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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Transcriptome analysis shows differential gene expression in the saprotrophic to parasitic transition of *Pochonia chlamydosporia*

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Abstract Expression profiles were identified in the fungus *Pochonia chlamydosporia*, a biological control agent of plant parasitic nematodes, through a cDNA-amplified fragment length polymorphism approach. Two isolates with different host ranges, IMI 380407 and IMI 331547, were assayed in conditions of saprotrophic-to-parasitic transition, through in vitro assays. Gene expression profiles from three different nutritional conditions and four sampling times were generated, with eggs of host nematodes *Globodera pallida* and *Meloidogyne incognita*. Expression of transcripts changed in RNA fingerprints obtained under different nutritional stresses (starvation in presence/absence of eggs, or rich growth media). Transcript derived fragments (TDFs) obtained from the expression profiles corresponded to 6,800 products. A subset was sequenced and their expression profile confirmed through RT PCR. A total of 57 TDFs were selected for further analysis, based on similarities to translated or annotated sequences. Genes expressed during egg parasitism for both IMI 380407 and IMI 331547 were involved in metabolic functions, cellular signal regulation, cellular transport, regulation of gene expression, DNA repair, and other unknown functions. Multivariate analysis of TDF expression showed three groups for IMI 380407 and one for IMI 331547, each characterized by expression of genes related to eggs parasitism. Common amplification profiles among TDF clusters from both isolates also reflected a pool of constitutive genes, not affected by the nutritional conditions and nematode associations, related to general metabolic functions. The differential expression of parasitism related genes suggest a network of induced/repressed products, playing a role in fungal signaling and infection, with partial overlaps in host infection and parasitism traits.

Keywords Transcriptomics · Nematode eggs · Parasitism · Differential gene expression · Saprotrophic · Pochonia chlamydosporia

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Introduction

Recent technical, economic, and conceptual innovations in agricultural practices, including organic cropping and sustainable soil management, consider the use of biological control agents as a possible alternative to the widespread application of chemicals and pesticides (Kerry 2000). For several reasons related to safety and environment protection, this approach appears as promising as it is difficult to apply. In fact, in spite of the large number of biological control agents identified and investigated in the last few decades and of the efforts aiming at developing suitable protocols for open field applications, applied biological control still appears in its infancy. Furthermore, as concerns

parasites of agronomically important plant pathogens or nematode pests, basic background knowledge about the biology, behavior, and biochemistry of useful antagonists occurring in soil is often lacking.

Given their richness in microbial species, soil habitats sustain a web of complex trophic networks. However, the effects of this complexity are often neglected, since a very large number of variables are active in soil systems. As a consequence, experimental studies have been constrained to simplified assays with two to three microbial and host species, tested in semicontrolled or artificial conditions, to gain insights into their relationships (Polesani et al. 2008). However, the deciphering of active biochemical signals may yield new insights to understand the conditions leading to pest suppression by a given organism, and may enable the exploitation of a promising biocontrol strategy, that has already been proven efficacious in controlled assays.

Pochonia chlamydosporia Gams & Zare 2001 is a widespread soil fungus as well as a true endophyte, colonizing epidermal and cortex root cells (Kerry 2000; Lopez-Llorca et al. 2010; Maciá-Vicente et al. 2009). The fungus is also hyperparasitic or antagonistic on other fungi, or parasitic on economically important plant or animal–parasitic nematodes, with consequent applications as a biopesticide (Kerry 2000). Furthermore, distinct variants of this fungus were found as associated with different host nematode species, suggesting a long-term, adaptive evolutionary process (Morton et al. 2003).

Studies of parasitic fungi revealed that the host surface chemical composition has an important role for production of specialized hydrolytic enzymes involved in the infection process (Tunlid and Jansson 1991). In the *P. chlamydosporia*–nematode interaction, the fungus molecular response involves the alkaline serine protease VCP1, an enzyme that specifically degrades the proteins from the outer egg shell layers, whose gene is expressed at the beginning of the host infection process (Morton et al. 2003; Segers et al. 1996). However, VCP1 release represents a single step in a more complex cascade of biochemical induction and gene activation processes largely unknown, and data on the global molecular response of this fungus to different soil factors or nutritional conditions are lacking.

Knowledge about the biochemical events leading to parasitism and the mechanisms involved in their regulation could suggest new strategies to improve the reliability of *P. chlamydosporia* and other parasitic fungi as biocontrol agents. It is already known that parasitic fungi behave differently in response to local nutrient availability (Mo et al. 2005). However, even distantly related parasitic species may exploit common genetic pathways during the host infection process. Given the importance of *P. chlamydosporia* as a potential biopesticide and the broad range of niches that it exploits, we implemented an analytical approach to study metabolic pathways in different nutritional conditions. Our aim was to gain insight on the biochemical signals affecting the behavior of this fungus through transcriptomic assays, identifying the genes activated or repressed in varying trophic conditions. The corresponding metabolic pathways, as well as their expression levels were investigated and analyzed, in view of their possible role in nematode biocontrol. The assays included the presence of nematode eggs and starvation, a condition affecting the fungus behavior, frequently observed in other invertebrate host–parasite studies (Freimoser et al. 2005; Wang et al. 2005).

Materials and methods

Nematodes

Two plant parasitic nematode species were used, the root knot nematode (RKN) *Meloidogyne incognita* and the potato cyst nematode (PCN) *Globodera pallida*. A RKN population was maintained on susceptible tomato plants (cv Tiny Tim), in greenhouse at 24–26°C. Roots infected by mature females were gently washed to eliminate soil and plant debris. The roots were then dissected to harvest mature females embedded in the galls and their egg masses. The eggs, stored in a gelatinous matrix attached to the protruding posterior end of the female on the root surface, were washed with NaOCl, vortexed for 30 s for egg release and washed several times with tap water. The egg suspension was then collected on a 50 μm sieve. The PCN population was provided by the Rothamsted Research (RRes) culture collection. The cysts were maintained in tap water for 1 h (in a watch glass), and then crushed to release the eggs and the suspension was screened through a sequence of sieves to eliminate cyst debris.

The eggs of both species were surface sterilized by shaking for 5 min with 0.1% $HgCl_2$ solution, then washed with sterile distilled water (SDW) and collected in an autoclaved 0.45 μm pore filter. Aliquots were spread on 1.5% water agar plates, and incubated at 25°C and 37°C and daily checked for microbial contamination, during 1 week.

Fungal isolates and test conditions

The two fungal isolates of *P. chlamydosporia* var. *chlamydosporia*, IMI 331547 and IMI 380407, used in the study were obtained from the freeze-dried culture collection maintained at RRes. The two isolates were originally isolated from different populations of nematode eggs, RKN and PCN, respectively, and showed higher VCP1 efficiency for their original host species (Morton et

al. 2003). The fungi were grown on potato dextrose agar (Oxoid Ltd, Basingstoke, UK) and incubated at 28°C for 7 days. Conidia were harvested from the plates by pouring 5 ml of SDW onto each plate followed by gentle scraping of the fungal colonies. For subculture, 650 conidia ml^{-1} were inoculated in Czapek Dox broth (CD: 30 g sucrose, 3 g $NaNO_3$, 0.5 g $MgSO_4$, 0.5 g KCl, 1.0 g K_2HPO_4, 0.01 g $FeSO_4$) l^{-1} and incubated at 28°C with constant shaking at 150 rpm for 5 days in the dark.

