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Endophytic *Trichoderma* Isolates from Tropical Environments Delay Disease Onset and Induce Resistance Against *Phytophthora capsici* in Hot Pepper Using Multiple Mechanisms

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Endophytic *Trichoderma* isolates collected in tropical environments were evaluated for biocontrol activity against *Phytophthora capsici* in hot pepper (*Capsicum annuum*). Six isolates were tested for parasitic and antimicrobial activity against *P. capsici* and for endophytic and induced resistance capabilities in pepper. Isolates DIS 70a, DIS 219b, and DIS 376f were *P. capsici* parasites, while DIS 70a, DIS 259j, DIS 320c, and DIS 376f metabolites inhibited *P. capsici*. All six isolates colonized roots but were inefficient stem colonizers. DIS 259j, DIS 320c, and DIS 376f induced defense-related expressed sequence tags (EST) in 32-day-old peppers. DIS 70a, DIS 259j, and DIS 376f delayed disease development. Initial colonization of roots by DIS 259j or DIS 376f induced EST with potential to impact *Trichoderma* endophytic colonization and disease development, including multiple lipid transferase protein (LTP)-like family members. The timing and intensity of induction varied between isolates. Expression of CaLTP-N, encoding a LTP-like protein in pepper, in *N. benthamiana* leaves reduced disease development in response to *P. nicotianae* inoculation, suggesting LTP are functional components of resistance induced by *Trichoderma* species. *Trichoderma* isolates were endophytic on pepper roots in which, depending on the isolate, they delayed disease development by *P. capsici* and induced strong and divergent defense reactions.

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Genus *Trichoderma* species have been described as "opportunistic avirulent plant symbionts" due to their abilities to benefit from and provide benefit in direct interactions with plants (Harman et al. 2004). Most of this discussion centered on *Trichoderma* spp. as soil inhabitants and root colonizers. The biocontrol capabilities of several *Trichoderma* species have been extensively documented (Harman et al. 2004). *Trichoderma* isolates employ several mechanisms in providing biocontrol of disease, including antibiosis, parasitism, and induced resistance. In the context of *Trichoderma* species limiting disease, antibiosis results from the production of antimicrobial metabolites by *Trichoderma* spp. (Harman et al. 2004; Howell 1998, 2003), while parasitism is evidenced by the direct penetration and parasitism of pathogens by *Trichoderma* spp. (Chet et al. 1998; Harman et al. 2004). Induced resistance results when plant defense is activated in response to *Trichoderma* colonization (Alfano et al. 2007; Djonovic et al. 2006; Harman et al. 2004; Korolev et al. 2007; Meyer et al. 1998; Segarra et al. 2009; Shoresh and Harman 2008a; Shoresh et al. 2005). A *Trichoderma* isolate may use more than one of these mechanisms in providing biocontrol of plant diseases.

We have been studying a group of *Trichoderma* isolates collected from highly competitive tropical environments (Bailey et al. 2006, 2008). They are unique because they were isolated as endophytes not of roots but of aerial plant tissues, including tree trunks, stems, and fruit (Bailey et al. 2006, 2008). In developing this collection, many new *Trichoderma* species have been described. Examples include *T. ovalisporum* (Holmes et al. 2004), *T. martiale* (Hanada et al. 2008), *T. stromaticum* (Samuels et al. 2000), *T. theobromicola*, and *T. paucisporum* (Samuels et al. 2006b), *T. koningiopsis* (Samuels et al. 2006a), and *T. evansii* (Samuels and Ismaiel 2009). The biocontrol potentials of these newly described species are being studied, but details on their activities are lacking. Primarily, they have been studied in the tropical crop *Theobroma cacao* (Bailey et al. 2006; Hanada et al. 2008; Holmes et al. 2004; Samuels et al. 2000).

Although collected from plants in tropical climates, we see no *a priori* reason why the biocontrol potential of these *Trichoderma* isolates should be limited to tropical cropping systems. Specificity may exist between microbes and their host plants through coevolution (Burdon and Thrall 2009). The possibility that plant host specificity has contributed to the development

of new *Trichoderma* species capable of colonizing plant canopies in a tropical environment is intriguing but yet unproven.

One of the diseases being targeted for biocontrol in cacao is black pod, caused by several *Phytophthora* species including *P. capsici*, *P. citrophthora*, *P. palmivora*, and *P. megakarya* (Guest 2007). *Phytophthora* species cause disease in many different plant species (Birch et al. 2006). *P. capsici* has a broad host range but isolates can show specificity (Tian and Babadoost 2004). For example, although some isolates of *P. capsici* cause disease on cacao, others cause a devastating disease on hot pepper (*Capsicum annuum*), causing severe yield losses (Kim and Hwang 1992).

In this study, the first objective was to determine if *Trichoderma* isolates collected as endophytes of plants in tropical climates can establish endophytic relationships with hot pepper and can provide control of disease in the *P. capsici*–pepper pathosystem. The second objective was to characterize the different biocontrol activities possessed by the *Trichoderma* isolates and the consequences of their endophytic associations with pepper. The third objective was to determine whether transient expression of *Trichoderma*-induced pepper expressed sequence tags (EST) related to resistance could reduce disease development in *N. benthamiana* leaves infected by *P. nicotianae*.

RESULTS

Parasitism.

The six *Trichoderma* isolates (Table 1) were evaluated for their abilities to parasitize *P. capsici* mycelia. Five of the six isolates (all but DIS 320c) completely colonized the *P. capsici* precolonized plates within 7 days (Table 2). Only three of the isolates, DIS 70a, DIS 219b, and DIS 376f, prevented *P. capsici* from being reisolated from the *P. capsici* side of the precolonized plates (Table 2). *P. capsici* was not reisolated, while DIS 320c was reisolated. Water agar slides were coinoculated with *P. capsici* and individual *Trichoderma* isolates. Intense coiling of DIS 259j mycelia on and around the *P. capsici* mycelia was observed but without penetration (Fig. 1H). DIS 70a (Fig. 1F) and DIS 376f (Fig. 1E) directly penetrated the *P. capsici* mycelia but without significant coiling. DIS 219b (Fig. 1C) and DIS 85f (Fig. 1G) were observed to form parallel associations with *P. capsici* mycelia, but penetration was not observed. DIS 320c repressed normal growth of *P. capsici*, resulting in densely stained mycelium tips (Fig. 1D), but neither coiling nor penetration was observed.

Antibiosis and phytotoxicity.

Extracellular metabolites were harvested from *Trichoderma* culture filtrates and were evaluated for their antibiosis activities against *P. capsici* and their phytotoxic activities against pepper. In the antibiosis study, DIS 320c completely prevented growth of *P. capsici* (Table 2). DIS 259j, DIS 70a, and DIS 376f produced metabolites that inhibited growth of *P. capsici* 56.5, 30.7, and 19.2%, respectively (Table 2). Metabolites produced by DIS 320c also damaged pepper roots, causing the tips to brown (data not shown). The metabolites produced by the other isolates did not damage pepper roots.

Trichoderma endophytic growth in hot pepper.

The six *Trichoderma* isolates (Table 1), DIS 70A, DIS 85f, DIS 219b, DIS 259j, DIS 320c, and DIS 376f, were 100% effi-

Table 1. *Trichoderma* isolates used, their species, host plant, country of origin, and reference to isolate or species description

Isolate	Species	Host	Origin	Reference
DIS 70a	*T. ovalisporum*	*Banisteriopsis caapi*	Ecuador	Holmes et al. 2004
DIS 85f	*T. theobromicola*	*Theobroma cacao*	Peru	Samuels et al. 2006b
DIS 219b	*T. hamatum*	*Theobroma gileri*	Ecuador	Bae at al. 2009
DIS 259j[z]	*T. stilbohypoxyli*	*Theobroma cacao*	Ecuador	Lu and Samuels 2003
DIS 320c	*T. caribbaeum* var. *aequatoriale*	*Theobroma gileri*	Ecuador	Samuels et al. 2006a
DIS 376f	*T. theobromicola*	*Cola praecuta*	Camaroon	Samuels and Ismaiel 2009

[z] Isolate DIS 259j has only recently been described. All of the strains were provided by G. J. Samuels, who identified them by sequencing a 0.65-kb region of the translation-elongation factor 1-alpha (*tef1*) gene. The GenBank number for isolate DIS 259j *tef1* is GQ862819.

Table 2. Endophytic growth, parasitism, and antibiosis activities of six selected endophytic *Trichoderma* isolates[w]

			Viable biomass[y]					
	Endophytic growth[x]		*Phytophthora capsici* side		*Trichoderma* side		Growth inhibition[z]	
Isolate	Root	Stem	T	P	T	P	Colony diameter	Inhibition
Control	0	0	–	100	–	100	2.6 ± 0.04	0
DIS 70a	100	4.2	100	0	100	0	1.80 ± 0.04	30.7
DIS 85f	100	4.2	100	100	100	100	3.08 ± 0.04	–18.5
DIS 219b	100	0	100	0	100	0	3.65 ± 0.06	–40.4
DIS 259j	100	11.1	100	100	100	100	1.13 ± 0.05	56.5
DIS 320c	100	6.9	33	67	100	0	0	100
DIS 376f	100	1.4	100	0	100	0	2.10 ± 0.07	19.2

[w] Endophytic growth was determined by the ability of *Trichoderma* isolates to internally colonize pepper roots or stems and survive surface sterilization. Parasitism was determined by the ability of *Trichoderma* isolates to colonize *P. capsici* precolonized plates from the *Trichoderma*-inoculated side to the opposite edge where the *P. capsici* was originally inoculated and to prevent *P. capsici* regrowth from subsamples. Antibiosis was determined by the ability of *Trichoderma* metabolites from culture filtrate to inhibit growth of *P. capsici* when incorporated into agar media.

[x] Seedlings grown for 35 days in *Trichoderma*-precolonized soil were dissected and surface-sterilized, and approximately 1-cm of surface-sterilized tissue sections of roots and stems were plated on cornmeal dextrose agar. For each seedling, four lower stem sections and one root section were plated and were observed for *Trichoderma* growth (percentage of sections showing growth).

[y] At 7 days after inoculation of *P. capsici*-precolonized plates with *Trichoderma* isolates, agar plugs were removed from the *P. capsici*- and *Trichoderma*-inoculated sides. Each plug was plated and observed for growth of *Trichoderma* (T) or *P. capsici* (P). Data are presented as percentage of plugs showing growth of *P. capsici* or *Trichoderma*.

[z] A 5-mm-diameter plug of *P. capsici* R198 was added to agar plates containing heat-treated *Trichoderma* culture filtrates in minimal salts broth agar media. The mean radial growth of *P. capsici* was observed after 5 days of incubation. Data are presented as colony diameter ± standard error and percent inhibition of growth relative to growth on control plates.

