Comparative study of *Trichoderma* gene expression in interactions with tomato plants using high-density oligonucleotide microarrays

M. Belén Rubio, Sara Domínguez, Enrique Monte and Rosa Hermosa

*Trichoderma* spp. are widely used as biopesticides and biofertilizers to control diseases and to promote positive physiological responses in plants. *In vitro* and *in vivo* assays with *Trichoderma harzianum* CECT 2413 (T34), *Trichoderma virens* Gv29-8 (T87) and *Trichoderma hamatum* IMI 224801 (T7) revealed that these strains affected the growth and development of lateral roots in tomato plants in different ways. The early expression profiles of these *Trichoderma* strains were studied after 20 h of incubation in the presence of tomato plants, using a high-density oligonucleotide (HDO) microarray, and compared to the profiles in the absence of plants. Out of the total 34 138 *Trichoderma* probe sets deposited on the microarray, 1077 (3.15%) showed a significant change of at least 2-fold in expression in the presence of tomato plants. The numbers of probe sets identified in the individual *Trichoderma* strains were 593 in *T. harzianum* T34, 336 in *T. virens* T87 and 94 in *T. hamatum* T7. Carbohydrate metabolism – the chitin degradation – the chitin degradation enzymes N-acetylglucosamine-6-phosphate deacetylase, glucosamine-6-phosphate deaminase and chitinase – was the most significantly overrepresented process commonly observed in the three *Trichoderma* strains in early interactions with tomato plants. Strains T7 and T34, which had similar positive effects on plant development in biological assays, showed a significantly overrepresented hexokinase activity in interaction with tomato. In addition, genes encoding a 40S ribosomal protein and a P23 tumour protein were altered in both these strains.

**INTRODUCTION**

*Trichoderma* is a fungal genus that includes species used as biocontrol agents owing to their ability to suppress plant diseases caused by phytopathogenic fungi (Howell, 2003). *Trichoderma* antagonism has been related to mechanisms of action such as the production of antibiotics (Vinale et al., 2006) and/or hydrolytic enzymes (Benitez et al., 2004) and competition for nutrients (Elad, 2000). These fungi also have the ability to interact with plants, inducing resistance to biotic and abiotic stresses and promoting plant growth (Shoresh et al., 2010).

Different 'omics-associated strategies have been used to study many still unresolved questions in our understanding of the biological basis of the actions of *Trichoderma* (Lorito et al., 2010). More global studies have applied 'omics to the understanding of the general aspects of biocontrol by *Trichoderma* spp. (Carpenter et al., 2005; Liu & Yang, 2005; Scherm et al., 2009; Vizcaino et al., 2006, 2007). Several researchers have explored the responses of plants to the presence of a root-colonizing *Trichoderma* strain (Alfano et al., 2007; Marra et al., 2006; Segarra et al., 2007; Shoresh & Harman, 2008), but the fungal component of this association has been less studied. Macroarray analyses have been applied to study the gene expression of four species of *Trichoderma* during their interaction with cacao seedlings (Bailey et al., 2006), and of *Trichoderma harzianum* during the early colonization of tomato roots (Chacón et al., 2007), although one limitation to such studies has been the low number of genes tested. In a recent study, a *Trichoderma* high-density oligonucleotide (HDO) microarray was designed to analyse the gene expression of *T. harzianum* CECT 2413 under different growth conditions, including tomato root colonization (Samolski et al., 2009).

In the present study, an HDO microarray composed of 374 824 60-mer probes was constructed by adding 11 643 genes of the annotated genome of the biocontrol strain *Trichoderma virens* (*Hypocrea virens*) Gv29-8 (Kubiček et al., 2011) to the previously described *Trichoderma* HDO microarray that encompassed 12 662 unique sequences derived from a *Trichoderma* expressed sequence tag (EST) collection obtained from 12 strains belonging to eight...
species (Vizcaíno et al., 2006, 2007), and 9129 genes of *Trichoderma reesei* (Samolski et al., 2009), and was used to compare the transcriptional responses of *Trichoderma hamatum* IMI 224801 and *T. harzianum* CECT 2413, which both promote tomato growth, and *T. virens* Gv29-8, which showed detrimental effects in our study, during the early stages of their interactions with tomato roots.

**METHODS**

**Micro-organisms.** *Trichoderma hamatum* IMI 224801 (CABI Bioscience, Egham, UK), referred to as T7, *Trichoderma harzianum* CECT 2413 (Spanish Type Culture Collection, Burjassot, Spain), referred to as T34, and *Trichoderma virens* Gv29-8, referred to as T87, were grown and maintained on potato dextrose agar medium (PDA, DiIco-Becton Dickinson). *Trichoderma atroviride* IMI 352941, *Trichoderma asperellum* IMI 296237, *T. harzianum* IMI 298372, *Trichoderma sp.* NBT 50 (Newbiotechnic SA, Sevilla, Spain), *Trichoderma longibrachiatum* NBT 52, *T. asperellum* IMI 20268 and *T. virens* NBT 59 were also used in an *in vitro* assay to select the *Trichoderma* strains included in microarray studies.

