedlings by colonization with each of the four *Trichoderma* isolates studied.

Several of the cacao ESTs induced due to colonization by these *Trichoderma* isolates share homology with genes reported to function in plant responses to environmental stresses. EST P1 shows close homology to the gene for ornithine decarboxylase (Table 3), a primary control point in polyamine biosynthesis. Ornithine decarboxylase and polyamines have been associated with developmental changes (Walden et. al. 1997), abiotic stresses such as drought (Capell et al. 2004), and resistance to biotic stresses including plant disease (Yoo et al. 2004; Walters 2000). EST P13 is related to zinc finger proteins (Table 3) commonly associated with responses to biotic and abiotic stresses including plant symbiont and plant pathogen interactions (Kim et al. 2004). EST P4 is related to a family of glutathione-S-transferase (GST)-like proteins (Table 3). Among their many activities GSTs have a broad role in protecting cells from oxidative injury by detoxifying compounds that would otherwise damage plant cells (Dean et al. 2005). The activity of GSTs contributes to resistance of plants to both biotic and abiotic stress (Dixon et al. 2002; Perl-Treves et al. 2004). Included within the GST family are the In2-related genes. Although the function of In2-related genes is unknown, they have been shown to carry GSH-dependent thiol transferase activity that could dethiolate *S*-glutathionylated proteins that accumulate during oxidative stress (Dixon et al. 2002; Dixon et al. 2005). EST P26 is nearly identical to a previously published cacao EST CA798633 (Table 3) that is related to a *Medicago sativa* gene (MSA248337) encoding a putative wound-induced protein (BLASTX, Identity = 61%, Expect = 5 E-15) with unknown function and associated with alfalfa nodule development (Jimenez-Zurdo et al. 2000) and general wound responses (Parsons and Mattoo 1991). EST P29 carries an EF-hand, calcium-binding motif (Table 3). EF-hand, calcium-binding motifs are found in a diverse super-family of proteins involved in calcium signaling leading to the targeted activation or inactivation of proteins. Proteins carrying EF-hand, calcium-binding motifs are often characterized as calmodulin-like proteins and are involved in various developmental processes and responses to stress (McCormack et al. 2005). P59 shares homology to a recently characterized family of carbohydrate oxidase encoding genes in plants that also share homology with berberine bridge-forming enzymes (Carter and Thornburg 2004; Hu et al. 2003). These carbohydrate oxidases produce hydrogen peroxide as a product and have been shown to be important in plant defense against microbes (Carter and Thornburg 2004; Hu et al. 2003). EST U4 has no known homology to established sequences.

Expression of plant ESTs P12 and P31 was repressed in cacao by colonization with the *Trichoderma* isolates studied although to a much lesser degree in seedlings colonized by DIS 70a and DIS 219f. EST P12 is nearly identical to *T. cacao* EST AM117766 where they overlap (BlastN, Identity = 97%, Expect = 5 E-80) and, EST CA796489 is closely related to a group of extensin-like proteins (Table 3). Extensins are typically associated with cell walls but the exact function of this group of extensin-like proteins is unknown (Hotze et al. 1994). P31 is closely related to the major intrinsic protein (MIP) superfamily (Table 3). Members of the MIP superfamily in plants, also called aquaporins, function as membrane channels that selectively transport water, small neutral molecules, and ions out of and between cells. A possible correlation comes from observations in tobacco (Smart et al. 2001) and *Arabidopsis* (Jang et al. 2004) that transcript levels for some aquaporins decline in response to drought and drought is known to induce polyamines biosynthesis in plants (Capell et al. 2004). The repression of MIP gene expression may reduce membrane water permeability and encourage water conservation during periods of drought (Smart et al. 2001). Enhanced drought tolerance is commonly associated with endophyte-colonized grasses (Schardl et al. 2004) and has been demonstrated with root-colonizing *T. harzianum* isolate T22 (Harman 2000). Enhanced drought tolerance would be a valuable trait in cacao since the crop is very sensitive to prolonged drought, a problem exacerbated by the cultivation of cacao in full sun (Belsky and Siebert 2003).