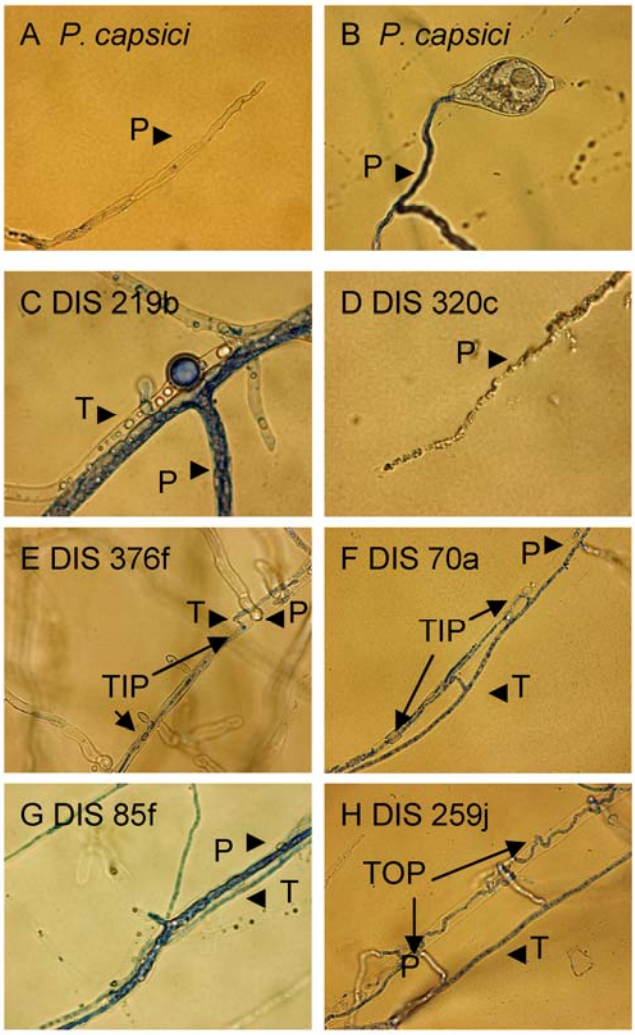

Fig. 1. Interactions between *Trichoderma* isolates and *Phytophthora capsici* on water agar slides. Glass slides covered with water agar were inoculated at opposite ends with agar plugs of *P. capsici* R198 and individual *Trichoderma* isolates. After 5 days of incubation, the slides were stained with lacto phenol cotton blue and were observed under the microscope (600× magnification) in areas at which the *Trichoderma* isolates had overgrown the *P. capsici*. **A,** *P. capsici* mycelium, **B,** *P. capsici* germinating sporangia, *P. capsici* in the presence of *Trichoderma*, **C,** DIS 219b, **D,** DIS 320c, **E,** DIS 376f, **F,** DIS 70a, **G,** DIS 85f , **H,** DIS 259j. P = *P. capsici*, T = *Trichoderma*, TIP = *Trichoderma* mycelium in *Phytophthora* mycelium, TOP = *Trichoderma* mycelium on *Phytophthora* mycelium. The size bar for the figure (H) represents 16.7 μm.

cient in colonizing pepper roots (Table 2). In contrast, the maximum endophytic colonization of pepper stems by any isolate was 11.1%.

Late-stage systemic induced resistance.

Depending on the isolate, *Trichoderma* colonization altered expression of candidate stress responsive genes and EST in 32-day-old pepper seedlings. Data for 12 genes and EST (Table 3) are presented for roots (Fig. 2) and leaves (Fig. 3). Genes *CaLTP1*, *CaCH21*, *CaPO1*, *CaPR4*, *CaSC1*, and *CaPEAS1* showed altered expression in pepper roots 32 days after *Trichoderma* colonization in an isolate-dependent manner (Fig. 2). DIS 259j, DIS 320c, and DIS 376f were the most consistent inducers of gene and EST expression in root. Inoculation of pepper roots with *Trichoderma* spp. altered the expression of genes and EST in leaves encoding pathogenesis-related (PR) proteins (*CaLTP1*, *CaPR1*, *CaCHI2*, CaPR4), enzymes involved in secondary metabolism (*CaSC1*, *CaHMG2*), hormone biosynthesis or action (*CaJA2*, *CaNPR1*, *CaGA2ox*), and gene regulation (*CaMAPK4*, *CaMK1*). The *Trichoderma* isolates altering gene and EST expression in leaves most consistently were DIS 259j, DIS 320c, and DIS 376f (Fig. 3).

Biocontrol of disease.

Pepper seedlings (42-day-old) precolonized by individual *Trichoderma* isolates were transplanted into *P. capsici*–infested soil and were observed for disease symptom development for 14 days (Fig. 4). Symptoms typically were expressed as a rapidly expanding black lesion at the stem base, emanating from below the soil line, followed by wilting within 24 h. The most consistent isolate in delaying symptom expression was DIS 376f, which delayed symptom expression by more than three days when averaged over the three independent disease assays (Table 4). At the end of the three disease assays (Fig. 4), 50, 60, and 25% of the pepper seedlings precolonized by DIS 376f remained asymptomatic while 0, 10, and 0% of control plants remained asymptomatic. DIS 259j and DIS 70a were next in efficiency at delaying symptom expression when considered over all three disease assays (Table 4).

Early induced responses to *Trichoderma* colonization in hot pepper.

In order to study the early interactions between the *Trichoderma* spp. and hot pepper, seedlings were grown on artificial media for 14 days and were then inoculated with individual *Trichoderma* isolates. Initially, a study was carried out using all six *Trichoderma* isolates and 24-day-old seedlings. The roots were harvested after 72 h of exposure to *Trichoderma*

Table 3. Pepper genes and expressed sequence tags (EST) used in evaluating the response of pepper seedlings to *Trichoderma* colonization

EST	Putative function	GenBank ID	Citation
CaAct	Actin	AY572427	
CaPR1	Pathogenesis related (PR1)	AF053343	Kim and Hwang 2000
CaLTP1	Lipid tranferase-like protein (LTP)-like (LTPI/PR2)	AF208832	Jung et al. 2003
CaPR4	Pathogenesis-related (PR4)	AF244122	Shin et al. 2001
CaPR5	Pathogenesis-related (PR5)	AY262059	
CaCHI2	Chitinase class II	AF091235	Hong et al. 2000
CaNPR1	NPR1	DQ648785	
CaJA2	JA2	GD080747.1	
CaMAPK4	MAPK4	BM064802.1	
CaSC1	Sesquiterpene cyclase	AF061285	Back et al. 1998
CaHMG2	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase	AF110383	Ha et al. 2003
CaPO1	Peroxidase (PO1)	AF442386	Do et al. 2003
CaPO2	Cell wall peroxidase	DQ489711	Choi et al. 2007
CaPEAS1	5-Epi-aristolochene	AJ005588	Zavala-Paramo et al. 2000
CaPR10	Pathogenesis-related (PR10)	AF44121	Shin et al. 2001
CaGA2ox	Gibberellic acid 2-oxidase	DQ465393	
CaMK1	Mitogen-activated protein kinase 1 (MK1)	AF247135	Shin et al. 2001

spp., and gene and EST expression was evaluated by quantitative polymerase chain reaction (qPCR). In these same samples, expression of the *Trichoderma* actin gene (*TriACT*) was determined. The data are presented in two ways: i) for fungal actin (*TriACT*) and hot pepper genes and EST, EST expression as a percent of hot pepper actin expression, and ii) for hot pepper genes and EST, the fold increase in EST expression relative to *TriACT* expression ($FI_{/\%Tact}$) as described below. All six *Trichoderma* isolates altered expression of one or more pepper genes and EST relative to plant actin expression, but the strongest inducers were DIS 219b and DIS 259j (Fig. 5, left panel). When pepper gene and EST fold induction is calculated in terms relative to *TriACT* expression, which proposes *Trichoderma* colonization, a different picture emerges. DIS 85f, DIS 259j, and DIS 376f were stronger inducers of pepper gene expression when the level of *Trichoderma* colonization was considered (Fig. 5, right panel). The expression levels for *TriACT* were 12.2, 2.7, 15.9, 1.5, 7.1, and 4.4% of pepper actin (*CaACT*) expression for DIS 70a, DIS 85f, DIS 219b, DIS 259j, DIS 320c, and DIS 376f, respectively. Based on the expression level of *TriACT* in root tissues 72 h after *Trichoderma* inoculation, DIS 219b and DIS 70a heavily colonized pepper