**Tomato seeds.** Tomato seeds (*Solanum lycopersicum* ‘Marmande’) were sterilized in 2% sodium hypochlorite for 20 min and washed thoroughly in sterile distilled water.

**Effect of the treatments with *Trichoderma* spp. on the development of tomato plants.** *Trichoderma* strains were evaluated *in vitro* for their ability to promote tomato root development. Inocula of 1 × 10^6* Trichoderma spores were placed at the opposite ends of plates of Murashige & Skoog (MS) medium (Duchefa), supplemented with 1% (w/v) sucrose and 0.8% agar, pH 5.7, containing 3-day-old germinated tomato seedlings (five seedlings per plate). Plates were incubated in a growth chamber under conditions of 40% humidity, 24°C and a 16 h light/8 h dark photoperiod. MS plates containing only tomato seedlings, without *Trichoderma* spores, were used as controls. Experiments were performed in triplicate and the plates were photographed 4 days after *Trichoderma* inoculation.

An *in vivo* assay was used to evaluate the ability of *Trichoderma* strains to promote tomato plant growth. Tomato seeds sterilized as described above were coated with an aqueous suspension containing 2 × 10^6 Trichoderma spores ml⁻¹ (1 ml of spore suspension per 30 seeds) and then air-dried overnight in an open Petri dish under a laminar flow hood. Treated tomato seeds were sown in pots (two seeds per pot and 12 pots per condition) containing commercial loamy field soil, previously autoclaved at 121°C for 1 h on two successive days. Pots with untreated tomato seeds were used as controls. The pots were incubated in a greenhouse at 22±4°C, and watered as needed. Measurements of stem height and main root length were taken after 4 weeks.

**Microarray–tomato interaction in hydroponic culture.** For microarray experiments, *Trichoderma–tomato* hydroponic cultures were used. Tomato seeds were placed inside Phytatray II boxes (Sigma) on a sterile gauze sheet over a sterile stainless steel screen (30 seeds per box), holding them 1 cm above 100 ml MS medium, and kept for 2 weeks in a plant growth chamber with controlled humidity, temperature and light conditions, as described above. Spores of *Trichoderma* spp. (10^6 spores) were used to inoculate 250 ml flasks containing 100 ml minimal medium (MM, Penttilä et al., 1987), supplemented with 2% glucose. Each strain was cultured at 28°C and 200 r.p.m. in the dark for 40 h. Then, mycelia were harvested by filtration, washed with sterile water, and used to inoculate Phytatray II boxes that contained 2-week-old tomato plants. These *Trichoderma–tomato* hydroponic cultures were maintained at 25°C and 80 r.p.m. for 20 h. Control mycelia were grown in the same hydroponic conditions but in the absence of tomato plants. Finally, *Trichoderma* mycelia were harvested by filtration (the mycelium on the plant roots was recovered with a direct jet of water), washed twice with sterile distilled water, frozen in liquid nitrogen, and lyophilized.

**Microarray assays.** Mycelia collected after *Trichoderma–tomato* tomato interactions were used for RNA extraction with TRIZOL reagent (Invitrogen), following the manufacturer’s instructions, and then purified using the RNeasy MinElute Cleanup kit (Qiagen). High-quality purified RNAs, 12 µg per microarray, were submitted to Roche-NimbleGen, where cRNAs were synthesized, amplified, and labelled, and then used for subsequent hybridizations. A self-designed *Trichoderma* HDO microarray (Roche-NimbleGen) was constructed similarly to a previous *Trichoderma* HDO microarray (Samolski et al., 2009). The microarray was designed against 12662 *Trichoderma* unique sequences (9510 singlets and 3152 contigs) (Samolski et al., 2009), 9129 genes of *T. reesei* QM6a, and 11643 genes of *T. virens* Gv29-8 (T87). The 12662 *Trichoderma* unique sequences came from 14237 *Trichoderma* spp. EST-derived transcripts obtained from 12 strains of eight different *Trichoderma* spp., including 991 ESTs from *T. virens* T39 (Vizcaíno et al., 2006, 2007). The microarray contained 374834 probes, corresponding to 34138 transcripts; 11 probes for 34026 transcripts and less than 11 probes for 112 that had passed the previous filters.

Digitization of the fluorescent signals emitted after the hybridization was performed by Roche-NimbleGen using an Axon GenePix 4000B scanner with NimbleScan 2.3 software. Then the images and the raw probe intensity values obtained from 18 microarrays examined [three replicates of three *Trichoderma* strains obtained from the ‘off and on’ presence (i.e. absence or presence) of tomato plants] were analysed, and the data were deposited in the GEO database with accession number GSE29171. A robust multichip average (RMA) convolution model was applied for background correction, and the corrected probe intensities were then normalized using a quantile-based normalization procedure, as performed by Irizarry et al. (2003).