The resulting mycelium was harvested on autoclaved 50 μm sieves and washed three times with SDW, then 2 g mycelium aliquots were used to inoculate 30 ml CD or nutrient-poor liquid minimal medium (MM: 1 mg sucrose, 14 mg $NaNO_3$, 0.25 g $MgSO_4$, 0.25 g KCl, 0.5 g K_2HPO_4, 0.06 g $FeSO_4$) l^{-1}, in 100 ml capacity conical flasks. Treatments for each isolate were: (I) nutrient-rich medium CD, predicted to repress parasitism; (II) minimal medium MM, predicted to de-repress genes associated with parasitism; and (III) MM with addition of nematode eggs from either PCN (to isolate IMI 380407) or RKN (to isolate IMI 331547), conditions to induce parasitism. The eggs were added in 2 ml SDW to (III) 24 h after inoculation to give 1 egg μl^{-1}; 2 ml SDW without eggs was added to (I) and (II) at the same time. Three replicate samples were taken from all three treatments at the point when eggs were added (24 h after the initial inoculation); this is referred to as 0 (control), and subsequent samples were taken 2, 4, 8, 24, and 48 h later. Samples were immediately frozen in liquid nitrogen and stored at −80°C until processed.

RNA isolation

Total RNA collected from replicates at the different sampling times was extracted from frozen samples (60–100 mg) with the RNeasy® Mini Kit (Qiagen®, Crawley, UK) following the manufacturer's protocol for plant tissues, and using and the RNAse-free DNAse in-column digestion option. For tissue disruption, the frozen samples were transferred into the Fast RNA tubes containing acid washed glass beads and were shaken in a Bio 101 FastPrep® homogenizer (Anachem Ltd, Luton, UK) twice for 30 s at 5 m/s speed, in the presence of the disruption buffer. The lysate was transferred to a Qiashredder® column (Qiagen®) and the homogenate from this was transferred to an RNeasy® column. Total RNA integrity was checked on an agarose gel.

cDNA–AFLP analysis

The cDNA-amplified fragment length polymorphism (AFLP) approach was chosen as an investigative tool since no microarray or whole genomic data have yet been made available for *P. chlamydosporia* or other closely related species. Analysis was performed as described by Bachem et al. (1996), and modified according to Polesani et al. (2008). The mRNA was reverse transcribed from total RNA using Superscript® II reverse transcription kit (Invitrogen™ Ltd, Paisley, UK) and a biotinylated oligo-dT primer. To supply sufficient cDNA template for the AFLP assay this, cDNA was preamplified using the Illustra™ GenomiPhi™ V2 DNA Amplification Kit (GE Healthcare kit, Little Chalfont, UK) as per the manufacturer's instructions. The fidelity of this preamplification was established by testing with a polymerase chain reaction (PCR) primer pair spanning introns to the VCP1 gene (Morton et al. 2003), to detect any carry-through of genomic DNA. The cDNA was digested with *Bst*YI (rare cutter), and the 3′ ends captured on streptavidin magnetic beads (Dynal A.S., Oslo, Norway). The enzyme used for the second digestion was *Mse*I (frequent cutter). Sequences of adapters and primers used for AFLP are as follows: *Bst*YI adapter top strand, forward: 5′-CTC GTA GAC TGC GTA GT-3′; *Bst*YI adapter bottom strand, reverse: 5′-GAT CAC TAC GCA GTC TAC-3′; *Mse*I adapter top strand, forward: 5′-GAC GAT GAG TCC TGA G-3′; *Mse*I adapter bottom strand, reverse: 5′-TAC TCA GGA CTC AT-3′. Preamplification was performed with an *Mse*I primer Mse0: 5′-GAT GAG TCC TGA GTA A-3′, combined with a *Bst*YI primer carrying either a T or a C at the 3′ end, BstT0: 5′-gac tgc gta gtg atc T-3′; BstC0: 5′-gac tgc gta gtg atc C-3′.

Preamplification and amplification were achieved according to Polesani et al. (2008). After preamplification, the mixture was diluted 600-fold and a 5 μl aliquot was used for selective amplification with several primer combinations, carried out with one selective nucleotide added on the ^{33}P-labeled *Bst*YI primer and two selective nucleotides on the *Mse*I primer (See Supplementary materials, Table S1). Touchdown PCR conditions for selective amplifications were as follows: 5 min denaturation at 94°C, followed by 30 s denaturation at 94°C, 30 s annealing at 64°C, 60 s extension at 72°C (13 cycles, scale down of 0.7°C per cycle); 30 s denaturation at 94°C, 30 s annealing at 54°C, 60 s extension at 72°C (27 cycles), and 5 min at 72°C. Selective amplification products were separated on a 5% polyacrylamide gel (33×42 cm) run at 60 W (1,400 V) at 45–50°C until the bromophenol blue reached the bottom. Gels were dried onto 3MM Whatman paper (Whatman®, Maidstone, UK) on a BioRad 583 gel dryer on vacuum 2 h at 80°C and positionally marked with radioactive ink before exposing to Kodak film (Sigma-Aldrich®, Gillingham, UK) for 2–7 days. Gene expression in the two fungal isolates was analyzed by comparing the electrophoretic profiles of transcript derived fragments (TDFs) produced for each combination of primers and treatments. The gels obtained for each isolate were inspected visually to identify each

TDF band by its position relative to all other bands, producing a virtual ladder. The similarity between isolates expression patterns was evaluated using the presence/absence and intensity of the bands in common, for each corresponding primer combination. The cDNA–AFLP protocol applied permits the visualization of one single cDNA fragment for each messenger originally present in the sample, thus reducing the redundancy of sequences obtained. To increase the procedure specificity, the same primers pairs of the preamplification step, with two additional 3′ terminal nucleotides (Table S1), were used in the second selective amplification (Bachem et al. 1996).

Isolation of fragments and sequencing

Differentially expressed bands were identified by superimposing the film on the gel and then marking either end of the corresponding gel band using a syringe needle. When present in all treatments, only the amplification product from treatments in rich or minimal media were isolated. The bands were cut out from the gel and eluted in 100 µl TE (10 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0) overnight at 4°C. An aliquot of the eluted band was used for reamplification by PCR using a standard method, with the same primers pairs as in the initial selective amplification. PCR fragments were purified with Qiagen® Mini Elute PCR purification or Qiaquick® (Qiagen®) gel extraction kits according to the manufacturer's instructions, cloned in a A-U* vector (StrataClone™ PCR Cloning Kit, Stratagene®) and the inserts sequenced using the Applied Biosystems™ (Warrington, UK) BigDye® Terminator v1.1 Cycle Sequencing Kit with 500 ng of DNA. Sequence traces were analyzed using the program Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and their identity confirmed through BLAST analyses (Altschul et al. 1997) at NCBI (http://www.ncbi.nlm.nih.gov/), searching protein databases using a translated nucleotide query (BLASTx), nucleotide databases using a nucleotide query (BLASTn), or translated nucleotide databases using a translated nucleotide query (tBLASTx).

For sequences arising from treatments with nematodes, the Parasite Genomes Database Query of the European Molecular Biology Laboratory–European Bioinformatics Institute (EMBL-EBI, http://www.ebi.ac.uk) was also interrogated to exclude the possibility of transcripts originated from nematode embryos.