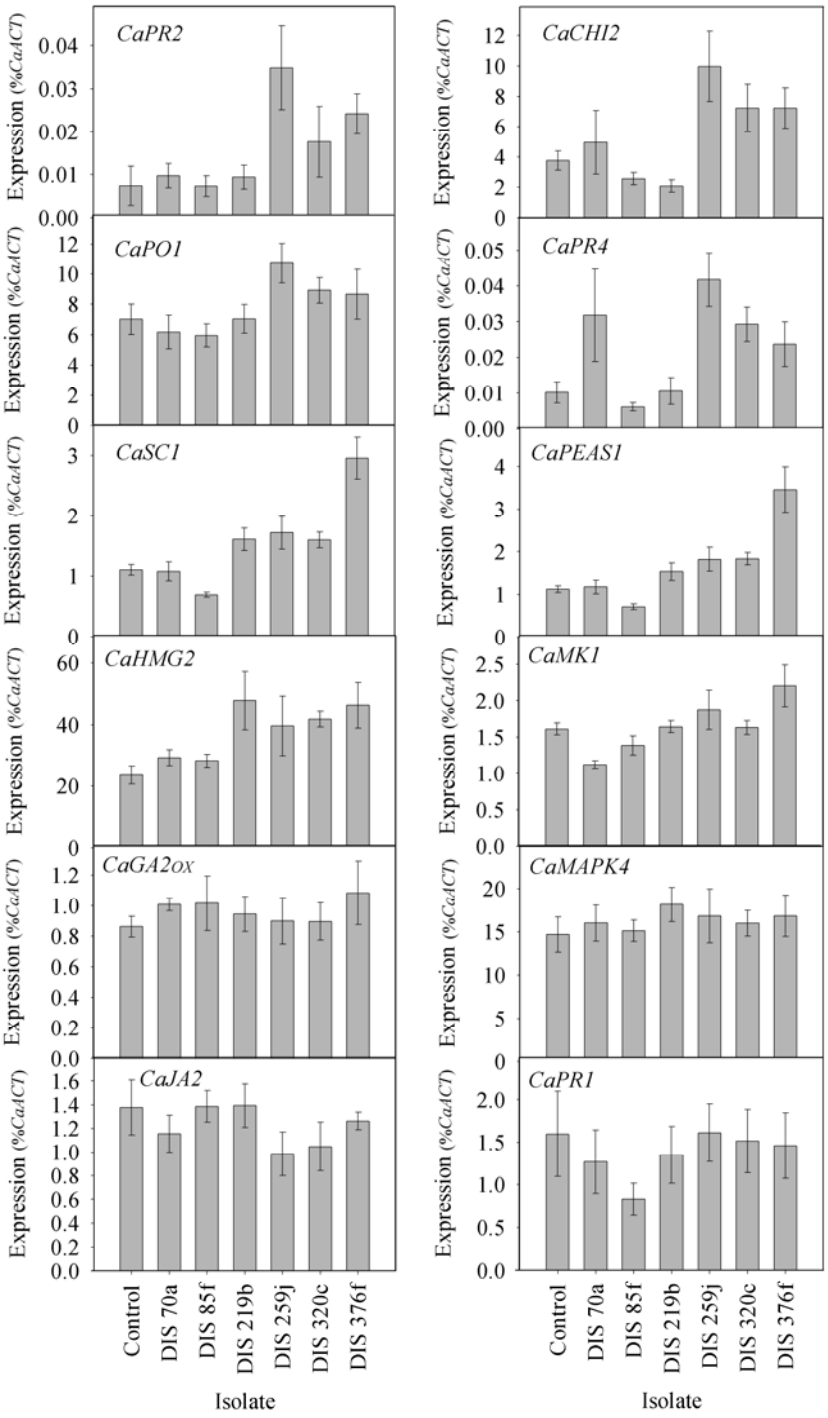

Fig. 2. Gene expression in pepper roots 32 days after inoculation with *Trichoderma* isolates. Pepper seeds were planted in Pro-Mix PGX inoculated with *Trichoderma* isolates. Seedlings were grown in a controlled environment chamber with a 12-h-light and 12-h-dark photoperiod at 25°C. After 32 days, roots were harvested for quantitative polymerase chain reaction. Genes and expressed sequence tags (Table 3) include *CaLTP1*, *CaCHI2*, *CaPO1*, *CaPR4*, *CaSC1*, *CaPEAS1*, *CaHMG2*, *CaMK1*, *CaGA2ox*, *CaMAPK4*, *CaJA2*, and *CaPR1*.

roots followed by DIS 320c and DIS 376f, followed by DIS 85f, and then DIS 259j.

Microarray analysis using a single biological replication was carried out (Supplementary Results) to identify candidate EST for further analysis of DIS 259j- and DIS 376f-induced samples by qPCR, using a 36- to 72-h timecourse fully replicated six times. The universal *TriACT* primer set (Supplementary Table S1) was used to assess the relative level of colonization of the pepper roots by DIS 259j and DIS 376f (Fig. 6). Visual observations of plate colonization indicate the two *Trichoderma* isolates completely colonized the plates the pepper seedlings were growing on, with less than a 12-h difference between the two isolates (Fig. 6A). The levels of *TriACT* transcript detected for each isolate were different from levels detected in the control samples but were not different between isolates regardless of the time sampled (Fig. 6B).

The expression levels of 12 EST highly induced in the microarray results were analyzed (Table 5, Supplementary Table S2 for statistical analysis). EST *CaAEC* (an auxin efflux carrier component) was similarly induced by DIS 259j and DIS 376f at 48 h, six EST were preferentially induced by DIS 259j, and seven EST were preferentially induced by DIS 376f (Fig. 7). All 12 EST studied to verify the microarray were induced by one or both of the *Trichoderma* isolates during the time-

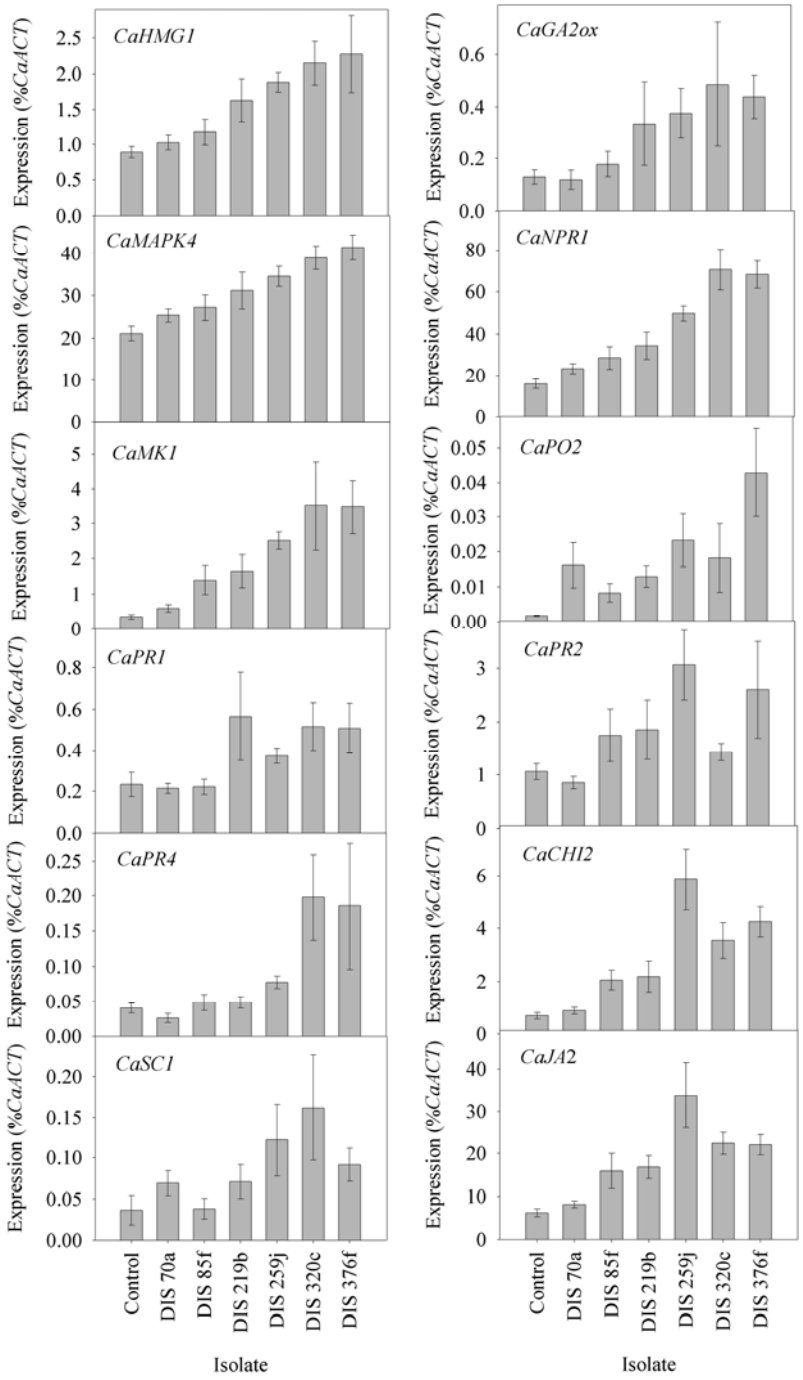

Fig. 3. Gene expression in pepper leaves 32 days after inoculation with *Trichoderma* isolates. Pepper seeds were planted in Pro-Mix PGX mix inoculated with *Trichoderma*. Seedlings were grown in a controlled environment chamber with a 12-h-light and 12-h-dark photoperiod at 25°C. After 32 days, leaves above the fifth node were harvested for quantitative polymerase chain reaction. Gene/expressed sequence tags include *CaHMG2*, *CaGA2ox*, *CaMAPK4*, *CaNPR1*, *CaMK1*, *CaPO2*, *CaLTP1*, *CaPR1*, *CaCHI2*, *CaPR4*, *CaJA2*, and *CaSC1*.

course, and all 12 of the EST followed the pattern of isolate-specific induction at 48 h indicated by the microarray analysis. DIS 259j induced *CaPOR* (porin/voltage-dependent anion channels), *CaPER* (a peroxidase), *CaTR* (a tropinone reductase), *CaZFP* (zinc finger protein), and *CaBKC* (beta-ketoacyl-CoA synthase/FIDDLEHEAD-like). DIS 376f induced *CaMLO* (seven transmembrane proteins), *CaCYP* (cytochrome P450), *CaGT* (1,6-glucosyltransferase), *CaACS* (1-aminocyclopropane-1-carboxylate [ACC] synthase), and *CaGAD* (glutamate decarboxylase).

An additional 12-member subset (Table 6) of lipid tranferase protein (LTP)-like family members was studied by qPCR analysis over the 36- to 72-h postinoculation timecourse (Fig. 8). The subset was assembled based on sequence similarity to *Arabidopsis* EST and differential expression in response to the two *Trichoderma* isolates in the microarray results. DIS 376f preferentially induced *CaLTP-I*, and *CaLTP-L*. DIS 259j preferentially induced *CaLTP-C*, *CaLTP-N*, *CaLTP-B*, *CaLTP-A*, *CaLTP-J*, and *CaLTP-E*. *CaLTP-M*, *CaLTP-F*, and *CaLTP-K* were induced by both DIS 259j and DIS 376f.

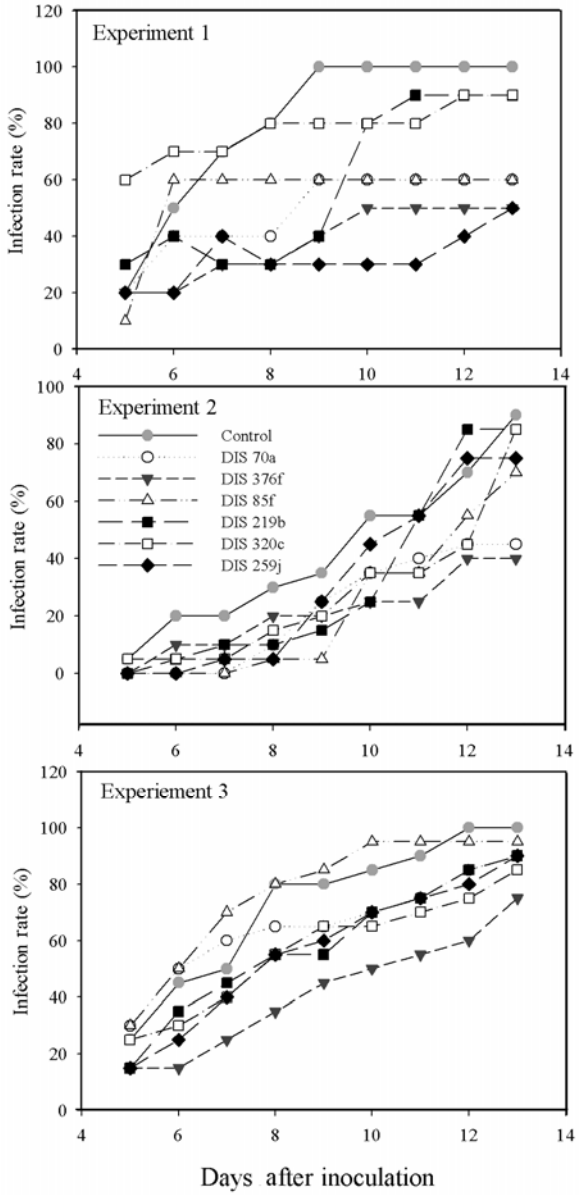

Fig. 4. Impact of *Trichoderma* spp. on disease development in pepper caused by *Phytophthora capsici*. Individual 42-day-old pepper plants (*Trichoderma*-inoculated or uninoculated controls) were transplanted into soil infested with *P. capsici*. Three experiments were carried out consisting of 10, 20, and 20 replicate pots per treatment, with one plant per pot, arranged in a completely randomized design. The percentage of diseased plants receiving each treatment was determined daily for 14 days after transplanting.