Fungal gene expression is altered during colonization of cacao seedlings

The pattern of detectable fungal gene expression varied with the *Trichoderma* isolate being studied (Table 4). The ESTs showing the greatest level of induction during colonization of cacao seedlings were EST F7 for DIS 70a, EST F11 for DIS 219b, EST F19 for DIS 219f, and EST F3 for DIS 172ai. It is notable that some fungal transcripts were detected *in planta* but not in liquid-culture-grown fungal mycelia, this occurring most often with DIS 70a. Several transcripts were detected in liquid-culture-grown mycelia but not *in planta*. At least 16-fold induction can be predicted for those fungal transcripts detected *in planta* and not detected in mycelia grown in clarified V8 broth. The primer sets used for amplifying fungal cDNAs for

Q-PCR were effective in most cases in amplifying the target cDNAs for DIS 219b (18) and DIS 70a (13) and less so for DIS 219f (8) and DIS 172ai (7). The majority of the fungal EST clones originated from DIS 219b-colonized cacao seedlings and, DIS 172ai was not used in the differential display from which the ESTs were derived. Some of the differences are likely due to sequence differences for the gene in question between the *Trichoderma* isolates used in combination with the specific primer sets used for Q-PCR. The primer sets used for each fungal-derived EST were developed from a single *Trichoderma* isolate (Table 3). The diversity of ESTs studied allowed identification of functional primer sets for all four *Trichoderma* isolates (species) studied.

The fungal ESTs identified can be loosely grouped into three groups: ESTs for enzymes potentially involved in nutritional support of the fungus and direct interaction with cacao tissue (F3, F7, F11, F12, and F19); ESTs for enzymes involved in basic cell functions (F2, F4, F5, F6, F12, F13, F15); and ESTs with unknown function (F21). Included in the first group are fungal genes associated with carbohydrate hydrolysis (F3, F7, F19), protein digestion (F11), and lipid metabolism (F12). These enzymes are of obvious importance in the establishment of endophytic associations, potentially functioning in acquisition of nutrients from the cacao seedling and aiding in the colonization process itself by *Trichoderma* and in the mycoparasitic interaction. Hydrolytic enzymes are important in the parasitism of other fungi by *Trichoderma* species facilitating the digestion of cell walls (Chet et al. 1998; Harman et al. 2004). Fungal ESTs F3 and F7 have homology with hydrolases with unspecified activities (Table 3). F3 is expressed by DIS 70a, DIS 219b, and DIS 172ai in liquid culture and induced during colonization of cacao by these isolates from 6 to 124-fold. EST F3 is related to genes encoding glucosyl hydrolase family 2 proteins that have diverse activities including β-galactosidase, β-mannosidase and β-glucuronidase activities. EST F7 was detected in cacao tissues colonized by DIS 70a and DIS 219b and was highly induced in both *Trichoderma* species/cacao interactions. EST F7 is most closely related to genes encoding a newly characterized xyloglucan hydrolase family that is thought to function in the digestion of cell wall carbohydrates (Gielkens et al. 1999; Grishutin et al. 2004). EST F11 encodes a serine protease (Table 3). Serine proteases have been shown to function both in mycoparasitism by *T. hamatum* (Steyaert et al. 2004) and *T. harzianum* (Geremia et al. 1993) and in the endophytic association between *Acremonium typhinum* and the grass, *Poa ampla* (Lindstrom et al. 1993). EST F12 is most closely related to genes encoding Delta3, Delta2-enoyl-CoA isomerase, an enzyme that is essential for the beta-oxidation of unsaturated fatty acids (Geisbrecht et al. 1998). EST F19 encodes an alcohol oxidase (Table 3). Alcohol oxidase is expressed by *Cladosporium fulvum* in tomato during the disease process (Segers et al. 2001). Coleman et al. (1997) observed that many genes expressed by fungi under starvation conditions, including the gene encoding alcohol oxidase, were also expressed by fungi during the plant disease process.

The *Trichoderma*/cacao interaction involves genetic cross talk

The *Trichoderma* isolates studied were able to grow on and in all cacao seedling tissues examined. During this colonization process the *Trichoderma* isolates produce enzymes that function in the growth and development of the fungus through interactions with the *T. cacao* seedling. Some of the fungal genes being preferentially expressed may also function in the biocontrol activity of the *Trichoderma* isolates. The *Trichoderma* isolate/cacao interaction was not detrimental to cacao since colonized cacao seedlings grew normally when transplanted into sterile soil. The result of this interaction on the plant side is the discoloration of roots and the induction of genes potentially involved in resistance to biotic and abiotic stresses. The pattern of altered gene expression observed in cacao and *Trichoderma* suggests a complex system of communication involving genetic cross talk. The interaction is *Trichoderma* isolate/species specific and results in the establishment of an endophytic association potentially beneficial to both participants in the plant microbe interaction. This is the first report, to our knowledge, showing complex isolate specific genetic cross talk between endophytes and host plants during colonization.

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