Real-time analysis

VCP1 expression profiles were initially used to validate and determine the most appropriate sampling times for differential gene expression using the primers R2 5′-ATGCAACTGTCTGTTCTTCTC-3′ and VCPrev1 5′ AGGTGGTGCTTCCCTTTTG3 specific for VCP1 (Morton et al. 2003). Gene-specific primers were designed based on the sequences of the selected TDFs (Table S2). Transcript levels were measured using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich®). The quantitative real-time (RT) PCR reactions (20 µl) consisted of approximately 10 ng cDNA, TDF-specific primers (500 nM) and fluorescent dye SYBR Green I. The thermal profile was performed in a Mx3000P™ (Stratagene®), one cycle at 94°C for 4 min and 40 cycles at 94°C for 30 s, at specific annealing temperatures for 30 s and 72°C for 20 s.

Melting curve analysis was performed after the PCR reaction to confirm that the signal was the result of a single-product amplification. Cycle threshold (Ct) values were determined for three biological assays, with two replicates each. Amplification efficiencies were calculated using the Miner program (Zhao and Fernald 2005), around 84% (76–97%) for target and 81% for normalized products. Data were analyzed using MxPro QPCR Software (Stratagene). The corresponding Ct values were applied to estimate the ratio of starting amounts DNA according to the comparative quantitation method, DDCt (Livak and Schmittgen 2001; Pfaffl et al. 2002).

TDF validation by real-time PCR

To validate the results of the expression patterns observed by cDNA–AFLP, RT-PCR assays were performed for some selected transcripts that showed differential gene expression for each isolate. The validation of the selected TDFs was carried out through a further biological assay with the same protocols and cDNA–AFLP conditions described above. TDFs that showed constant band intensity levels during treatments were used as endogenous controls (normalizers). For validation of the selected TDFs through RT-PCR, specific time points were chosen based on the gel profiles to show most clearly any induction or repression due to treatments.

Statistical analyses

To analyze gene expression profiles, a matrix was constructed for each *P. chlamydosporia* isolate and nematode combination using presence/absence on the gels of the TDF bands, that were scored as 0 (band absent or faint), 1 (present), or 2 (bold). Each treatment and hour lane was considered as a variable, and each transcript as an observation. The data were analyzed using a multivariate statistical procedure, in order to identify groups of TDFs coexpressed or putatively related to common metabolic pathways, reflecting differences due to the

nutritional conditions applied. A principal component analysis (PCA) was performed from the correlation matrix of each dataset, followed by average linkage hierarchical cluster analysis (HCA; Dopazo et al. 2001). Clusters were separated on the trees using as a criterion the mean cluster similarity distance plus one standard deviation unit (Sonderegger et al. 1986), starting from a 0.5 distance threshold. All analyses were performed using SAS® ver. 8.02 software.

Results

Time course

Several time-course RNA samplings from the isolate/egg-challenge assays were checked to select the best exposure times required to analyze their transcriptome. The comparative transcript profiling of the synchronized cultures revealed differential patterns of gene expression, most relevant at 0–8 h (Fig. 1). The pattern of genes expressed at time 0, (the point at which eggs or SDW were added to cultures, which had recovered for 24 h in CD or MM after harvesting and washing of the mycelium), served as a control for each of the treatments. Temporal and nutritional responses were apparent and subsequently the VCP1 transcript (data not shown) proved effective as a marker to identify the time needed to elicit induction of other genes that responded to the different challenges.

c-DNA and gel analysis

A total of 12 primer combinations were used for the AFLP analyses of the cDNA from each combination of *P. chlamydosporia* isolate and nematode species, at the four different times selected. The cDNA–AFLP patterns were screened for newly expressed fragments, after challenging with eggs. On average, each primer combination generated 50 clear and unambiguous bands per lane, which yielded a total 6,800 TDFs for each isolate and nematode interaction, with a 65–500 bp size range (Fig. 1). Only one primer combination (n. 4) resulted in no bands. From the other combinations a total of 73 TDFs (38 for IMI 380407 and 35 for IMI 331547) were selected and isolated from the cDNA–AFLP gels, in relation to the differential gene expression profiles observed, for further sequencing and analyses. Of these TDFs, 57 were subsequently selected (28 from IMI 380407 and 29 from IMI 331547) for annotation (Tables 1–2).

Visual inspection of gels for band comparison between isolates showed that, of the 28 TDFs identified for IMI 380407, 23 were present in IMI 331547 gels and five could not be found in the corresponding gel expression profiles. Conversely, 18 TDFs of IMI 331547 were present in IMI

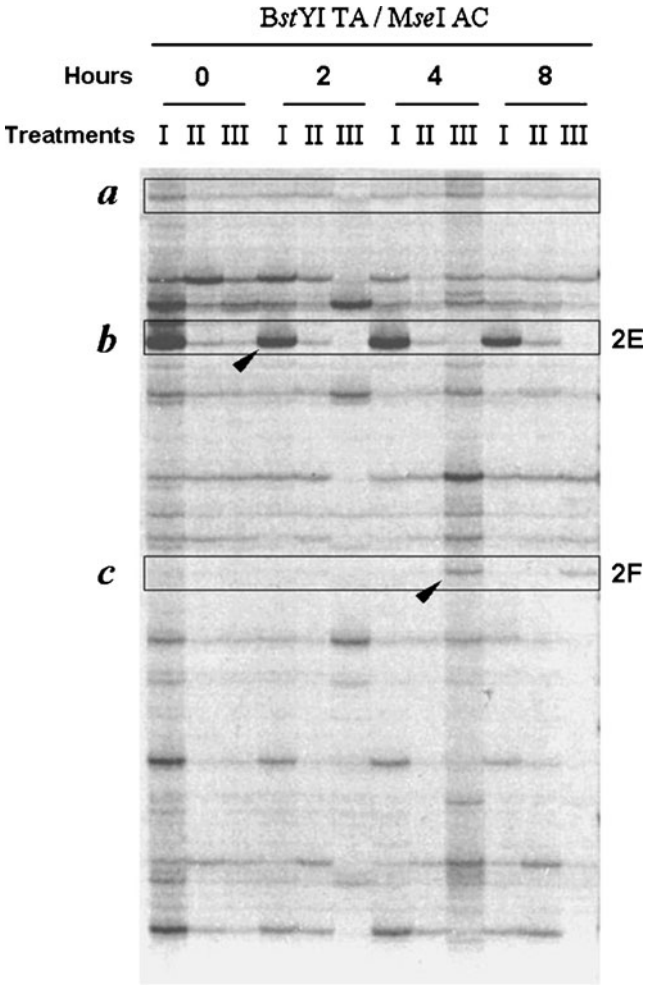

Fig. 1 A selected area representative for the whole gel produced for *P. chlamydosporia* isolate IMI 380407. Primers pair B*st*Y1TA/M*se*I AC used for this gel correspond to primers combination n. 2 (see Supplementary materials for all primers combinations). Treatments I, rich medium; II minimal medium (MM); III, MM with PCN nematode eggs at different sampling times. Bands in *frames* show examples of genes constitutively expressed (**a**), repressed in treatment III (**b**), and induced in treatment III at 4 and 8 h after eggs exposure (**c**). Sequenced TDFs are identified by their primers combination and letters assigned to their sequential position on the gel (i.e., *arrowheads* show bands of transcripts 2E and 2F)

380407 gels (Table 2). A comparison for similar amplification patterns for both *P. chlamydosporia* isolates revealed that five bands of IMI 331547 had the same expression of IMI 380407 in the rich (treatment I) and minimal medium (treatment II), and three bands in the MM with egg parasitism (treatment III). The IMI 380407 bands with corresponding TDFs in IMI 331547 gels were 11 for treatment I, nine for treatment II, and eight for treatment III (Table 2).