Expressing *Trichoderma*-induced pepper genes in *N. benthamiana* leaves.

Expression of CaLTP-N, CaPOR, and CaMLO 3 days after agroinfiltration of *N. benthamiana* leaves was detected at an average 3.7, 6.2, and 22.6% of *N. benthamiana* actin expression, respectively. The area under the disease progress curve (AUDPC) for leaves infiltrated with pGD-CaLTP-N was reduced by 42% in comparison with the pGD-empty vector control (Table 7). The results for the pGD-CaPOR and pGD-CaMLO were inconsistent between experiments, even though their mean AUDPC were lower than the empty vector controls.

DISCUSSION

Isolates DIS 70a, DIS 219b, and DIS 376f completely eliminated *P. capsici* growth when cocultured. DIS 70a and DIS 376f penetrated *P. capsici* without coiling. Coiling, a common recognition response of parasitic *Trichoderma* (Chet et al. 1998; Harman et al. 2004), is not a requirement for parasitism. DIS 70a is also a parasite of the basidiomycete *Moniliophthora roreri*, causal agent of frosty pod rot on *Theobroma cacao*, demonstrating a broad host range across kingdoms and plant hosts (Bailey et al. 2008). *Trichoderma* species produce many metabolites with antimicrobial activity (Howell 1998) and also produce metabolites that inhibit plant growth (Bailey and Lumsden 1998). DIS 320c produced metabolites that strongly inhibited growth of *P. capsic*i. The DIS 320c metabolites inhibiting *P. capsici* growth may be related to the metabolites in those same samples, causing damage to pepper roots. *Phytophthora* species are globally spread and cause disease on many different plant species (Birch et al. 2006), including species from which some of the *Trichoderma* isolates were collected (Bowers et al. 2001; Guest 2007). The *Trichoderma* isolates have been exposed to many pathogenic and beneficial microbes during their evolution into unique species, and antibiosis and parasitism capabilities against *Phytophthora* and other plant pathogens may have proven advantageous.

Trichoderma isolates collected from aerial plant tissues in the tropics were inefficient colonizers of hot pepper stems, although they efficiently internally colonized hot pepper roots. Five of the isolates studied can be considered endophytes, since they penetrated and survived in pepper roots without causing observable damage (Stone et al. 2000). Isolate DIS320c is a

Table 4. Increase in the average number of days without symptoms (DWS) in response to *Trichoderma* treatment[y]

Treatment	DWS	Means separation
Control	7.2	C[z]
DIS 70a	9.3	AB
DIS 85f	8.6	ABC
DIS 219b	8.7	ABC
DIS 259j	9.7	AB
DIS 320c	8.5	BC
DIS 376f	10.5	A

[y] Three experiments were carried out. *Trichoderma*-colonized seedlings and controls were transplanted into *Phytophthora capsici*–infested soil and were monitored daily for symptom development for 14 days. Symptoms were sunken necrotic lesions on the stem at the soil line, followed by wilting of the plant. DWS values represent the average over three experiments.

[z] Means not followed by the same letters are significantly different ($P \leq 0.05$).

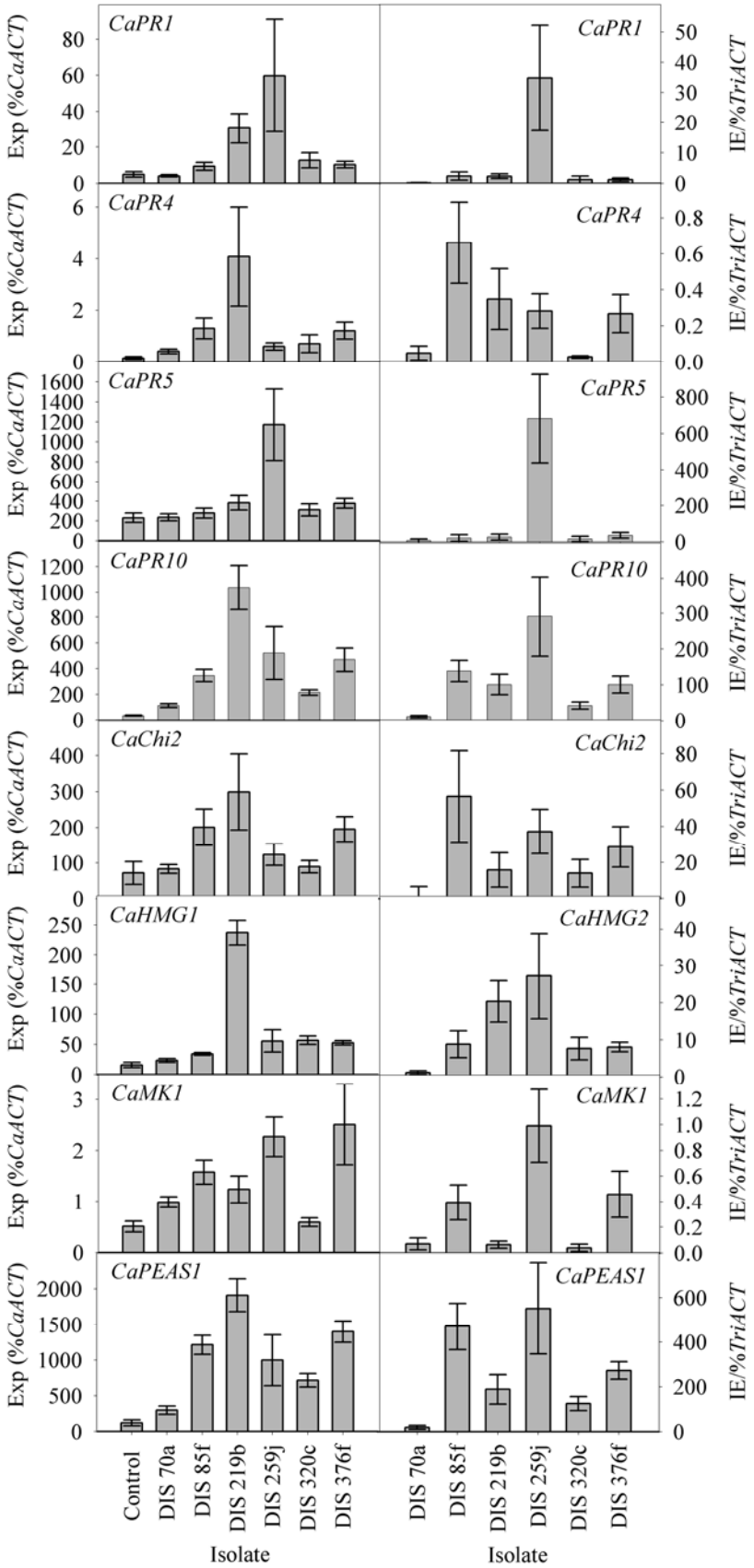

Fig. 5. The early response of pepper root gene expression after inoculation with *Trichoderma* isolates. Pepper seedlings were grown for 14 days and were inoculated with *Trichoderma* isolates on agar plates to determine the early responses in the pepper-*Trichoderma* interaction. Pepper roots were harvested 72 h after inoculation, and RNA was extracted for quantitative polymerase chain reaction analysis. The expression of eight pepper expressed sequence tags (EST) responsive to stress were analyzed, i.e., *CaPR1*, *CaPR5*, *CaMK1*, *CaPEAS1*, *CaCHI2*, *CaPR10*, *CaPR4*, and *CaHMG2*. The data are expressed as a percentage of pepper actin expression for each sample (graphs on the left) and the increase in EST expression (fold induction) relative to *Trichoderma* actin expression (graphs on the right).

possible exception, since it produced compounds that damaged roots. The endophytic nature of two of the isolates, DIS 70a and DIS 219b, has been studied in detail on cacao (Bae et al. 2009; Bailey et al. 2006, 2008). They are aggressive endophytes of cacao, being reisolated at high frequencies from the xylem of stems in addition to roots, cotyledons, and plumules (Bailey et al. 2008). Both isolates intensely colonized hot pepper roots but were poor colonizers of pepper stems, when grown under conditions similar to those used in the cacao studies (Bailey et al. 2008). The observations demonstrate that some aspects of specificity in regards to endophytic capability are due to tissue type and general host suitability rather than host identity. Roots are more easily colonized by *Trichoderma* spp. than stems, seemingly regardless of the plant species (Harman et al. 2004). In terms of host suitability, the developing woody stem of cacao (Bailey et al. 2006, 2008) is a more suitable niche for *Trichoderma* growth than the stem of pepper. *Trichoderma* spp. can actively influence their own ability to colonize plant tissues, as demonstrated by the discovery that the *T. asperellum* class 1 hydrophobin *TasHyd1* supports colonization of plant roots, possibly by protecting the hyphal tips from plant defense compounds (Viterbo and Chet 2006).

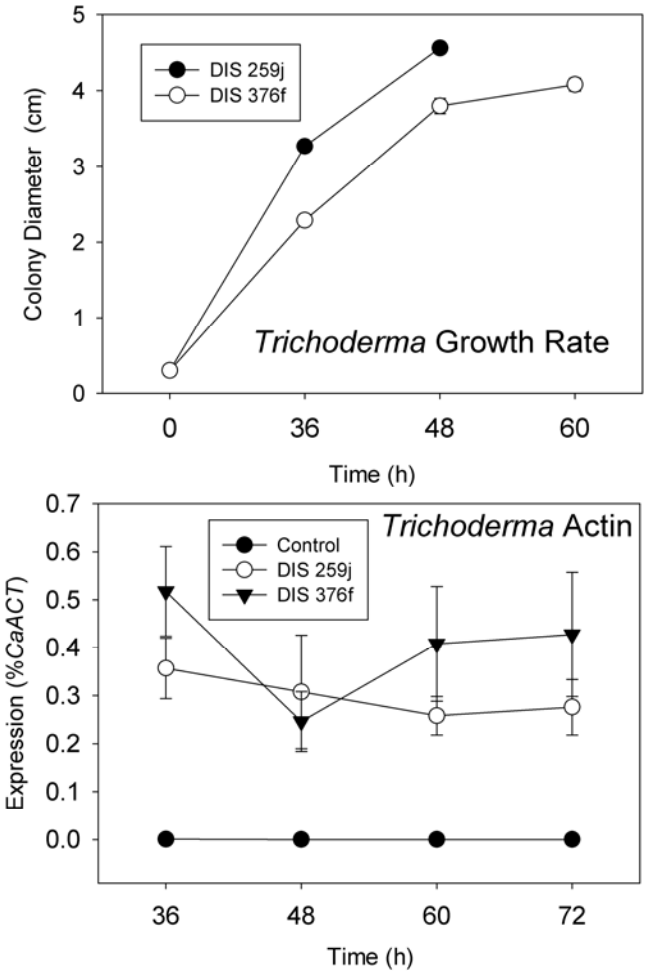

Fig. 6. Initial colonization of pepper roots by *Trichoderma* isolates DIS 259j and DIS 376f as measured by relative amounts of *Trichoderma* actin (*TriACT*). Pepper seedlings were grown 14 days and were inoculated with *Trichoderma* isolates on agar plates. Top, The percentage of the agar plates colonized by *Trichoderma* isolates was estimated. Bottom, A universal actin primer pair was used to monitor relative *Trichoderma* activity in the pepper root zone. Pepper roots were harvested 36, 48, 60, and 72 h after inoculation, and RNA was extracted for quantitative polymerase chain reaction analysis. The data are expressed as *TriACT* as a percentage of pepper actin (*CaACT*).