Following this, the normalized values for each probe obtained from the 18 microarrays were scaled in the 0–1 range to compensate for sequence-specific sensitivity. Finally, the processed data for the different probes within a probe set were summed to produce an expression measure. To identify probe sets showing a significant difference in expression level in at least one of the strains compared to another one in the tomato plant interaction, a multi-class significance analysis of microarray (SAM) test was carried out on the expression values using a false discovery rate (FDR) of 0.25. The analysis was performed using the Gene Spring GX program through the R software. Transcripts showing significant differential expression (fold-change ≥2 and FDR 0.25) were annotated according to gene ontology (GO) terms (Ashburner et al., 2000), and these GO terms were based on the Blast definitions, applying an E-value < 10⁻5 level.

**Quantitative PCR.** cDNAs were synthesized from 1 µg total RNA, obtained as described above, using the Transcriptor First Strand cDNA Synthesis kit (Roche) with an oligo(dT) primer. Then 1 µl of the cDNA was used in the subsequent PCR.

Quantitative real-time PCR was performed using the AB PRISM 7000 Sequence Detection System with Brilliant SYBR Green QPCR Master Mix (Roche). All PCRs were performed in triplicate in a total volume of 10 µl for 40 cycles under the following conditions: denaturation, 95°C, 30 s; annealing, 57°C, 1 min; extension, 72°C, 1 min. Threshold cycles (Cₜ) were determined using the 7000 SDS System Software, and Cₜ values were calculated using the β-tubulin gene as an endogenous control. Data are expressed using the 2⁻^ΔΔCₜ method (Livak & Schmittgen, 2001). The specific primers used are shown in
RESULTS AND DISCUSSION

Effect of Trichoderma spp. on the growth and development of tomato plants

To check the effect of different Trichoderma strains on the development of tomato roots, an in vitro assay with 10 different strains was carried out. Trichoderma inoculations affected the root system architecture in tomato seedlings; both positive and negative effects were observed, depending on the strain. The most evident effects were observed for strains T7, T34 and T87, which were selected for further studies (Fig. 1). Strains T7 and T34 produced the most beneficial effects in tomato seedlings in terms of root elongation and lateral root development. In a previous in vitro study (Chacón et al., 2007), strain T34 was also seen to promote the growth of tomato and tobacco plants, increasing leaf area and lateral root formation. In our study, strain T87 had detrimental effects on tomato roots, and the plants also had a smaller leaf area and a lower true leaf number than the controls in three independent experiments. These results are in disagreement with previous studies indicating that strain T87 (Gv29-8) promoted Arabidopsis seedling growth through production of auxin-like compounds (Contreras-Cornejo et al., 2009) and induced higher photosynthetic rates and systemic increases in the uptake of CO2 in leaves (Vargas et al., 2009). Interestingly, 18-mer peptaibols from this strain elicited systemic defences against a leaf pathogen in cucumber (Viterbo et al., 2007) and the T87-secreted elicitor Sm1 systematically stimulated defence mechanisms in maize (Djonovic et al., 2007). Since the cross-talk between Trichoderma and plants is dynamic, the negative effects that we observed in T87–tomato interactions might be related to the fungal concentration used, the plant material, the developmental stage of the plant, or the timing of the interaction. Moreover, it has been described that T87 is a ‘Q’ strain of T. virens able to produce gliotoxin, a phytotoxic metabolite, under several culture conditions (Howell, 2006); this may have negatively affected the development of tomato plants in our study.

Table 1. Effect of treatment with three different Trichoderma strains on the growth of tomato plants

Root and stem length values correspond to 4-week-old tomato plants developed from untreated (control), T. hamatum T7-treated, T. harzianum T34-treated or T. virens T87-treated seeds. Data are the means ± SEM of 24 measurements. Values in columns with the same letters are not significantly different (P<0.05) according to Fisher’s test.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Root length (cm)</th>
<th>Stem length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.19 ± 2.72a</td>
<td>8.73 ± 2.05a</td>
</tr>
<tr>
<td>T7</td>
<td>9.70 ± 2.15a,b</td>
<td>9.79 ± 1.93a</td>
</tr>
<tr>
<td>T34</td>
<td>11.43 ± 1.48a</td>
<td>9.07 ± 1.08a</td>
</tr>
<tr>
<td>T87</td>
<td>8.06 ± 2.11b</td>
<td>6.99 ± 1.84b</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of Trichoderma on tomato seedlings. Three-day-old germinated tomato seedlings grown on MS medium, supplemented with 1% (w/v) sucrose and 0.8% agar, pH 5.7, were inoculated with (A) water, (B) T. hamatum T7, (C) T. harzianum T34 or (D) T. virens T87. Photographs were taken 4 days after inoculating 1×10⁶ Trichoderma spores.
Our in vivo results also showed that strain T87 significantly reduced the stem and root lengths of tomato plants and that tomato plant growth was unaffected by strains T7 and T34 (Table 1). It has recently been reported that the genetic background of the plant affects Trichoderma–tomato interactions, and treatments with biocontrol agents exerting negative effects in some tomato genotypes were also observed (Tucci et al., 2011). Moreover, evidence is being accumulated that the significant advantages of Trichoderma treatments to plants occur when they are under stress (Mastouri et al., 2010).