The majority of sequences did not show any significant similarity with nucleotide data proceeding from the genera *Meloidogyne* or *Globodera*. In a few cases (results not shown), when the nematode protein

Table 1 Homology of *P. chlamydosporia* transcripts with sequences in Genbank, for IMI 380407 vs. *Globodera pallida* and IMI 331547 vs. *Meloidogyne incognita*

TDF	Length (bp)	Sequence homology organism[a]	Annotation	BLAST scores	Acc. number[b]
Globodera pallida					
			Metabolism/signal regulation		
2C1	292	*Pyrenophora tritici-repentis* Pt-1 C-BFP	Mg dependent phosphatase 1	2e-20	XM_001931899
7A1	353	*Gibberella zeae* PH-1	Chaperonin GroEL (HSP60 family)	3e-43	XP_380734
1F1	219	*Neurospora crassa* OR74A	Glycosyltransferase family A (GT-A)	9e-25	XP_960725
7Q1	116	*P. chlamydosporia* ATCC 16683	Reducing polyketide synthase (radicicol gene cluster)	2e-35	EU520419
11A1	409	*Schizosaccharomyces japonicus* yFS275	GTPase activating protein Rga6	0.55	XM_002171293
1B1	451	*Aspergillus nidulans* FGSC A4	Patatin-like serine hydrolase, putative	0.029	XP_660922
9B1	465	*Phaeosphaeria nodorum* SN15	Chromosome segregation protein, putative	0.001	XM_001794805
			Transport		
10B2	228	*Neurospora crassa*	Nuclear pore protein, related	9e-12	CAF06074
6K1	165	*Ajellomyces capsulatus* G186AR	Metal homeostatis protein bsd2	2e-07	EEH09092
7G1	238	*Gibberella zeae* PH-1	ABC transporter (ABC-type multidrug resistance-associated protein, domain 2)	2e-12	XP_389787
3G1	73	*Aspergillus terreus* NIH2624	Similar to glucose transporter, putative	2e-06	XP_001208397
10A1	296	*Neurospora crassa*	Major facilitator (MFS1) transporter, related	0.93	CAE76439
3H1	72	*Penicillium marneffei* ATCC 18224	MFS glucose transporter, putative	6e-13	XM_002143781
			Regulation of gene expression		
3A1	364	Nectria haematococca mpVI 77-13-4	GAL4-like transcription regulator (hypothetical protein NECHADRAFT_77346)	1e-08	EEU47161
9H1	233	*Gibberella zeae* PH-1	MIZ/SP ring zinc finger domain	5e-08	XP_390191
2D1	291	*Aspergillus clavatus* NRRL 1	Fungal specific transcription factor, putative	2e-05	XP_001267928
2G1	131	*Aspergillus flavus* NRRL3357	Transcriptional regulator, putative	1e-13	XM_002376115
10D1	118	*Neurospora crassa*	Transcriptional regulatory protein pro-1, putative	3e-12	AJ238440
			Transposable elements		
11B1	392	*Phaeosphaeria nodorum*	Polyprotein (protease, integrase, reverse transcriptase)	6e-09	CAB91877
7E1	286	*Fusarium oxysporum* f. sp. *lycopersici*	Fot5 transposase	6e-29	CAE55867
			Unknown function		
9C1	383	*Aspergillus terreus* NIH2624	Predicted protein	1.0	XM_001218612
3E1	124	*Gibberella zeae* PH-1	Hypothetical protein FG07937.1	5e-06	XM_388113
9G1	238	*Gibberella zeae* PH-1	Hypothetical protein	3e-17	XM_381709
5A1	297	*Nectria haematococca* mpVI 77-13-4	Hypothetical protein	2e-11	EEU_39983
1A1	487	*Podospora anserina* DSM 980	Hypothetical protein	5e-26	XP_001909636
1E1	260	*Gibberella zeae* PH-1	Hypothetical protein	0.48	XM_385470
6G1	224	*Chaetomium globosum* CBS 148.51	Hypothetical protein	0.82	XM_001226472
6D1	379	*Sclerotinia sclerotiorum* 1980	Hypothetical protein	0.2	XM_001596580
Meloidogyne incognita					
			Metabolism/signal regulation		
3G1	118	*Aspergillus niger* CBS 513.88	Acid phosphatase aph (3-phytase phyB)	4e-06	XM_001393169
11I1	89	*Pyrenophora tritici-repentis* Pt-1 C-BFP	Phospholipase D1	4e-05	XM_001935238
7A1	242	*Gibberella zeae* PH-1	Arrestin (or S-antigen), N-terminal domain	1e-14	XP_390197
7F1	133	*Pyrenophora tritici-repentis* Pt-1 C-BFP	Monoxygenase	7e-06	XM_001940586
5B1	416	*Ajellomyces capsulatus* NAm1	Cystathionine-γ-lyase	0.12	XM_001542489
9E1	238	*Ajellomyces capsulatus* NAm1	Dihydrolipoamide dehydrogenase	0.032	XM_001536111
8I2	74	*Gibberella zeae* PH-1	RNA-dependent RNA polymerase (hypothetical protein)	0.42	XM_388892
			Transport		
8B1	403	*Aspergillus flavus* NRRL3357	NCS family nucleoside transporter, putative	4e-28	XP_002384546
8F1	159	*Gibberella zeae* PH-1	E1-E2 ATPase (hypothetical protein FG04995)	6e-18	XP_385171
11G1	148	*Aspergillus nidulans* FGSC A4	Amino acid transporter (hypothetical protein AN1659.2, amino acid permease)	0.44	BN001307
			Regulation of gene expression		
1D2	261	*Nectria haematococca* mpVI 77-13-4	b-ZIP transcription factor (related, predicted protein)	3e-14	EEU47875

Table 1 (continued)

TDF	Length (bp)	Sequence homology organism[a]	Annotation	BLAST scores	Acc. number[b]
8A1	403	*Hypocrea spinulosa*	Frequency clock protein	5e-19	Q00586
11A1	430	*M. anisopliae*	Mmc protein (microcycle conidiation rel.)	5e-23	FJ827121
			DNA repair		
5C1	400	*Verticillium albo-atrum* VaMs.102	Tyrosyl-DNA phosphodiesterase	3e-15	EEY14778
			Unknown function		
11H1	123	*Podospora anserina*	Unnamed protein product	2e-03	CU633900
2A2	214	*Neurospora crassa* OR74A	Hypothetical protein (DNA linkage group V)	4e-15	AL513445
6F1	143	*Gibberella zeae* PH-1	Hypothetical protein FG04793	9e-10	XM_384969
8C1	413	*Gibberella zeae* PH-1	Hypothetical protein FG09687	1e-06	XP_389863
9D1	354	*Talaromyces stipitatus* ATCC 10500	Conserved hypothetical protein	2e-22	XP_002481203
10E1	441	*Gibberella zeae* PH-1	Hypothetical protein FG04002	9e-31	XP_384178
9G1	165	*Magnaporthe grisea* 70-15	Hypothetical protein	0.52	XM_363878
6A1	362	*Penicillium chrysogenum* Wisc. 54-1255	Hypothetical protein	0.26	XM_002568065
1C1	200	*Claviceps purpurea*	mRNA sequence	0.052	FM986884
6C1	192	*Saccharomyces cerevisiae* EC1118	Putative protein, similar to YMR221C	0.72	FN393082
6D1	164	*Chaetomium globosum* CBS 148.51	Hypothetical protein	0.17	XM_001225238
10A1	348	*B. fuckeliana* strain T4	cDNA	0.092	AL111838
1E1	109	*Cryptococcus neoformans* var. *neoformans* B-3501A	Hypothetical protein	1.0	XM_766851
3E1	194	*Coprinopsis cinerea* okayama7#130	Hypothetical protein	0.083	XM_001838635
9A1	158	*Yarrowia lipolytica* strain CLIB122	Hypothetical protein	1.6	CR382132