In 32-day-old seedlings, the *Trichoderma* isolates altered expression of gene and EST, some with known functions in pepper (Table 3) and others with strong sequence similarity to other disease resistance and stress-response genes. This includes genes and EST previously demonstrated to be induced by *P. capsici* (*CaLTP1*, *CaCHI2*, *CaHMG2*, *CaPEAS1*), viruses (*CaPR4*, *CaPR10*), and bacteria (*CaPR1*, *CaCHI2*, *CaPO2*, *CaPO1*, *CaGA2ox*). Genes involved in the hypersensitive response (*CaPR4*, *CaPO2*, *CaPO1*, *CaPR10*), responsive to UV exposure (*CaMK1*, *CaSC1*), and sesquiterpene phytoalexins biosynthesis (*CaSC1*, *CaHMG2*, and *CaPEAS1*) were induced. Genes involved in hormone metabolism and action were also induced, i.e., ethylene (*CaPR1*, *CaCHI2*), salicylic acid (*CaNPR1*), jasmonic acid (*CaNPR1*, *CaJA2*), and gibberellic acid (*CaGA2ox*). There is significant overlap in the local (roots) and systemic (leaves) responses to *Trichoderma* spp. at this late stage of colonization despite the diversity of isolates studied. DIS 259j, DIS 320c, and DIS 376f were the most consistent in altering gene expression (Figs. 2 and 3).

The potential for *Trichoderma* spp. providing protection against disease caused by *Phytophthora* spp. has been demonstrated in many cropping systems, although with isolates of relatively few *Trichoderma* species (Ahmed et al. 2000; Etebarian et al. 2000; Ezziyyani et al. 2005; Hoitink et al. 2006; Khan et al. 2004; Orlikowski 1995; Porras et al. 2007; Smith et al. 1990). Our disease assay included five *Trichoderma* species (Table 1), four recently identified as new species (*Trichoderma caribbaeum* var. *aequatoriale*, *T. theobromicola*, *T. ovalisporum*, and *T. stilbohypoxyli*) from three countries (Cameroon, Ecuador, and Peru), isolated from the canopy of four tropical plant species (*Cola praecuta*, *Theobroma cacao*, *Theobroma gileri*, and *Banisteriopsis caapi*). *T. theobromicola* isolate DIS 376f, provided a consistent delay in

Table 5. Expressed sequence tags (EST) used to verify the microarray results and evaluate the early response of pepper seedlings to *Trichoderma* colonization

EST	Putative function	GenBank ID	tBLASTX	Species	Relatedness[y]
CaUNK[z]	Unknown	NIDB			
CaMLO	MLO-like protein	BM068042.1	EU812235	*Vitis vinifera*	4E-15/71%
CaGT	1,6-Glucosyl-transferase	GD057261.1	AB443871	*Catharanthus roseus*	1E-40/46%
CaACS	1-Aminocyclopropane-1-carboxylate synthase	GD053246.1	AB434927	*Capsicum chinense*	1E-161/98%
CaAEC	Auxin efflux carrier component	CA526298.1	XM_002328968	*Populus trichocarpa*	2E-41/51%
CaGAD	Glutamate decarboxylase	GD057472.1	L16977.1	*Petunia hybrida*	1E-25/87%
CaCYP	Cytochrome P450	GD096370	EU955363	*Zea mays*	6E-74/63%
CaPER	Anionic peroxidase	BM061283.1	Y19023	*Lycopersicon esculentum*	8E-61/89%
CaPOR	Voltage-dependent anion channel	GD111311.1	AB286178	*Nicotiana tabacum*	2E-65/68%
CaTR	Tropinone reductase	GD070148.1	DURTROPR	*Datura stramonium*	1E-89/65%
CaBKC	Beta-ketoacyl-CoA synthase	GD064384.1	AJ310739	*Antirrhinum majus*	4E-28/73%
CaZFP	C3HC4-type Zinc ring finger	GD124721.1	NM_123708.2	*Arabidopsis thaliana*	3e-09/82%

[y] Relatedness values represent expected value to percent identity for each EST.
[z] *CaUNK* is an EST included in the microarray (caKS13046E01.ab1_rev) with no equivalent sequence in GenBank.

disease development (Table 4). *T. stilbohypoxyli* isolate DIS 259j and *T. ovalisporum* isolate DIS 70a also significantly delayed disease development (Table 4). DIS 70a is an endophyte of *T. cacao* and a parasite of *M. perniciosa* and *M. roreri* (Holmes et al. 2004, 2006). Isolates of *T. stilbohypoxyli* (but not isolate DIS 259j) were shown to be endophytes of cacao and produced metabolites that inhibited *M. roreri* in vitro and on cacao pods but were not parasites of *M. roreri* (Lu and Samuels 2003).

Although DIS 259j, DIS 320c, and DIS 376f were the most consistent in altering gene expression, only DIS 259j and DIS 376f consistently delayed disease development. DIS 320c failed to protect against *P. capsici*, despite the ability to systemically induce defense responses in pepper. The root damage caused by DIS 320c metabolites may have provided a point of entry for *P. capsici*, negating the benefit of defense-gene induction. DIS 70a significantly delayed disease development but induced very few defense-related genes and EST among of the gene repertoire selected here. As DIS 70a and DIS 376f are both parasites of *P. capsici*, it is possible that parasitism contributes to their abilities to delay disease.

In the two volume book series "Trichoderma & Gliocladium" (Harman and Kubicek 1998), induced resistance by *Trichoderma* was cited as a needed future work without reference to diseases caused by *Phytophthora* spp. A review by Harman and associates (2004) discusses *Trichoderma*-induced resistance to disease in detail but cited only one reference to induced resistance to *Phytophthora* species (Ahmed et al. 2000). The importance of induced resistance in some *Phytophthora*-plant pathosystems has been demonstrated (Ahmed et al. 2000; Ezziyyani et al. 2005; Hoitink et al. 2006; Khan et al. 2004) but detailed molecular studies are lacking. The recent detailed molecular studies of resistance induced by *Trichoderma* species do not address induced resistance in *Phytophthora*-plant pathosystems (Alfano et al. 2007; Korolev et al. 2007; Segarra et al. 2009; Shoresh and Harman 2008a; Shoresh et al. 2005).

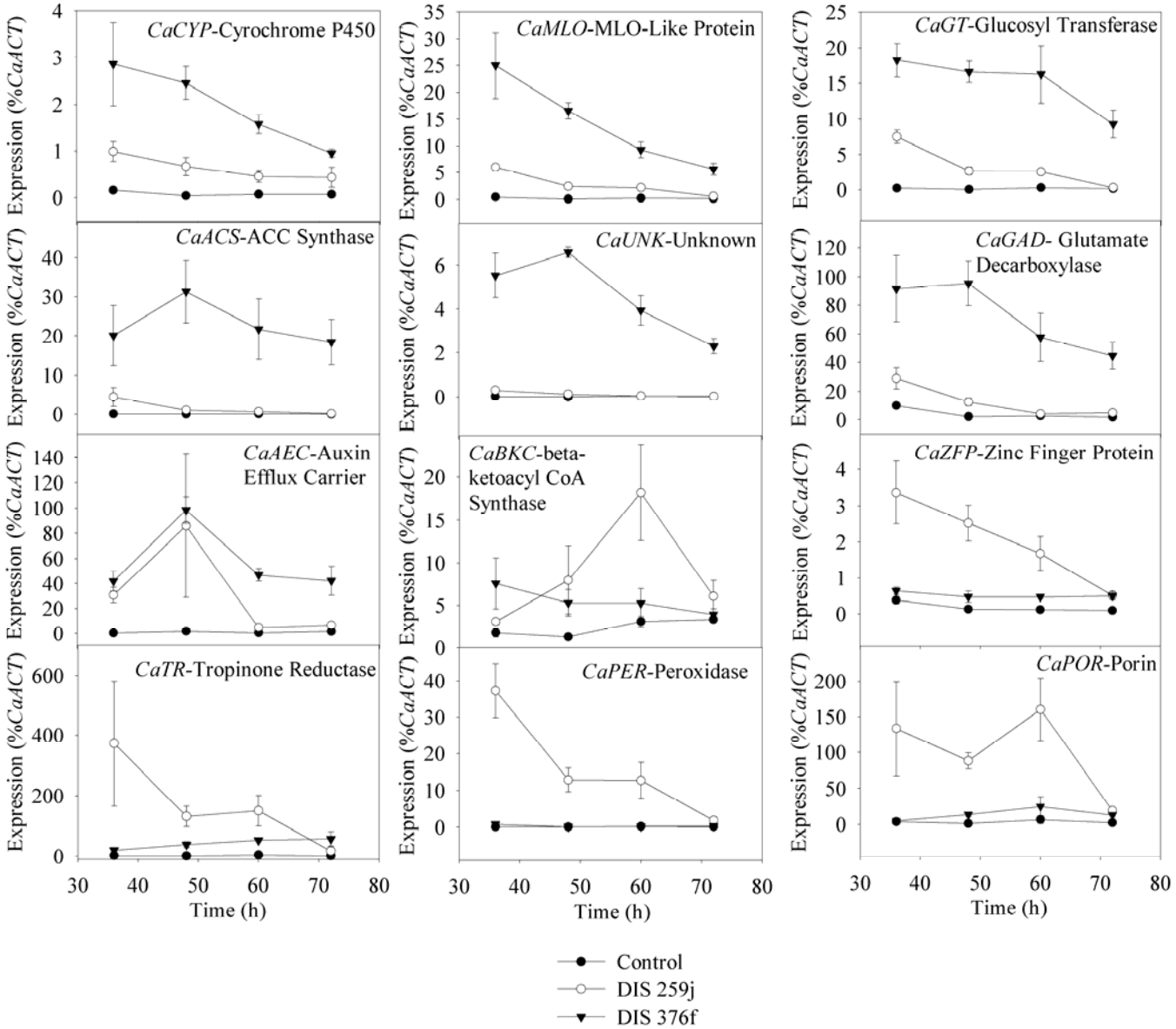

Fig. 7. Expressed sequence tags (EST) encoding stress-related proteins are differentially induced by *Trichoderma* isolates DIS 259j and DIS 376f. quantitative polymerase chain reaction (qPCR) was carried out, using primers for 12 EST that were indicated to be highly induced, by microarray analysis of RNA samples collected 48 h after *Trichoderma* inoculation. Pepper seedlings were grown 14 days and were inoculated with *Trichoderma* isolates on agar plates. Pepper roots were harvested 36, 48, 60, and 72 hours after inoculation, and RNA was extracted for qPCR analysis. EST include *CaCYP*, *CaMLO*, *CaGT*, *CaACS*, *CaUNK*, *CaGAD*, *CaAEC*, *CaBKC*, *CaZFP*, *CaTR*, *CaPER*, and *CaPOR*.