Overview of the Trichoderma gene expression data from microarray analysis

Plants benefit from the symbiotic interaction with Trichoderma mainly by increases in growth potential, resistance to diseases and tolerance to abiotic stresses (reviewed by Hermosa et al., 2012, this issue) whereas Trichoderma uses the nutrients provided by the plants. It has been demonstrated that plant-derived sucrose is an important nutrient source for Trichoderma and is also involved in the control of root colonization (Vargas et al., 2009). However, little is known about the changes influenced by plants in the transcriptome of Trichoderma. To assess the effects of the presence of tomato plants on the

![Venn diagram representing the number of probe sets on Trichoderma HDO microarrays that showed significant common or different changes (up or down) in expression in T. hamatum T7, T. harzianum T34 and T. virens T87 in response to the presence of tomato plants in the culture medium in comparison to the basal medium without plants.](image)

**Fig. 2.**

**Table 2. Summary of BLAST definitions for 54 probe sets that afforded a significant difference (≥2-fold) in expression levels in at least two of the culture conditions**

The culture conditions considered were *T. hamatum* T7, *T. harzianum* T34 and *T. virens* T87 in response to the presence of tomato plants in the culture medium in comparison to the basal medium alone. An E-value of <10^{-5} and an FDR of 0.25 were applied.

<table>
<thead>
<tr>
<th>Hit description</th>
<th>Triplet</th>
<th>T7–T34 pair</th>
<th>T7–T87 pair</th>
<th>T34–T87 pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coenzyme A transferase</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose permease</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosamine-6-phosphate deaminase</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>N-Acetylglucosamine-6-phosphate deacetylase</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Without homology</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td>3</td>
<td>2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>TCTP*</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosaminidase</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitinase</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transcriptional regulator</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multidrug transporter</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precursor of endo-α-1,4-polygalactosaminidase</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Oxidoreductase</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Aminobutyrate permease</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABC transporter</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol-3-phosphate dehydrogenase</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Isocitrate lyase</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Aminobutyrate transaminase</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*TCTP, translationally controlled tumour protein.
global gene expression of three *Trichoderma* strains, selected in an *in vitro* assay as described above, we hybridized *Trichoderma* HDO microarrays with cRNAs from *T. hamatum* T7, *T. harzianum* T34 and *T. virens* T87 obtained from cultures in the presence and absence of tomato plants. Of the total 34 138 probe sets deposited on the microarray, 1077 (3.15%) showed at least a 2-fold significant change (FDR 0.25) in expression in the presence of tomato plants. The distribution of the 1077 probe sets across the three sample groups is shown in Fig. 2. Most (1023) of the selected probe sets exhibited expression change (up- or downregulated) in only one culture condition as compared with their control condition, with 593, 336 and 94 selected probe sets displaying changes only in T34–, T87– and T7–tomato interactions, respectively.

One of the most striking findings seen in the Venn diagram (Fig. 2) is that only six differentially expressed probe sets were common to the three *Trichoderma*–tomato conditions (Table 2). Genes encoding the *N*-acetylglucosamine-6-phosphate (GlnNac-6P) deacetylase and glucosamine-6-phosphate (Gln-6P) deaminases proteins, involved in carbohydrate metabolism and also related to cell wall biosynthesis, were upregulated in all three *Trichoderma* strains. A gene encoding a protein related to lipid metabolism (coenzyme A-transferase) was upregulated in T34 and T87 but was downregulated in T7. Independently of the effects provoked in tomato plants by the different *Trichoderma* strains, carbohydrate metabolism and transport processes were commonly overrepresented in the three *Trichoderma*–tomato interactions, which could indicate an active adaptation of the fungus to the rhizosphere niche. A previous study with different endophytic *Trichoderma* strains also identified an induction of ESTs for enzymes potentially involved in nutritional support of tomato plants. The distribution of the 1077 probe sets deposited on the microarray, 1077 (3.15%) showed at least a 2-fold significant change (FDR 0.25) in expression in the presence of tomato plants. Of the total 34 138 probe sets deposited on the microarray, 1077 (3.15%) showed at least a 2-fold significant change (FDR 0.25) in expression in the presence of tomato plants. The distribution of the 1077 probe sets across the three sample groups is shown in Fig. 2. Most (1023) of the selected probe sets exhibited expression change (up- or downregulated) in only one culture condition as compared with their control condition, with 593, 336 and 94 selected probe sets displaying changes only in T34–, T87– and T7–tomato interactions, respectively.