[a] TDFs are identified through their primers combination (see Supplementary materials) and ordered by the annotated functional roles. Scores determined using BLAST (BLASTn, BLASTx or tBLASTx) algorithms for homologies and annotations are shown for closest accessions found in GenBank

[b] Nucleotide or protein accessions

database was interrogated, using a translated nucleotide query, similarity was greater than to the fungal databases, and these sequences were not used in the subsequent analyses.

The percent GC contents of the selected TDFs were 51.4± 3.4 (mean±SD) for IMI 380407, and 51.6±5.0 for IMI 331547. Of the 38 TDF sequences selected from isolate IMI 380407, 20 showed similarity with known functions, eight with hypothetical proteins, and ten had no homologous sequence in the databases searched. Of the 35 TDFs selected from isolate IMI 331547, 14 matched to sequences with known function, 13 corresponded to sequences coding for conserved, predicted or hypothetical proteins, two showed similarities for cDNA or mRNA sequences, and six had no homologous sequence in the databases searched. The selected TDFs showed varying levels of homology with known genes or ESTs of other fungal species (*E* value range: 1.6–1e-42). Clones identified and submitted to the NCBI database for each isolate (accession numbers: GW868519-546 for IMI 331547; GW874603 and GW868547-574 for IMI 380407) are shown in Table 1, with the corresponding homology accession numbers.

Differentially expressed transcripts

Of the 28 TDFs identified for IMI 380407, 16 appeared related to egg parasitism, due to a different pattern of expression observed in relation either to treatment I or II (rich and minimal medium), respectively. Twelve of these genes were upregulated during the last 4 h of the assay only, two were downregulated, and a further two were expressed at all times. Of these TDFs, 13 corresponded to bands in the IMI 331547 gels, with varying levels of expression similarity. In the IMI 331547 assay, among the 29 selected TDFs, nine appeared linked to parasitism, four of which were not matched in the IMI 380407 expression gels. Of the expressed genes, some were regulated early (two appeared at 0–2 h, two at 2 h, one at 4–8 h) and three were upregulated later (at 8 h). Only two transcripts were observed over the entire 8-h period of the assay (Table 2).

TDF validation by real-time PCR

The RT-PCR validation analysis carried out to check selected TDFs from IMI 380407 and IMI 331547 showed that the transcript quantities were in agreement with the profiles detected by cDNA–AFLP, supporting the reproducibility and reliability of this approach (Fig. 2). TDFs that showed constant band intensity levels during treatments (9H1 from IMI 380407 and 9A1 from IMI 331547, coding for a MIZ zinc finger domain protein and a hypothetical protein, respectively), were selected as endogenous controls (normalizers), since

their expression was not affected by the variable treatments and times applied. The selection of a stable reference gene for data normalization represents a critical task, since several genes commonly considered as constitutively expressed, such as tubulin, ubiquitin, and glyceraldehyde 3-phosphate dehydrogenase, were differentially expressed in other studies (Polesani et al. 2008). Data from our experiments were therefore normalized against genes whose expression levels were stable during the times tested.

TDFs annotation by functional group, isolate, and treatment

Annotation and BLAST analyses showed that most of the TDFs could be included in functional groups related to metabolic functions or involved in mechanisms of cellular signals regulation, cellular transport, regulation of gene expression as well as DNA repair. Other TDFs showed similarities with fungal transposable elements or hypothetical proteins not yet characterized. The TDF annotations as inferred by the closest fungal sequences and the corresponding BLAST scores are shown, for each isolate, in Table 1. The expression profiles per nutritional conditions and sampling times are shown, for each isolate and TDF, in Table 2.

Expression analysis

Comparison of groupings obtained by PCA and HCA showed distinct clusters of putatively coexpressed TDFs, for both *P. chlamydosporia* isolates (Fig. 3a, b). At least five groups could be identified in isolate IMI 380407. The first cluster included an Mg-dependent phosphatase that was coexpressed with a MSF1 transporter and a fungal specific transcription factor. Cluster II showed a first subgroup formed by metabolism-related TDFs and a transposon-related polyprotein, associated with a second subgroup including mainly transport related products and three hypothetical proteins. Clusters I and II included all TDFs identified as involved in *G. pallida* egg parasitism (up- or downregulated), with the unique exception of a hypothetical protein (1E1), which was grouped in cluster V. Transposable elements did

Table 2 Expression of *P. chlamydosporia* putative genes with unknown or predicted functions at four sampling times, for IMI 380407 vs. potato cyst nematode (PCN) *G. pallida* and IMI 331547 vs. root knot nematode (RKN) *Meloidogyne incognita*, in rich (I) or minimal medium (starvation, II) and minimal medium with nematode eggs (III)

IMI 380407/PCN	Treatments												S [b] IMI 331547
Putative genes [a]	I				II				III				
Time course (hours)	0	2	4	8	0	2	4	8	0	2	4	8	
Mg-dependent phosphatase 1 (2C1) [c] π													
Chaperonin GroEL (HSP60 family) (7A1)											+	+	I, II, III
Glycosyltransferase family A (1F1)													I
Reducing polyketide synthase (7Q1)	+	+	+	+									I, II, III
GTPase activating protein Rga6 (11A1) π													
Patatin-like serine hydrolase, putative (1B1)													n
Chromosome segregation protein, putative (9B1) π													I
Nuclear pore protein, related (10B2)													
Metal homeostatis protein bsd2 (6K1) π											+	+	I, II, III
ABC transporter (7G1) π											+	+	
Glucose transporter (3G1), putative π											+	+	n
Major facilitator (MFS1) transporter, related (10A1) π													
MFS glucose transporter, putative (3H1) π											+	+	n
GAL4-like transcription regulator (GAL4-like) (3A1) π													n
MIZ/SP ring Zn finger protein (9H1) (constitutive, all bands faint)													I, II, III
Fungal specific transcription factor, putative (2D1) π													
Transcriptional regulator, putative (2G1)													II, III
Transcriptional regulatory protein pro-1, putative (10D1)													
Polyprotein (protease, integrase, rev. transcriptase) (11B1) π													
Fot5 transposase (7E1)													
Predicted protein (9C1) π													
Hypothetical protein FG07937.1 (3E1)			+	+				+			+		II, III
Hypothetical protein (9G1) π													I, II
Hypothetical protein (5A1) π											+	+	I, II
Hypothetical protein (1A1)													n
Hypothetical protein (1E1) π													I
Hypothetical protein (6G1) π											+	+	I, III
Hypothetical protein (6D1)													I, II, III