In the cases in which detailed molecular analysis of the interaction has taken place, ethylene- and jasmonic acid–dependent induced systemic resistance (ISR) has been implicated (Korolev et al. 2007; Segarra et al. 2009; Shoresh et al. 2005) as opposed to salicylic acid–associated systemic acquired resistance (SAR). It is unclear if this is true in all cases in which *Trichoderma* spp. induce systemic effects in plants. Alfano and associates (2007) found that, although many aspects of the response of tomato to *Trichoderma hamatum* isolate 382 were similar to those previously reported in other *Trichoderma*-plant interactions, expression of genes involved in the jasmonate and ethylene signaling pathways of ISR (*Lox1*, *Pal1*, *ETR1*, and *CTR1*) was not altered. *Trichoderma* spp. also alter expression of genes involved in plant cell metabolism (Alfano et al. 2007), and in the case of *T. harzianum* isolate T22, proteins involved in carbohydrate metabolism and photosynthesis are up-regulated in association with enhanced plant growth (Shoresh and Harman 2008b).

Depending on the *Trichoderma* isolate, many different peptides have been identified as elicitors of plant defense. The *Trichoderma virens* hydrophobin-like (cerato-platanin protein family) elicitor SM1 induces resistance in plants, and its action has been extensively studied in cotton and maize (Djonovic et al. 2006, 2007). SM1-related EST were expressed at high levels by all the *Trichoderma* isolates studied (data not shown), but when cloned and sequenced, the SM1-related EST lacked the glycosylation site critical to maintaining *Trichoderma* SM1-related peptides in active form (Vargas et al. 2008). This would suggest that SM1-related peptide elicitors are not involved in the resistance induced by DIS 259j or DIS 376f. Additional *Trichoderma* elicitors include the 10-kDa class II hydrophobin Hytra1 (Ruocco et al. 2004, 2007), the 22-kDa xylanase EIX (Bailey et al. 1990; Lotan and Fluhr 1990), the short-chain peptide peptaibols (Viterbo et al. 2007), and the protein Swollenin (Brotman et al. 2008).

DIS 259j and DIS 376f delayed disease development and demonstrated divergent induction patterns for many EST when examined by qPCR analysis (Figs. 7 and 8). Some of these EST have putative functions with potential importance to the *P. capsici*–pepper pathosystem and *Trichoderma*-induced plant defense. For example, *CaMLO* is related to a class of seven transmembrane proteins, the most relevant member of which, in recessive form, confers resistance to powdery mildew (Miklis et al. 2007). The gene product appears to delay cell death in response to powdery mildew, which allows infection-allowing colonization to take place. An obvious question is whether *CaMLO* impacts the ability of *Trichoderma* spp. to colonize plant tissues. *TcACS* putatively encodes an ACC synthase. ACC synthase is a primary control point in ethylene biosynthesis (Fluhr and Mattoo 1996), and ethylene induces many plant defense genes, including PR proteins, as has been demonstrated for *PR1* in pepper (Kim and Hwang 2000). *CaPOR* encodes a protein related to mitochondrial porin and voltage-dependent anion channels (VDAC). In *Nicotiana tabacum*, VDAC are being studied for their importance in programmed cell death (Tateda et al. 2009). *CaGAD* putatively encodes a glutamate decarboxylase, a primary enzyme leading to the production of γ-amino-n-butyric acid (GABA) in plants (Bouché and Fromm 2004). DIS 219b confers drought tolerance in *Theobroma cacao* through enhanced root growth (Bae et al. 2009). In that study, GABA was shown to accumulate in response to *Trichoderma* colonization (Bae et al. 2009).

DIS 376f and DIS 259j also differentially induced EST-encoding members of a lipid transfer protein (LTP)-like family of proteins (Fig. 8). LTP play a role in plant responses to biotic and abiotic stress. Plant LTP are a class of small, soluble, and mostly basic proteins found in large gene families in plants (Yeats and Rose 2008). Although the nucleotide and amino-acid sequences diverge, several features are conserved, including eight cysteine residues to form four disulphide bonds, an N-terminus signal peptide that likely directs the proteins to the endoplasmic reticulum, and a retention signal at the C-terminus allowing entry into the secretory pathway. Several functions have been proposed for LTP-like proteins in plants, including facilitating the transfer of lipids between membranes, involvement in cuticle biosynthesis, and fatty acid and acyl coenzyme A carrier proteins. They are generally secreted and associated with the plant cell wall. LTP share a number of structural similarities with oomycetous elicitins. Elicitins are thought to be a class of hydrophobins specific to the oomycetes, including *Phytophthora* spp. (Yu 1995). The elicitins and tobacco LTP1 compete for the same plasma membrane receptors (Buhot et al. 2004). Additionally, LTP1 binds jasmonic acid and, together, they compete with stronger affinity for the elicitin-binding site and are capable of inducing resistance at a distance from the point of application. The LTP sequences in pepper are diverse, with the most closely related EST to LTP1 in pepper being *CaLTP-M*, which was induced by both isolates. Jung and associates (2003) found LTP1 to be induced in the incompatible interaction between *P. capsici* and pepper but not the compatible interaction. Of the LTP studied, *CaLTP-N* is most closely related to *DIR1* (Lascombe et al. 2008; Maldonado et al. 2002). When *DIR1* is mutated, long-distance induction of SAR-derived resistance is lost. *CaLTP-N* has a distinct pattern of induction, depending on the isolate being studied. DIS 259j causes a bimodal induction, with a peak at 48 h after inoculation and a second rise in expression 72 h after inoculation. DIS 376f

Table 6. Lipid tranferase protein (LTP)-like related expressed sequence tags (EST) used to verify microarray results and evaluate the early response of pepper seedlings to *Trichoderma* colonization

EST	GenBank ID	tBLASTX	Species	Relatedness[z]
CaLTP-A	CA516668.1	FJ603280	*Tamarix hispida*	6E-28/59%
CaLTP-B	GD122697	NM_117322	*Arabidopsis thaliana*	1E-37/64%
CaLTP-C	CA519148.1	AF208833	*Capsicum annuum*	4E-32/64%
CaLTP-E	GD092083.1	AY554167	*Nicotiana tabacum*	2E-44/74%
	GD129887.1			
CaLTP-F	BM066625	Z14088.1	*Solanum lycopersicum*	2E-40/74%
CaLTP-G	CA523984	NM_124927	*Arabidopsis thaliana*	0.00002/47%
CaLTP-I	GD122016	AY496100	*Capsicum annuum*	4E-56/68%
CaLTP-J	BM063340.1	AB041516.1	*Nicotiana tabacum*	4E-43/83%
CaLTP-K	GD091697	AB061266	*Solanum tuberosum*	2E-32/72%
CaLTP-L	GD090271	NM_112712	*Arabidopsis thaliana*	6E-26/61%
CaLTP-M	GD092868.1	AF118131	*Capsicum annuum*	1E-61/84%
		AF208832	*Capsicum annuum*	5E-52/87%
CaLTP-N	GD084957	NM_124224	*Arabidopsis thaliana*	4E-25/43%

[z] Relatedness values represent expected value to percent identity for each EST.

does not induce *CaLTP-N* expression until 72 h after inoculation. When *CaLTP-N* is expressed in *N. benthamiana* leaves, it provides protection against *P. nicotianae*, reducing AUDPC by 42% over 5 days (Table 7). The reduction in disease caused by *CaLTP-N* expression in *N. benthamiana*, taken together with early and systemic induction of *CaLTP1*/*CaLTP-M* in pepper seedlings responding to *Trichoderma* isolates DIS 259j and DIS 376f, provides evidence for the function of LTP in resistance induced by *Trichoderma* spp. in pepper.

When isolates DIS 259j and DIS 376f were applied to cacao seedlings using previously published methods (Bailey et al. 2006), the molecular responses observed were similar to those observed with isolate DIS 70a (Bailey et al. 2006) and much less intense than those observed in pepper (*unpublished data*). Although induced resistance to *Phytophthora* spp. has been demonstrated in cacao (Arnold et al. 2003; Melnick et al. 2008), only muted molecular responses have been observed. Strong induction of classical plant defense genes, such as genes encoding PR-related proteins, has not yet been observed in cacao. Cacao is heavily colonized by a divergent endophytic fungal community in above- and below-ground tissues (Arnold et al. 2003; Evans et al. 2003; Rubini et al. 2005), and we suggest cacao may promote endophytic colonization by suppressing its induced-resistance responses. On the other hand, annual plant species have been shown to respond to root colonization by specific *Trichoderma* isolates with a strong defense response (Harman et al. 2004), which limits colonization of plant tissues (Metcalf and Wilson 2001; Yedidia et al. 1999, 2000). The divergent responses of cacao and pepper to colonization by the same endophytic *Trichoderma* species leads us to suggest the two species may have taken different paths in response to endophytic microbial species.