Despite the similar beneficial effects on tomato seedlings observed for strains T7 and T34, only seven probe sets were commonly altered in this strain pair (Fig. 2, Table 2), the highest number of them corresponding to genes encoding hypothetical proteins and genes that had no matches in databases. The other probe sets corresponded to genes encoding a 40S ribosomal protein, related to translation, and a protein homologous to the translationally controlled tumour protein (TCTP), also called fortillin or P23, a protein with chaperoning activity related to mitotic proliferation during the early stages of cell differentiation (Felts & Toft, 2003). TCTP/P23 overexpression in human cells leads to cell elongation resembling hyphal growth in fungi. In a previous study, the induction of the gene encoding the protein homologous to TCTP in *T. harzianum* T34 in contact with tomato roots was also observed (Samolski et al., 2009). These results suggest that P23 could be related to hyphal growth and the plant growth benefit derived from T7 and T34 root colonization. We also observed that a hexokinase activity, which phosphorylates hexoses and is mainly involved in the glycolytic pathway, leading to energy and metabolic building block availability, was commonly overrepresented in the T7 and T34 strain pair. However, the differences in plant development observed in biological assays between this strain pair and T87 may not be attributable only to such activity.

The largest number of common probe sets between strain pairs was observed between T34 and T87, transport and redox metabolism being the best-represented processes. This could be due to the closer phylogenetic distance between these two strains, although very different effects were observed in tomato seedlings when these strains were tested *in vitro* and *in vivo* assays.

To check the reliability of the microarray-based data, we used quantitative real-time PCR to analyse the expression of selected genes: one upregulated gene and one downregulated gene in the T7–tomato interaction, two upregulated genes in the T34–tomato interaction, and three upregulated genes and one downregulated gene in the T87–tomato interaction (Fig. 3). The Gln-6P deaminase gene was upregulated in all three *Trichoderma*–tomato interactions. We also observed an upregulation of the 4-aminobutyrate transaminase gene in the T34–tomato interaction, and of cutinase and cellobiohydrolase I in the T87–tomato interaction, but a downregulation of the VIR1 chitinase gene in the T7–tomato and of the hydrophobin II gene in the T87–tomato interaction in comparison to their controls.

Fig. 3. Expression analysis of selected genes by RT-PCR. *T. hamatum* T7, *T. harzianum* T34 and *T. virens* T87 were cultured in basal medium alone or basal medium in the presence of tomato plants, as described in Methods. Gene abbreviations: *g6pd*, glucosamine-6-phosphate (Gln-6P) deaminase (XP_001657190); *vir1*, endochitinase VIR1 (BAB40593); *gab*, 4-aminobutyrate transaminase (EFQ31201); *cut*, cutinase (ADQ27862); *cbhl*, cellobiohydrolase I (ACF93800); *hfbll*, hydrophobin II (ABS59374).

Our study may have certain limitations and it should be noted that several probe sets could correspond to the same
gene; that GO categories were only assigned to some 45–52% of the probe sets with significant expression change; that the expression changes were analysed under hydroponic culture conditions; and that with the present approach genes that rapidly and transiently change during \textit{Trichoderma}–tomato interaction may have been missed. Nevertheless, several strengths of our study could be mentioned. Firstly, our chip contained 374 824 probes representative of a total of 34 138 gene transcripts from 14 \textit{Trichoderma} strains. Although no \textit{T. hamatum} genes were included in the microarray, a substantial representation of the \textit{Trichoderma} transcriptome can be expected, considering that already sequenced \textit{Trichoderma} genomes have been estimated to contain 9143–12 518 predicted genes (Martinez et al., 2008; Kubicek et al., 2011). Secondly, the robustness of our experimental approach was tested by using three biological replicates for each of the six conditions considered, and a hierarchical clustering of selected probe sets (Supplementary Fig. S1), based on the different expression patterns, grouped the three biological replicates of each experimental condition together, indicating a high quality of samples and overall comparability. Thirdly, eight quantitative real-time PCR results confirmed the microarray data (Fig. 3). Furthermore, we used a corrected $P$-value of $<0.05$ to identify the significantly overrepresented biological processes within the differentially expressed (FDR 0.25) probe sets in each sample.

\textbf{T. hamatum T7 genes differentially expressed in response to tomato plants}

A total of 113 probe sets differed significantly (FDR 0.25) in expression by at least 2-fold in 20 h \textit{T. hamatum} T7–tomato plant interactions: 90 were upregulated whereas 23 were downregulated. This was the lowest number of differentially expressed probe sets among the three \textit{Trichoderma}–tomato interactions analysed. Since the \textit{T. hamatum} genome was not present on the HDO microarray, it could be expected that only a partial representation of the changes occurring in its transcriptome was being detected. However, we found that most of the differentially expressed probe sets (Table 3), 68% from upregulated and 74% from downregulated probe sets, were due to hybridization with EST-based unique sequences derived from 12 \textit{Trichoderma} strains. Since these percentages were higher than those found for \textit{T. harzianum} T34 (65% from upregulated and 50% from downregulated probe sets), represented by 3826 EST-based transcripts on this chip, we consider that this HDO microarray is an adequate tool for obtaining information about gene expression in \textit{T. hamatum} T7.