Table 2 (continued)

IMI 331547/RKN Putative genes [a]	Treatments												S [b] IMI 380407
	I				II				III				
Time course (hours)	0	2	4	8	0	2	4	8	0	2	4	8	
Acid phosphatase aph (3-phytase phyB) (3G1) [c]					+	+	+	+	+	+	+		
Phospholipase D1 (11I1)										+			n
Arrestin (or S-antigen) N-terminal domain (7A1)											+		
Monoxygenase (7F1) π												+	
Cystathionine-γ-lyase (5B1) π												+	n
Dihydrolipoamide dehydrogenase (9E1)												+	II, III
RNA-dependent RNA polymerase (8I2)									+				n
NCS family nucleoside transporter, putative (8B1) π												+	n
E1-E2 ATPase (8F1) π											+	+	n
Amino acid transporter (11G1) π										+			n
bZIP transcription factor, related (1D2)													
Frequency clock protein (8A1)												+	n
Mmc protein (microcycle conidiation, related) (11A1)												+	
Tyrosyl-DNA phosphodiesterase (5C1)							+	+			+	+	n
Unnamed protein product (11H1) π										+			n
Hypothetical protein, DNA linkage group V (2A2)													
Hypothetical protein FG04793 (6F1)										+	+	+	I, II
Hypothetical protein FG09687 (8C1)		+	+	+									n
Conserved hypothetical protein (9D1)	+	+	+	+	+	+							II
Hypothetical protein FG04002 (10E1)	+	+	+	+									n
Hypothetical protein (9G1) π												+	I, II
Hypothetical protein (6A1)	+	+	+	+									I, III
mRNA sequence (1C1)								+					
Putative protein, similar to YMR221C (6C1) π										+			
Hypothetical protein (6D1)					+	+	+	+	+	+	+	+	I
cDNA (10A1) π												+	
Hypothetical protein (1E1)													
Hypothetical protein (3E1)											+		
Hypothetical protein (9A1)													I, II, III

[a] □, downregulated gene gel band absent or faint; ▒, upregulated gene band visible; (+), bold bands. π, up- or downregulated TDFs differentially involved in parasitism

[b] Similarity, treatments showing the same expression patterns in gel of the isolate shown, at all sampling times, for the same primers pair (n: bands not found or not identified)

[c] Primer combinations

not appear coexpressed, since the second transposon-related product, identified as a Fot5 transposase, was grouped in cluster V with a member of the HSP60 family (chaperonin GroEL) and three further hypothetical proteins. Finally, clusters III and IV included mainly TDFs related to transcription and metabolism (Fig. 3a).

Similarly, four clusters accounting for distinct metabolic pathways could be identified in isolate IMI 331547. The first cluster included a phytase and a hypothetical protein. TDFs involved in *M. incognita* egg parasitism were all grouped in cluster II, that included metabolism and transport related products, the only exception being an E1-E2 ATPase, grouped in cluster III. Cluster II also included a phospholipase, a microcycle conidiation-related protein and a putative RNA-dependent RNA polymerase, which did not appear specifically involved in the parasitic metabolism. Finally, cluster III included TDFs related to metabolism, DNA repair and gene regulation, whereas cluster IV showed only six different hypothetical proteins which could not be characterized (Fig. 2b).

Discussion

In the present study, a modulation of transcriptional activity between the *P. chlamydosporia* isolates was ascertained, providing a first comparative insight on a number of genes involved in metabolism as well as parasitism. The TDFs examined showed a partial overlap between isolates, with 15% of the IMI 380407 transcripts not matched in IMI 331547 gels and 40% from IMI 331547 not found in IMI 380407. This difference might be due to faint band resolution at gel edges in some combinations. However, the two isolates showed expression profiles and induced transcriptional changes only partially similar, thus indicating a differential reaction to the presence of eggs. Of the

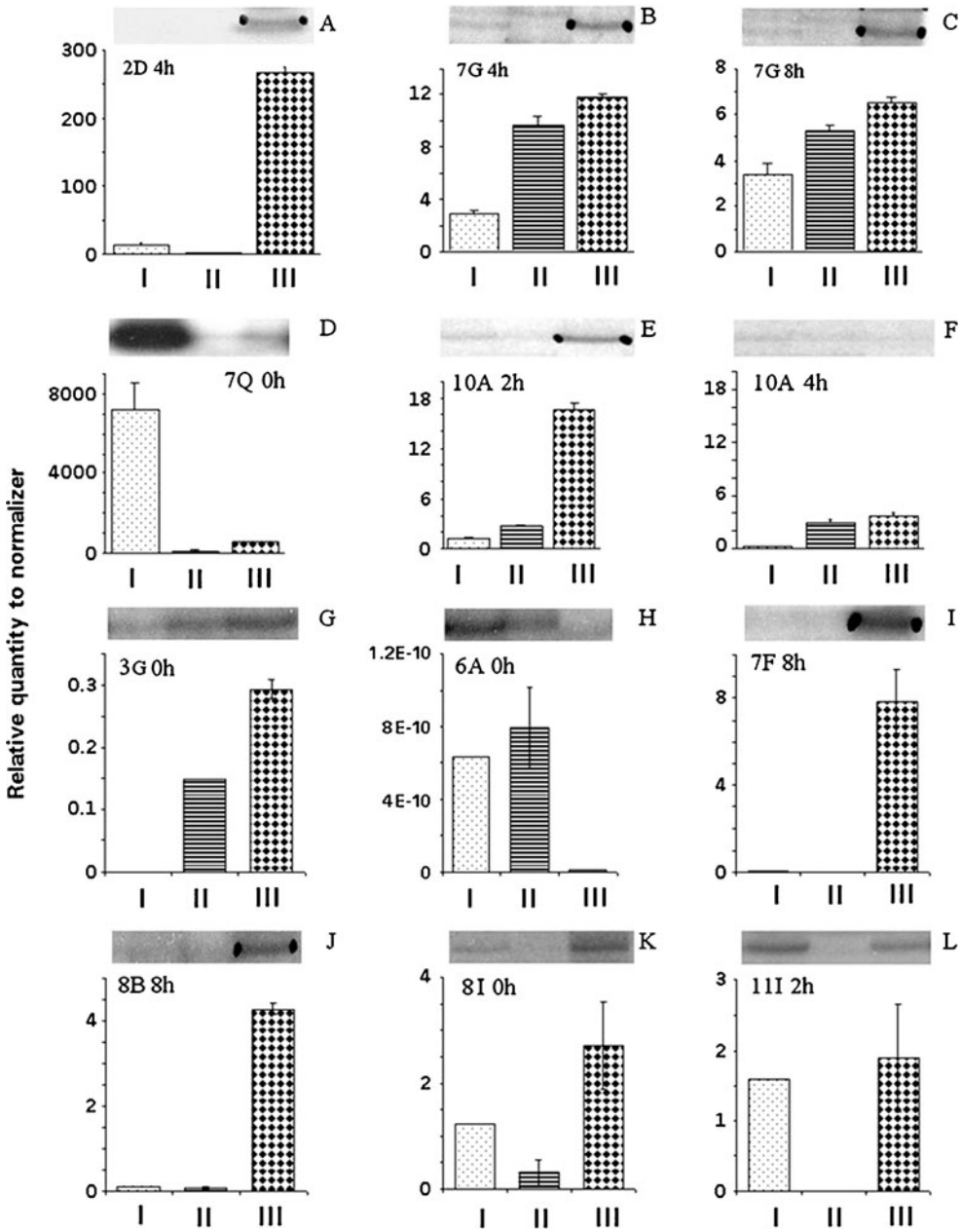

Fig. 2 Real-Time qPCR validation analysis of transcript levels. Bands from the cDNA–AFLP gels of IMI 380407 (**a**–**f**) and IMI 331547 (**g**–**l**) for selected specific time points and primers combinations. Corresponding histograms of selected TDFs show differential gene expression among treatments, relative to the normalizers endogenous controls (9H1 for IMI 380407 and 9A1 for IMI 331547). Treatments I, II, and III represent rich medium (CD), minimal medium (MM) and MM with eggs, respectively. Values represent means ratios of transcripts values relative to normalizers (*error bars* show 1 SD on biological replicates)