A complex picture emerges when, under the same conditions, the impact of multiple endophytic *Trichoderma* isolates on the *P. capsici*–pepper pathosystem are considered. Direct interactions between *Trichoderma* spp. and pathogens through metabo-

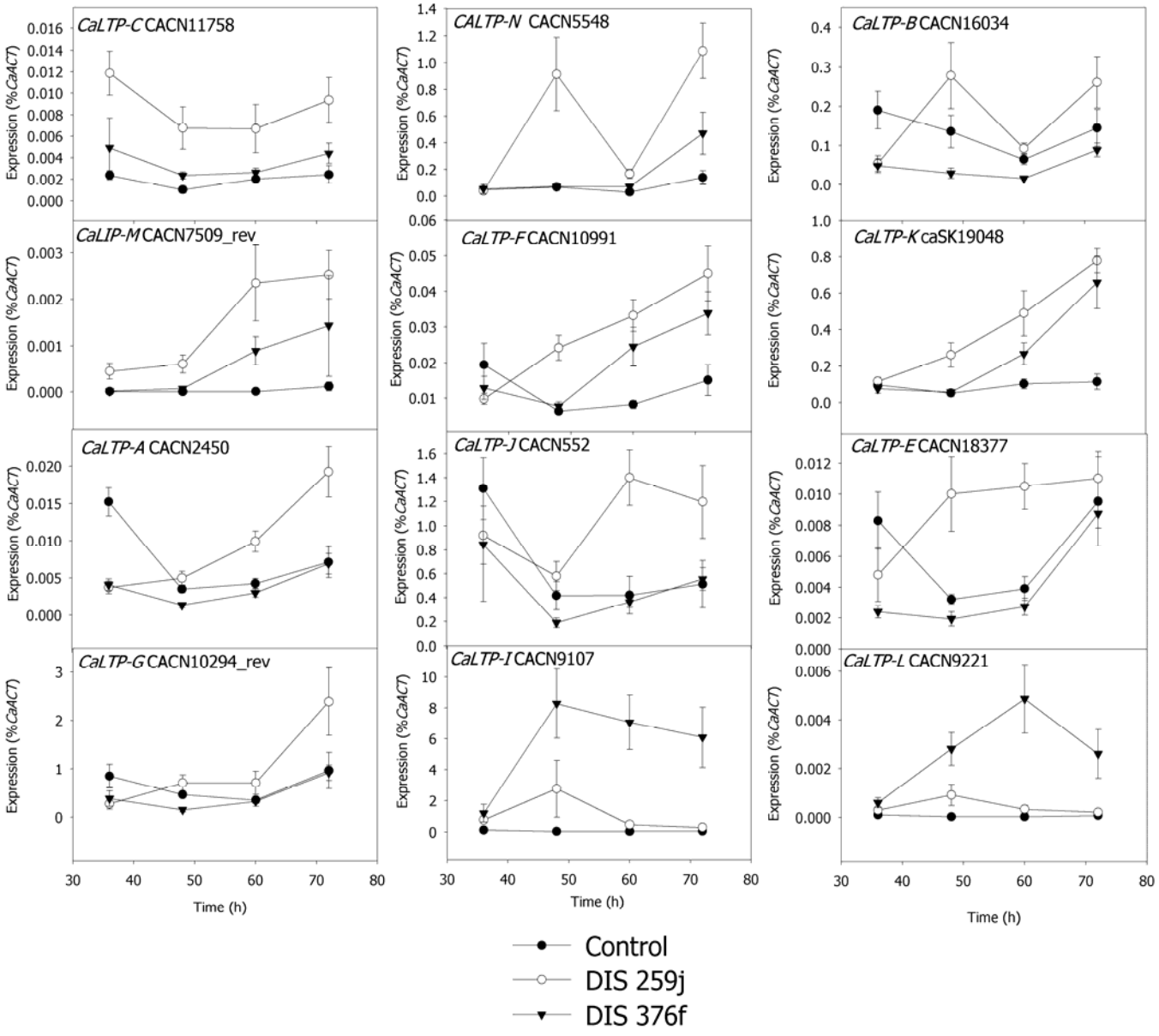

Fig. 8. Expressed sequence tags (EST) encoding members of the lipid tranferase protein (LTP)-like protein family are differentially induced by *Trichoderma* isolates DIS 259j and DIS 376f. Quantitative polymerase chain reaction (qPCR) was carried out, using primers for LTP-like protein EST that were indicated as being induced, by microarray analysis of RNA samples collected 48 h after *Trichoderma* inoculation. Pepper seedlings were grown 14 days and were inoculated with *Trichoderma* isolates on agar plates. Pepper roots were harvested 36, 48, 60, and 72 h after inoculation, and RNA was extracted for qPCR analysis.

lite production and parasitism require the active metabolism of *Trichoderma* isolates and may benefit from aggressive colonization of plant tissues by endophytic *Trichoderma* isolates. Endophytic colonization by *Trichoderma* spp. is dependent on both the tissue and the plant species being colonized.

Of the six *Trichoderma* isolates studied, DIS 70a, DIS 259j, and DIS 376f delayed disease development. Isolate 70a, an aggressive root colonizer, was a poor inducer of pepper gene expression both early and late in the *Trichoderma*-pepper interaction. DIS 259j and DIS 376f were not highly aggressive colonizers of pepper roots, but they strongly induced defense-response genes as early as 36 h after inoculation and as late as 32 days after inoculation. The altered gene expression occurred both in the roots at the site of colonization and systemically in pepper leaves. A unique isolate-dependent pattern of pepper EST induction was observed during initial root colonization, but the systemic pattern of induction tended to converge after 32 days. Transient expression of *CaLTP-N*, a LTP-like protein encoding pepper EST, in *N. benthamiana* leaves reduced disease development in response to *P. nicotianae* inoculation, providing evidence LTP function in *Trichoderma*-induced resistance to infection by *Phytophthora* spp.

MATERIALS AND METHODS

Trichoderma and *P. capsici* isolates and growth.

Six isolates of *Trichoderma* spp. were used in this study (Table 1). The *Trichoderma* isolates were maintained on potato dextrose agar (PDA) (Difco, Sparks, MD, U.S.A.) at 25°C. *P. capsici* isolates R198, RA4, 223, and R8 (Bowers et al. 2007) were maintained in clarified V8 agar (cV8) at 25°C.

P. capsici parasite screening.

Trichoderma isolates were screened for parasitic ability against *P. capsici* as previously described (Evans et al. 2003; Holmes et al. 2004). *P. capsici* R198 was inoculated on the edge of 9-cm-diameter plates of cV8, and the plates were maintained at 25°C in the dark for 12 days. A strip of *Trichoderma* inoculum (2.5 × 0.5 cm) was excised from a growing edge of a 5-day-old culture on PDA and was placed at the opposite edge of a 9-cm-diameter cV8 plate precolonized with *P. capsici*. Three replicate plates were used for each *Trichoderma* isolate. After 7 days in the dark at 25°C, samples were removed with a 5-mm-diameter cork borer, starting at the *P. capsici* inoculum side moving to the *Trichoderma* inoculum side. Each sample was plated on a minimal salts broth agar plate (MIN) (5-cm diameter) (Srinivasan et al. 1992), and the plates were observed over a period of 10 days for growth of the *Trichoderma* spp., *P. capsici*, or both.

A water-agar microscope slide assay was also performed to screen the *Trichoderma* isolates for parasitic ability against *P. capsici*. Glass slides were covered with water agar (1.5%). The slides were inoculated 3 cm apart with 5-mm-diameter plugs of *P. capsici* R198 and *Trichoderma* spp. from the growing edge of a 5- to 7-day-old cV8 plate and PDA, respectively. Three replicate slides were used for each *Trichoderma* isolate. The slides were maintained at room temperature, and the interaction between the two organisms was observed in the area of contact after 5 days. The slides were stained with lacto phenol cotton blue and were photographed with a Nikon Eclipse E600 compound scope (Nikon, Inc., Melville, NY, U.S.A.) equipped with a Nikon digital camera DXM1200 at 600× magnification.

Metabolite production and their impact on *P. capsici* and hot pepper growth.

The conidia of *Trichoderma* isolates were harvested from 2-week-old cultures grown on PDA at 25°C, as previously described (Samuels et al. 2006b). Sterile distilled water (4 ml) was added to the PDA plate, and the plate was scrubbed gently with a bent glass rod. The conidia suspension was filtered through four layers of sterile cheese cloth. Samples of MIN media (50 ml) in 125-ml flasks were inoculated with 1 ml of a 1×10^6 conidia suspension and were incubated in a shaker at 25°C and 110 rpm for 7 days. Mycelia were removed using four layers of sterile cheese cloth, and the filtrates were sterilized using a 0.22-µm membrane disposable 30-ml syringe filter system (Millipore, Billerica, MA, U.S.A) and were stored at –20°C until used. The filtrates were placed in a 90°C water bath for 2 h to inactivate enzyme activity. An equal volume of the heat-treated filtrate was added to strengthened agar, 3% MIN. Controls were prepared by replacing the filtrate with the corresponding uninoculated MIN broth. The plates were inoculated centrally with a 5-mm-diameter plug of *P. capsici* R198 and were incubated at 25°C. Three replicate plates were used for each isolate. The mean radial growth of *P. capsici* was observed after 5 days.

The effect of *Trichoderma* metabolites on pepper seedlings was also studied. Metabolites in MIN broth were mixed with an equal volume of 2× strength Murashige-Skoog (MS) basal salts medium supplemented with 2× vitamin B5, 0.4% phytagel, and 0.2 mM morpholineethanesulfonic acid (MES)-KOH (pH 5.7). Fourteen-day-old pepper seedlings grown from surface-sterilized seed on 1× MS basal salts medium supplemented with 1× vitamin B5, 0.2% phytagel, and 0.1 mM MES-KOH (pH 5.7) were transferred to the solidified agar plates containing *Trichoderma* culture filtrates. The plates were sealed with parafilm, were incubated under fluorescent lights (16-h-light period) at 23°C for 4 days, and were observed for root damage. Roots were photographed under a Nikon SMZ1500 dissecting scope (Nikon, Inc.) equipped with a Nikon digital camera (DXM1200) at 50 and 100× magnification. Cross sections of the roots were made and photographed with a Nikon Eclipse E600 compound scope (Nikon) equipped with a Nikon digital camera DXM1200 at 200× magnification.

Endophytic growth–Magenta box study.

Two agar plugs (0.5 cm in diameter) of each *Trichoderma* isolate were added to a sterilized soil-less mix (Pro-Mix PGX mix; Premier Horticulture Inc., Quakertown, PA, U.S.A.) in double Magenta boxes (77 × 77 × 194 mm; Magenta, Chicago). The Magenta boxes contained 9 cm of sterile soil-less mix and had four holes (0.5 cm diameter) sealed with tape on the bottom. Sterile water (25 ml) was added to the soil-less mix at the time of inoculation. The Magenta boxes were maintained in growth chambers as described below for 14 days before being planted with pepper seed (*Capsicum annuum* L. cv.

Table 7. Area under the disease progress curve (AUDPC) for *Phytophthora nicotiana*–challenged *Nicotiana benthamiana* leaves after infiltration with *Agrobacterium tumefaciens* EH105 carrying *Trichoderma*-induced pepper genes[y]

Plasmid construct	AUDPC	Means separation
pGD-Empty Vector	4,102	B[z]
pGD-CaLTP-N	2,397	A
pGD-CaPOR	3,252	AB
pGD-CaMLO	4,020	B

[y] Three days after infiltration, detached leaves were inoculated with zoospores (approximately 5×10^4 zoospores/ml). Necrosis measurements (% under droplet) were made for 5 days or until the empty vector control reached a rating of 400%. AUDPC was determined as described by Shanner and Finney (1977), and the data were analyzed by PROC MIXED followed by Tukey's analysis ($P \leq 0.05$).

[z] Means not followed by the same letters are significantly different ($P \leq 0.05$).