GO categories were assigned to 59 of the 113 probe sets. Next we examined whether these differentially expressed probe sets were associated with similar GO categories. This analysis revealed that six categories were significantly overrepresented in \textit{T. hamatum} T7 in the presence of tomato plants. Because several GO terms overlapped, based on similar groups of the probe sets we identified 14 different overrepresented probe sets (Supplementary Table S2) that were grouped into two physiological processes: carbohydrate metabolism and transport. As indicated above, both processes were overrepresented in the three \textit{Trichoderma} strains in interaction with tomato plants. Strain T7 showed the highest number of differentially expressed probe sets related to chitinases. We identified an upregulation of genes with homology to glycoside hydrolase family 18 class III chitinases G1, 18-13 and 18-17 (phylogenetic group B), whereas a downregulation of a gene encoding the glycoside hydrolase family 18 class V chitinase VIR1 (phylogenetic group A) (Seidl et al., 2005) was only observed in this strain. Considering that group A chitinases have been associated with mycelial autolysis (Brunner et al., 2003), downregulation of proteins of this kind in the early \textit{Trichoderma}–plant interaction could be expected. Similarly, group B chitinases with a typical cellulose-binding domain, such as 18-13 and 18-17, have been related to \textit{Trichoderma}–plant recognition and attachment (Seidl et al., 2005), and their upregulation could be

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
\textbf{Condition} & \textbf{Total} & \textbf{Probe sets} \\
\hline
\textit{T. hamatum} T7–tomato & 90 (79.6%) & \\
& 61 (67.8%) spp & \\
& 7 (7.8%) \textit{T. reesei} & \\
& 22 (24.4%) \textit{T. virens} & \\
\hline
\textit{T. harzianum} T34–tomato & 519 (81.0%) & \\
& 336 (64.7%) spp & \\
& 19 (3.7%) \textit{T. reesei} & \\
& 164 (31.6%) \textit{T. virens} & \\
\hline
\textit{T. virens} T87–tomato & 205 (53.5%) & \\
& 57 (27.8%) spp & \\
& 15 (7.3%) \textit{T. reesei} & \\
& 133 (64.9%) \textit{T. virens} & \\
\hline
\end{tabular}
\caption{Summary of numbers and percentages of probe sets on \textit{Trichoderma} HDO microarrays showing significant changes in expression in strains T7, T34 and T87 in response to the presence of tomato plants}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|}
\hline
\textbf{Condition} & \textbf{Total} & \textbf{Probe sets} \\
\hline
\textit{T. hamatum} T7–tomato & 23 (20.4%) & \\
& 17 (74.0%) spp & \\
& 3 (13.0%) \textit{T. reesei} & \\
& 3 (13.0%) \textit{T. virens} & \\
\hline
\textit{T. harzianum} T34–tomato & 122 (19.0%) & \\
& 61 (50.0%) spp & \\
& 29 (23.8%) \textit{T. reesei} & \\
& 32 (26.2%) \textit{T. virens} & \\
\hline
\textit{T. virens} T87–tomato & 178 (46.5%) & \\
& 152 (85.4%) spp & \\
& 8 (4.5%) \textit{T. reesei} & \\
& 18 (10.1%) \textit{T. virens} & \\
\hline
\end{tabular}
\caption{Summary of numbers and percentages of probe sets on \textit{Trichoderma} HDO microarrays showing significant changes in expression in strains T7, T34 and T87 in response to the presence of tomato plants}
\end{table}
related to the beneficial effect of strain T7 on plants. A peptide with a cellulose-binding domain has been identified in T. asperelloides as a microbe-associated molecular pattern able to induce defence responses in cucumber (Brotman et al., 2008).

Chitinases are also involved in the metabolism of chitin, a polysaccharide not present in plant roots. However, only a few chitinase genes are induced by chitin in Trichoderma, suggesting that non-chitinous cell wall components can also act as inducers (Gruber et al., 2011). This is in agreement with the upregulation of genes encoding GlnNac-6P deacetylase, Gln-6P deaminase and chitinase activities in hydroponic Trichoderma–plant interaction observed in the present study and may indicate that chitooligomers, able to trigger plant defence responses (Woo et al., 2006), could be generated by the chitin-hydrolytic system of Trichoderma without the need for chitin induction.

Regarding transport processes, it could be thought that an upregulation would be needed for nutrient exchange between the fungus and the plant. However, we observed a downregulation of major facilitator superfamily (MFS) transporters, which could indicate that after hyphal root attachment and colonization, Trichoderma nutrient uptake, at least at an early stage, is limited.

**T. harzianum T34 genes differentially expressed in response to tomato plants**

We identified 641 probe sets that differed significantly (FDR 0.25) in expression by at least 2-fold in 20 h T. harzianum T34–tomato plant interaction (Fig. 2); 65% of the 519 upregulated and 50% of the 122 downregulated probe sets were from EST-based unique sequences (Table 3). GO categories were assigned to 325 of the 641 probe sets. Subsequent analysis revealed that 235 GO categories were significantly overrepresented in T34 in the presence of tomato plants vs T34 alone. We identified 186 different overrepresented probe sets (Supplementary Table S3) that were grouped into several physiological processes, the transport and carbohydrate metabolism processes being those most evidently affected.