five selected TDFs of IMI 380407 not matched in the IMI 331547 gels, three were related to parasitism. Similarly, of the 11 TDFs of IMI 331547 not observed in IMI 380407 gels, five appeared related to parasitism (Table 2). The screening of two isolates and host associations confirmed the utility of a broad categorization of parasitism genes, since changes reflected isolate-specific expression patterns (Johannesson et al. 2006).

Gene expression and TDFs abundance suggest differential regulation. Disparity between isolates was expected, since functional differences exist among them (Morton et al. 2003). Furthermore, it did not appear related to the methodology applied, as failed AFLP reactions typically yield few bands due to under-amplification or incomplete restriction–ligation. Changes in expression can therefore be assigned to variations in synthesis of extracellular enzymes, in response to the same biological (i.e., egg shell) or ecological (i.e., starvation) stimuli, albeit of the differences in PCN and RKN eggs (Morton et al. 2003). Further possibilities include specific host recognition, genome variations, and structural or functional changes of products. The discovery of transposable elements adds a further source of genetic recombination, previously unexpected.

The *P. chlamydosporia* parasitic behavior revealed specific metabolic pathways, as shown by the genes activated only in the presence of eggs. When comparing treatments switching from saprophytism to parasitism, *P.*

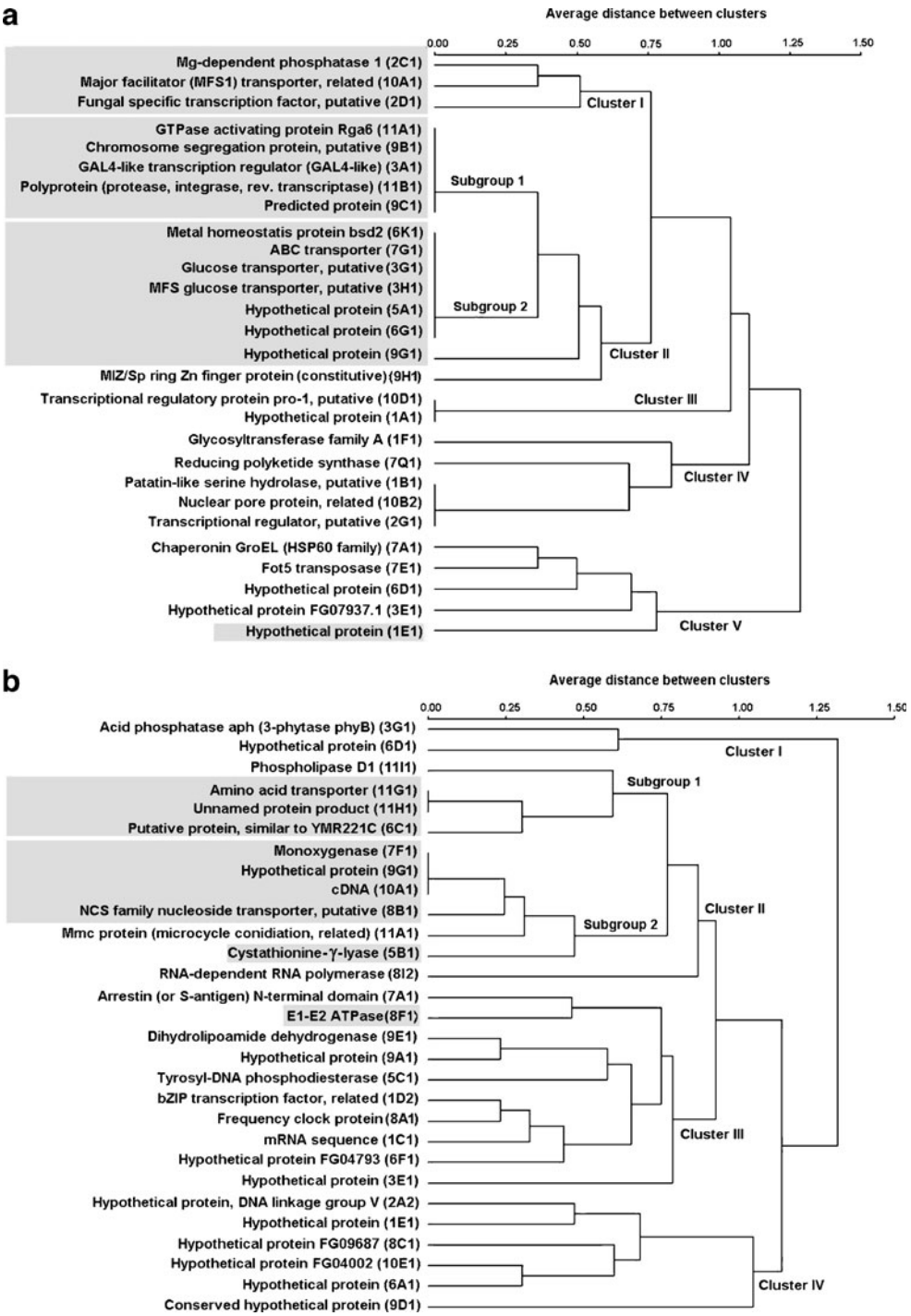

Fig. 3 Average linkage hierarchical cluster analysis (HCA) dendrograms showing clusters of annotated TDFs for isolates IMI 380407 (**a**) and IMI 331547 (**b**), identified by their primers combinations. Transcripts involved in nematode eggs parasitism are shaded in *gray* for both isolates

chlamydosporia showed significant transcriptional reprogramming, as shown by the differences in expression of upregulated genes involved in detoxification, metabolism, or cellular transport (Fig. 2; Table 2). Some genes were induced/expressed or repressed suggesting a concerted regulation, especially when activated only after eggs exposure. However, common amplification profiles also reflected expression of basic metabolic genes, not affected by the assay conditions.

Genetic reprogramming reflects evolutionary adaptive processes in fungus–host interactions and has been observed in nematode and insect parasitic species, in mycoparasites or in human pathogens (Fekete et al. 2008; Johannesson et al. 2006; Wang et al. 2005; Zhang et al. 2009). The transcriptome of the nematode-trapping fungus *Monacrosporium haptotylum* showed a significant reprogramming during the adhesion, penetration, and digestion of its test prey, *Caenorhabditis elegans*, with changes

concerning subtilisins or pathogenesis genes, conidial germination and morphogenesis (upregulated), a glycogen phosphorylase (downregulated) or abundance of noncoding RNAs (Ahrén et al. 2005; Fekete et al. 2008).