Bugang). Seeds were surface-sterilized in 30% sodium hypochlorite for 5 min, followed by five washes in sterile distilled water. Sterilized seeds were germinated on 1.5% water-agar plates under fluorescent lights for 4 days at 25°C. Three germinated seeds were planted in each Magenta box 3 cm deep into the sterile soil-less mix with or without *Trichoderma* spp. Seedlings were grown with a 12-h-light and 12-h-dark cycle at 25°C. The irradiance was 50 μmol m^{-2} s^{-1} photosynthetically active radiation. After 35 days, the pepper seedlings (two per Magenta box) were dissected, and approximately 1-cm tissue sections of roots and stems were plated on 1× corn meal dextrose agar (Becton Dickinson and Company, Sparks, MD, U.S.A.). For each seedling the lower stem was cut into four sections and one root section was plated for each seedling. Treatments consisted of six replications. The plated plant sections were incubated on the lab bench for 5 to 7 days, until the *Trichoderma* isolates grew out of the pepper-tissue sections and were assessed as positive or negative for colonization.

Induced resistance and disease suppression assay.

Preparation of Trichoderma inoculum. Hot pepper (*Capsicum annuum* L. cv. Bugang) seeds were sown into *Trichoderma*-amended Pro-Mix PGX mix. The *Trichoderma* inocula were prepared using Biodac granular carrier (20/50; Kadant Gran Tek, Inc., Green Bay, WI, U.S.A.). Biodac (50 g) was aliquoted into mycobags (Unicorm Imp. & Mfg. Corp., Commerce, TX, U.S.A.) with 50 ml of diluted V8 juice (10%). The bags were sealed and were autoclaved for 60 min twice over 2 days. Four plugs (0.5-cm diameter) of each *Trichoderma* isolate, grown on PDA agar plates for 3 to 5 days, were added to the bags. The bags were resealed and were incubated at room temperature for 3 weeks.

Preparation of P. capsici *inoculum.* Petri dishes containing cV8 juice agar (Bowers and Mitchell 1990) were inoculated with *P. capsici* isolates R1198, RA4, 223, and R899 (Bowers et al. 2007). The plates were incubated at room temperature for 2 days in the dark and an additional 3 days under light. Biodac (100 g) was mixed with 100 ml of cV8 and added to a mycobag, and the mycobag was autoclaved for 60 min on two consecutive days. For each *P. capsici* isolate, four 1-cm^2 agar plugs were added to the mycobag and the mycobag was incubated at room temperature for 2 to 4 weeks.

Inoculation of hot pepper with Trichoderma *spp. Trichoderma*-inoculated Biodac was added into Pro-Mix PGX mix (0.1%, vol/vol), and seeds were sown in square pots (5 × 5 × 6 cm). Tap water was applied until the seeds germinated, after which the seedlings were watered with Miracle-Gro (Scotts Miracle-Gro Co., Marysville, OH, U.S.A.) twice a week. Seedlings were grown in a controlled environment chamber (model M-2; EGC Corp., Chagrin Falls, OH, U.S.A.) with a 12-h-light and 12-h-dark photoperiod at 25°C. The irradiance was 100 μmol m^{-2} s^{-1} photosynthetically active radiation. Relative humidity was not controlled in this experiment, but relative humidity was always above 50%. After 32 days of growth, leaves above the fifth node and roots were harvested for qPCR.

Real-time reverse transcriptase qPCR. Induced resistance was also observed by real-time reverse transcriptase qPCR. Total RNA and cDNA synthesis, qPCR conditions, and data analysis were performed as described by Bailey and associates (2006). Relative transcript levels of each gene were normalized with respect to pepper actin transcript levels (*%CaACT*). Mean values were obtained from six biological replications.

Transplanting Trichoderma-*colonized hot pepper into* P. capsici*–inoculated soils. P. capsici* in Biodac was added to moistened soil at a rate of 0.15 g per liter of soil 2 days prior to transplanting pepper. Individual pepper plants (42-day-old *Trichoderma*-inoculated or uninoculated controls) were transplanted into empty 7.62-cm pots and the remaining volume of the pot was filled with soil infested with *P. capsici*. Three experiments were carried out consisting of 10, 20, and 20 replicate pots per treatment, with one plant per pot, arranged in a completely randomized design. Days to symptom expression was determined for each plant for the 14 days after transplanting. The number of days without symptoms was calculated for each seedling as the number of days until symptoms appeared minus one, with the minimum being zero and the maximum being 13. The data for days without symptoms was combined over the three experiments for analysis. The data were analyzed as a two-way factorial with treatment (control plus six *Trichoderma* isolates) and experiment (experiments one, two and three) as factors, using the PROC MIXED procedure of SAS 9.1 (SAS Institute Inc., Raleigh, NC, U.S.A.), and means were separated using Tukey's studentized range test.

The early responses in the hot pepper–*Trichoderma* interaction.

Seedling growth and inoculation on artificial media. Pepper seedlings were grown and inoculated with *Trichoderma* spp. on agar plates to determine the early responses in the pepper-*Trichoderma* interaction. Pepper seeds were surface-sterilized in 30% sodium hypochlorite for 10 min, followed by three washes in sterile distilled water. Sterile seeds were placed on plates containing 1× strength MS basal salts medium supplemented with 1× vitamin B5, 0.2% phytagel, and 0.1 mM MES-KOH (pH 5.7), and the plates were sealed with parafilm. The plates were incubated under fluorescent lights (16-h light period) at 23°C for 14 or 24 days, depending on the study. The plates were inoculated with PDA plugs (0.5-cm diameter) of the *Trichoderma* isolates, and the *Trichoderma* isolates were allowed to colonize the pepper seedlings. Four PDA plugs were applied per plate. PDA plugs without *Trichoderma* were used on control plates. Pepper roots were harvested 36, 48, 60, or 72 h after inoculation, depending on the study. Total RNA was extracted as described above.

Pepper gene expression in response to initial Trichoderma *colonization.* Eight EST listed in Table 3 were studied by qPCR analysis of pepper root RNA harvested from plate-grown seedlings 72 h after inoculation with all six *Trichoderma* isolates. Each treatment combination included six independent replications. Expression of *TriACT* was also determined using consensus primers generated from sequences available in GenBank. The data are presented in two ways: i) for fungal actin (*TriACT*) and hot pepper genes and EST, EST expression as a percentage of hot pepper actin expression and ii) for hot pepper genes and EST, the fold increase in EST expression relative to *TriACT* expression ($FI_{/\%Tact}$). This was accomplished by subtracting the mean expression level relative to plant actin for each EST in the controls (Exp_{ct}) from each EST expression level relative to plant actin in individual treated samples (Exp_{trt}) and then dividing the increase in expression level in individual *Trichoderma* treated samples ($Exp_{trt} - Exp_{ct}$) by the mean expression level relative to plant actin in the control samples (Exp_{ct}), yielding the fold induction for each sample relative to plant actin. The fold induction for each sample relative to plant actin was divided by the percentage of *TriACT* relative to plant actin ($\%Exp_{Tact}$) in each sample, yielding the fold increase relative to the percent *TriACT* ($FI_{/\%Tact}$):

$$(FI_{/\%Tact}) = [(Exp_{trt} - Exp_{ct})/(Exp_{ct})]/(\%Exp_{Tact}).$$

In order to identify new candidate EST for study of early gene expression in the pepper-*Trichoderma* interaction, experiments were performed with a single replication pepper microarray. EST identified in the microarray results were studied by qPCR analysis of pepper root RNA harvested from plate-

grown seedlings 36, 48, 60, and 72 h after inoculation with *Trichoderma* isolates DIS259j and DIS376f. Each treatment combination included six independent replications. A total of 24 EST (Tables 5 and 6) were evaluated over the full time-course, including 12 members of the pepper LTP-like gene family. Expression of *TriACT* was determined by qPCR analysis, as described above. Analysis of variance was conducted on the data for *TriACT*, and each pepper EST was expressed as percent plant actin, using PROC GLM followed by a Tukey test using SAS 9.2 (SAS Institute Inc.).

Cloning of CaLTP-N, CaPOR, and CaMLO and the impact of their *Agrobacterium tumefaciens*–mediated protein expression on the *N. benthamiana–P. nicotianae* interaction.

The full-length mRNAs for *CaLTP-N* and *CaPOR* were available in GenBank. In order to clone the full-length of *CaMLO*, 5′ rapid amplification of cDNA ends (RACE) was performed, using the RACE system according to manufacturer's recommendation (Invitrogen, Carlsbad, CA, U.S.A.). The RACE products were cloned into pCR2.1-TOPO vector (Invitrogen) for sequencing. DNA sequences were determined by COSMO Genetech (Seoul, Korea). A full length of *CaMLO* (1,745 bp), consisting of a 1,236-bp open reading frame encoding 411 amino acids, was obtained. The sequence of *CaMLO* was deposited in GenBank (HQ324112). The deduced amino-acid sequences of *CaMLO* showed highest identity (67%) with the MLO-like protein 6 of *Vitis vinifera* (E-value = 2e-140).

The pGD binary vector system was used for expressing *CaLTP-N*, *CaPOR*, and *CaMLO* in *N. benthamiana* leaves as described by Goodin and associates (2002). *CaLTP-N*, *CaPOR*, and *CaMLO* were amplified from pepper cDNA and were cloned into TOPO vector (Invitrogen). After sequence confirmation, the correct size fragments from *Xho*I/*Bam*HI (*CaLTP-N*, *CaPOR*) and *Xho*I/*Hin*dIII (*CaMLO*) digests were inserted into the *Xho*I/*Bam*HI and *Xho*I/*Hin*dIII sites of pGD vector, respectively. pGD-CaLTP-N, pGD-CaPOR, and pGD-CaMLO were transformed into *Agrobacterium tumefaciens* EH105 and were infiltrated into the abaxial side of *N. benthamiana* leaves, as described by Lim and associates (2009). A final EH105 concentration of 0.4 optical density at 600 nm was applied in this experiment. Three to four leaves were infiltrated on each plant, and two plants were infiltrated with each of two independent isolates of each construct.

After 3 days, zoospores were liberated from 7- to 10-day-old cultures of *P. nicotianae*, following modification from Lawrence (1978) and two to four 20-µl drops (approximately 5×10^4 zoospores/ml) were applied to the abaxial side of freshly detached previously infiltrated leaves maintained on wetted Whatman paper in petri dishes. Percent necrosis measurements were made for each drop on each leaf over the next 5 days or until the empty vector control reached a necrosis rating of 400% of the original drop area. AUDPC was determined as described by Shanner and Finney (1977). The experiment was repeated four times, and the combined data were analyzed by PROC MIXED followed by Tukey analysis using SAS 9.2 and a significance level of $P \leq 0.05$. The expression of the pepper genes in *N. benthamiana* leaves was monitored by qPCR, using the methods described above. Total RNA was isolated from *N. benthamiana* leaves 3 days after agroinfiltration, using the Qiagen RNeasy plant mini kit with the optional on-column DNase step (Qiagen Inc., Valencia, CA, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

GreenGene Bio Tech Inc. database: www.ggbio.com

Erratum

Some authors' affiliations were identified incorrectly. Corrections were made to the list of authors on April 4, 2011.