The relevance of transport processes in the T34–tomato interaction was highlighted by the upregulation of 67 probe sets related to proteins involved in such processes, e.g. MFS domain proteins (24), amino acid transporters (18), mitochondrial carrier proteins (7) and the maltose ABC transporter (1). An upregulation of the genes involved in sugar and amino acid transport, using macroarray analysis (Chacón et al., 2007), and of the genes related to mitochondrial carrier proteins, using an HDO microarray analysis (Samolski et al., 2009), was also observed in the T34–tomato interaction. Moreover, this strain showed upregulation of the highest number of genes encoding enzymes related to carbohydrate metabolism and involved in nutritional support of the fungus (β-galactosidase, cellubiohydrolase, endo-1,4-β-glucanase, xylanase, etc.) and in basic cell functions (glycerol-3P-dehydrogenase, alcohol dehydrogenase, phosphoenolpyruvate synthetase, etc.). The data indicate activation of glycolysis/glucogenesis, the tricarboxylic acid cycle, the pentose phosphate pathway, and other processes related to the generation of building-block compounds and energy and also involved in cell wall synthesis; these responses support the notion that nutrient exchange between the fungus and the plant is increased during the T34–tomato interaction. Since cuticle is not present in roots and since cutinase probe sets were upregulated in T34, a global regulation of the Trichoderma hydrolytic system could occur in order to improve the acquisition of carbon for saprophytic growth. Moreover, it has also been reported that the ethylene-inducing xylanase EIX1 elicits hypersensitive responses and other plant defence responses independently of its xylanolytic activity (Rotblat et al., 2002).

An upregulation of genes related to amino acid, nucleotide, energy and vitamin metabolism, detoxification and regulation, as well as of several genes involved in signalling, transcription, translation and post-translational processes was observed in T34–tomato microarrays, as shown in previous studies addressing this interaction (Chacón et al., 2007; Samolski et al., 2009). Although the upregulated genes were different from those observed in previous studies, our data confirm that modifications in the above physiological processes occur during the early stages of interaction between T34 and tomato in order to support the fungus’s higher demand for metabolic energy.

Although at least one gene encoding a β-ketoacyl synthetase, an enzyme that catalyses the condensation of malonyl-acyl carrier protein with the growing fatty acid chain, was downregulated, other genes involved in the biosynthesis of lipids and fatty acids (acytrol-CoA acetyltransferase, lipoic acid synthetase and 3-ketoacyl-acyl carrier protein reductase) were upregulated. These data are consistent with the upregulation of genes involved in lipid biosynthesis and catabolism observed in the T34–tomato interaction (Chacón et al., 2007). However, genes encoding laccases, which are enzymes with polyphenol oxidase activity related to melanin-like pigment synthesis in conidiospores, the induction of fruiting bodies and pathogenic interactions with plants (Hölker et al., 2002; Kiiskinen et al., 2004), were downregulated in T34.

An additional feature of the T34–tomato interaction observed in this work was the upregulation of genes encoding two enzymes involved in γ-aminobutyric acid (GABA) metabolism (succinate-semialdehyde dehydrogenase and 4-aminobutyrate transaminase). The GABA carbon is converted into succinic semialdehyde and then to succinate in reactions catalysed by these mitochondrial enzymes. An induction of both enzymes has been observed previously in Cladosporium fulvum–tomato and Pseudomonas–Arabidopsis interactions (Solomon & Oliver, 2002; Park et al., 2010), and in the symbiosis of Rhizobium leguminosarum with the pea host plant (Prell et al., 2002). The authors suggested that...
during the interactions the micro-organisms could alter the physiology of the plant, resulting in an enhanced production of GABA, which in turn plays a signalling role in the induction of the fungal enzymes responsible for its degradation. Thus, *T. harzianum* T34 could use GABA of plant origin as a source of nutrients.

Although one gene related to hydrophobin proteins was upregulated, another three hydrophobin genes were downregulated, as discussed below for the T87–tomato interaction.

**T. virens** T87 genes differentially expressed in response to tomato plants

We identified a total of 383 probe sets that showed at least 2-fold significant (FDR 0.25) changes in expression in the 20 h *T. virens* T87–tomato plant interaction. Whereas 65% of the 205 upregulated probe sets were from the *T. virens* genome, the majority (85%) of the 178 downregulated probe sets were from EST-based unique sequences (Table 3). It could be expected that the majority of the downregulated probe sets from the *T. virens* T87 genome but a high level of cross-hybridization with heterologous probe sets was detected. We know that 991 ESTs from *T. virens* T39 were also used in the microarray design and this could explain some of the high percentage of downregulated probe sets coming from the 12,662 *Trichoderma* unique sequences in strain T87. GO categories were assigned to 173 of these 383 differentially expressed probe sets. Further analysis revealed that 58 GO categories were significantly overrepresented in *T. virens* T87 in the presence of tomato plants vs *T. virens* T87 alone. We identified 67 different probe sets (Supplementary Table S4) and then, based on similar groups of probe sets, we reduced the 58 overrepresented GO categories to six physiological processes. The highest proportions of the overrepresented probe sets corresponded to genes encoding proteins involved in carbohydrate metabolism and cell communication processes.