Substrate-dependent shifts in gene expression were also observed in *Metarhizium anisopliae* (Wang et al. 2005). Changes during growth on the host hemolymph concerned proteases, expressed in presence of cuticle, or metabolic genes (cell wall reorganization, carbohydrate and lipid metabolism, detoxification, and synthesis of toxic metabolites). Stress response genes were expressed on root exudates, whereas energy related and transposable elements were not affected. In this study, HCA was reliable in identifying expression changes induced by the nutritional conditions, with groups reflecting changes in transcription between pathogenic and saprophytic growth (Wang et al. 2005). In our HCA analysis, performed from PCA results, specific regulatory systems of coexpressed TDFs were identified. Remarkably, the transcriptome of both isolates showed that products regulated by nematode eggs clustered in distinct HCA cohorts, three for IMI 380407 and two for IMI 331547 (Fig. 3).

For detailed properties and biochemical determinants of the transcripts, see Table S3. The first group of IMI 380407 (cluster I, Fig. 3a) included an upregulated Mg-dependent phosphatase-1, involved in a physiological repair mechanism (Fortpied et al. 2006). The closest GenBank entry to a second TDF, a major facilitator transporter domain MFS1 (CAE76439) showed similarity to the DHA14-like major facilitator of *Botryotinia fuckeliana* (acc. AAF64435.2), an antiporter catalyzing drug efflux (Pao et al. 1998). A BLAST search with the closest accession to the third transcript of cluster I in the Fungal Transcription Factor Database (http://ftfd.snu.ac.kr) showed similarity (e-score: 7e-21) with a tubby (membrane anchored) transcription factor from *Neurospora discreta*. TDFs of cluster I hence suggest detoxification and cell proteins protection mechanisms.

The second group of IMI 380407 (cluster II, subgroup 1, Fig. 3a) showed coexpression of two transcripts related to cell proliferation, Rga6 involved in cell wall formation in *Schizosaccharomyces pombe* (Nakano et al. 2001), and a chromosome segregation protein domain, active in yeast mitosis, sensing DNA double-strand breaks and repair (Borde 2007). A further member, GAL4, is a fungal specific activator of the GAL system, a glucose-repressible and galactose-initiated catabolic path (Johnston 1987). GAL4 is involved in several biochemical pathways (Table S3). Galactose, a suitable carbon source for *P. chlamydosporia* (Liu and Chen 2003), acts as promoter of cellulase in *Hypocrea jecorina* (anamorph *Trichoderma reesei*) (Karaffa et al. 2006), of filamental growth in *Candida albicans* (Brown et al. 2009), and is present in the fungus wall glycoprotein (Ruiz-Herrera 1992). Nematode cuticle bears galactose residues including D-galactose and *N*-acetyl-D-galactosamine, which are recognized by nematode trapping fungi (Nordbring-Hertz et al. 1982; Premachandran and Pramer 1984). Galactosamine was also found in the RKN egg masses (Sharon and Spiegel 1993) and in *Heterodera schachtii* cyst wall (Clarke 1970), whereas galactose and *N*-acetyl-D-galactosamine were found on *Globodera* spp. (Forrest and Robertson 1986). In a parallel with other pathosystems (Chung and Liao 2008; Dufresne et al. 2000), GAL4 analysis may identify promoter(s) steering *P. chlamydosporia* metabolism. However, galactose should be released from the eggs coat to act as a molecular switch.

Further transcripts included a polyprotein whose closest entry (CAB91877) corresponds to a retrotransposable element from *Phaeosphaeria* (*Septoria*) *nodorum* (Rawson 2000). As for other transposons of fungi (Basnayake et al. 2009), it may recombine pathogenicity or virulence factors. Together with the Fot5 transposase, clustered in a different group, these are the first transposable elements found in *P. chlamydosporia.* Subgroup I of cluster II suggests regulation of genes involved in cellular proliferation, through DNA replication, recombination, and repair, with expression of a retrotransposable element.

The third group of IMI 380407 TDFs associated to parasitism (subgroup 2, cluster II) included a metal homeostasis protein, involved in heavy metal ions homeostasis and detoxification (Liu and Culotta 1994). Also the ATP-binding cassette transporters are involved in detoxification and drug resistance (Sipos and Kuchler 2006; Table S3). This subgroup appears as a detoxification and glucose-mediated process, due to the coexpression of glucose transporters. The two subgroups of the IMI 380407 cluster II were joined at a higher distance by the MIZ/SP ring Zn finger domain, a gene regulator always faintly expressed, used as a constitutive control, and by cluster III, with a transcription regulator and a further hypothetical protein (Fig. 3a).

The two remaining clusters of IMI 380407 were not related to parasitism and showed similar expression patterns (Table 2). Cluster IV showed secondary metabolism and transport TDFs, mainly expressed in rich medium and downregulated in starving conditions or in presence of eggs, including a glycosyltransferase (GT-A type superfamily) and a reducing polyketide synthase (RPS), expressed only in rich nutritional conditions, with high affinity with an RPS from the *P. chlamydosporia* radicicol gene cluster (Table 1). The latter catalyzes a resorcilic acid lactone that inhibits the HSP90 chaperone (Table S3). In other host–parasite associations, a polyketide synthase was expressed during saprophytic metabolism (Johannesson et al. 2006), whereas in the mycoparasite *Chaetomium cupreum* it was associated with biological control functions (Zhang et al. 2009). IMI 380407 cluster V included mostly constitutive

products, like a chaperonin GroEL (HSP60 family), three hypothetical proteins and a Fot5 transposase (Table 2).

The fourth parasitism group (IMI 331547 cluster II), included a subgroup 1, with a phospholipase D1, an amino acid transporter similar to a membrane permease, and two undefined proteins. Subgroup 2 included a monoxygenase (Table 2) whose closest gene entry (6348590) is a binding domain involved in K^+ channels regulation (Anantharaman et al. 2001), and an NCS family nucleoside transporter. A further transcript showed partial similarity to cystathionine-γ-lyase, a key enzyme in cephalosporin biosynthesis in *Acremonium chrysogenum* (Kosalková et al. 2001), found also in *M. anisopliae* (Wang et al. 2005).

Subgroup 2 also included a microcycle conidiation protein (Table 2) which is induced by environmental stress in plant parasitic or entomopathogenic fungi (Zhang and Xia 2008) and a RNA-dependent RNA polymerase (RdRP; Fig. 3b), which is involved in fungi in a silencing mechanism related to the amount of transgenic RNAs (Forrest et al. 2004). This is the first report of an RdRP from *P. chlamydosporia*.

IMI 331547 showed two other clusters (III and IV) not related to parasitism. Cluster III contained constitutive or starvation TDFs including an arrestin, an E1-E2 ATPase (the only IMI 331547 parasitism-induced transcript), and TDFs related to metabolism, DNA repair (tyrosyl-DNA phosphodiesterase), gene expression regulation (bZIP transcription factor), with a frequency clock protein and three undefined products (Fig. 3b). A dihydrolipoamide dehydrogenase LPD1 and the DNA repair protein tyrosyl-DNA phosphodiesterase were always expressed as constitutive genes. The last two groups included cluster IV (hypothetical proteins), and cluster I, formed by an acid phosphatase 3-phytase (phyB) and a hypothetical protein (Fig. 3b), which putatively account for a further distinctive metabolic pathway.

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