The relevance of carbohydrate metabolism was reflected by the upregulation of genes corresponding to enzymes such as GlnNAC-6P deacetylase, Gln-6P deaminase and chitinase, also observed in strains T7 and T34 during the colonization of tomato roots as discussed above, as well as β-glucosidase, xylanase, glyceral-3P-dehydrogenase and cutinase enzymes, also observed in T34 during the colonization of tomato roots. A major characteristic of the T87–tomato interaction was the large number of downregulated hydrophobin probe sets (24). Hydrophobins are small proteins that are found on the outer surfaces of both hyphal and conidial cell walls, where they mediate interactions between the fungus and the environment (Seidl-Seiboth *et al.*, 2011), and some *Trichoderma* hydrophobin genes have been implicated in hyphal development and conidiation (Askolin *et al.*, 2005; Mendoza-Mendoza *et al.*, 2007; Muñoz *et al.*, 1997) and root colonization (Viterbo & Chet, 2006). In previous studies the upregulation of the Hfb2 gene of *T. reesei* in media containing complex plant polysaccharides (Nakari-Setälä *et al.*, 1997), the activation of a hydrophobin of *T. atroviride* in interactions with bean roots (Marra *et al.*, 2006) and the upregulation of a hydrophobin homologous gene in *T. harzianum* T34 interacting with tomato roots (Samolski *et al.*, 2009) have been reported. We might thus have expected to see an enhanced expression of one or more fungal hydrophobin genes during the *Trichoderma*–tomato interaction in our experiments. However, the hydrophobin genes appeared to be downregulated due to the presence of the plants. A differential regulation of 10 different class II hydrophobin genes in *T. atroviride* has been also documented (Mikus *et al.*, 2009). Whether the downregulation of hydrophobin genes observed by us might be due to a direct influence of the plant or beneficial for the root colonization by the fungus remains to be further investigated. The redundance of hydrophobin genes raises the question of different physiological functions. In this context, the downregulation of hydrophobin genes observed in T87 in this study could be involved in the negative effects caused by this strain in tomato seedlings in terms of root elongation and lateral root development.

Genes involved in lipid metabolism and in transport of phosphate, amino acids and general substrates were also upregulated. Our data are in agreement with the facilitation of nutrient exchange between the fungus and the plant and the reorganization of the membranes and cell wall of the fungus (Chacón *et al.*, 2007).

However, we also observed downregulation of a gene encoding glutamine synthetase, an enzyme of importance in ammonia assimilation and the regulation of nitrogen metabolism, and one related to redox processes (N-lactate dehydrogenase, which modulates pyruvate flow when glucose is required) – both involved in fungal growth – and genes with homology to myosin I-binding proteins with important roles in the movements of organelles (Lee *et al.*, 2000). The three probe sets assigned to the myosin I-binding GO category showed homology with genes encoding an L-amino acid oxidase, an oxidoreductase that catalyses the oxidative deamination of L-amino acids. Taking the T87 data together, it is difficult to understand the role played by the downregulation of genes involved in amino acid metabolism, although the turn-off of glutamine synthetase and L-amino acid oxidase could indicate a sufficient availability of nitrogen. In bacteria, lactate dehydrogenases can be regulated by the fructose 1,6-bisphosphate/phosphate ratio (van Niel *et al.*, 2004). The downregulation of lactate dehydrogenase in T87 could be due to the accumulation of fructose 6-phosphate obtained via the activation of Gln-6P deaminase observed in the T87–tomato interaction.

**Conclusions**

During early *Trichoderma*–tomato interactions, effects on tomato seedling development varied depending on the *Trichoderma* strain. The transcriptomic responses of *Trichoderma* to the presence of tomato plants in the medium were also strain-dependent. Although few differ-
entially regulated genes were common to *T. hamatum* T7, *T. harzianum* T34 and *T. virens* T87 in the presence of tomato plants, the overall transcriptomic changes detected in the three strains pointed to a significant activation of fungal carbohydrate metabolism and transport processes in the mutualistic *Trichoderma*–tomato interaction. The gene encoding TCTP/P23 protein was upregulated in the plant-growth-promoting T7 and T34 strains, relative to T87; this could be related to active hyphal growth in the contact with tomato roots. By contrast, the high number of down-regulated hydrophobin probe sets observed in T87 could indicate that, under the experimental conditions of our study, this strain had a limited root attachment, affecting its capacity to promote plant growth.

ACKNOWLEDGEMENTS

Research project funding was from the Regional Government of Castilla y León (GR67) and the Spanish Ministry of Science and Innovation (AGL2008-0512/AGR and AGL2009-13431-C02-01). S. D. had a fellowship from Castilla y León Education Council and the European Social Fund.

REFERENCES


Bailey, B. A., Bae, H., Strem, M. D., Roberts, D. P., Thomas, S. E., Crozier, J., Samuels, G. J., Choi, I. Y. & Holmes, K. A. (2006). Fungal and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings and plant gene expression during the colonization of cacao seedlings.


M. R. Rubio and others


Edited by: A. H. Herrera-Estrella