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RESEARCH ARTICLE

Isolation of mycoparasitic-related transcripts by SSH during interaction of the mycoparasite *Stachybotrys elegans* with its host *Rhizoctonia solani*

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Abstract Mycoparasitism by antagonistic fungi involves changes in the biochemistry and physiology of both partners. Analysis of genes that are expressed during mycoparasite–host interaction represents a powerful strategy to obtain insight into the molecular events underlying these changes. The aim of this study is to identify genes whose expression is upregulated when the mycoparasite *Stachybotrys elegans* is in direct confrontation with its host *Rhizoctonia solani*. Suppression subtractive hybridization (SSH) was used to create a subtracted cDNA library, and differential screening was applied to identify the over-expressed transcripts. We report the analysis of 2,166 clones, among which 47% were upregulated during mycoparasitism. Two hundred and sixty-one clones were sequenced that corresponded to 94 unique genes. Forty-four of these were identified as novel genes, while the remainder showed similarity to a broad diversity of genes with putative functions related to toxin production, pathogenicity, and metabolism. As a result of mycoparasitism, 15 genes belonged to *R. solani* among which 9 genes were assigned putative functions. Quantitative RT-PCR was used to examine the upregulation of 12 genes during the course of mycoparasitism. Seven genes showed significant upregulation at least at one-time point during interaction of the mycoparasite with its host. This study describes a first step toward knowledge of *S. elegans* genome. The results present the useful application of EST analysis on *S. elegans* and provide preliminary indication of gene expression putatively involved in mycoparasitism.

Keywords Mycoparasitism · SSH · Differential screening · Gene expression · Mycoparasitism-induced genes · ESTs

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Introduction

The development of EST libraries associated with differential gene expression technologies provides a panoramic view of many biological processes (Green et al. 2001). Initially developed within the animal and medical fields, transcriptome approaches are now integrated into plant–microbe interaction analysis (Guilleroux and Osbourne 2004; Felitti et al. 2006; Torregrosa et al. 2006) and to a lesser extent into microbe–microbe interaction (Carpenter et al. 2005; Vizcaino et al. 2006). Recent studies involving EST approaches have been carried out on model strains of fungal mycoparasites belonging to *Trichoderma* species. These studies have increased our knowledge on genes encoding cell wall degrading enzymes, proteolytic enzymes, and to a limited extent on genes encoding various biological processes (Liu and Yang 2005; Vizcaino et al. 2006; Suarez et al. 2007). The cDNA libraries of these studies were constructed from *Trichoderma* strains that were grown under different nutrient conditions, in the

presence of cell wall preparation (i.e. simulated mycoparasitism), or in confrontation with a host but without direct contact.

The cellular interaction between a pathogenic microorganism and its host starts at the molecular interaction between the two interacting partners and the expression of some of the genes involved in mycoparasitism may require the direct contact of a live host. Carpenter et al. (2005) applied suppression subtractive hybridization (SSH), and explored differential gene expression of the mycoparasite *Trichoderma hamatum* LU593 in the presence of the living host *Sclerotinia sclerotiorum*. Only 19 genes were identified resulting in an incomplete view of genetic regulation during mycoparasitism. Despite this recent finding, differential expression studies on mycoparasites other than *Trichoderma* strains and in direct contact with the hosts have not been reported to our knowledge.

Stachybotrys elegans is a typical mycoparasite that colonizes its host, *Rhizoctonia solani*, by accomplishing several successive steps: recognition and production of fimbrial extracellular matrix that surrounds the host cell (Benyagoub et al. 1996), coiling and the formation of appressoria that aid in penetrating the host cell wall followed by intracellular colonization (Benyagoub et al. 1994). This process is accompanied by the secretion of cell wall-degrading enzymes (CWDEs), including chitinases (Morissette et al. 2003; Taylor et al. 2002) and glucanases (Archambault et al. 1998). An endochitinase gene *sechi44* was cloned and characterized (Morissette et al. 2003), and evidence for its participation in the mycoparasitic process over 12 days of interaction, its stimulation by purified host cell wall fragments, and its regulation by nitrogen and carbon availability was recently provided (Morissette et al. 2006).

Our aim in this work was to contribute to the wider picture of mycoparasitism, by characterizing genes preferentially expressed over a 12-day period of mycoparasitic interaction between *S. elegans* and *R. solani*. SSH is a PCR-based method (Diatchencko et al. 1996) that was developed to enrich rare transcripts and low abundance genes in several plant–microbe interactions (Beyer et al. 2002; Thara et al. 2003; Kong et al. 2005). Here the SSH was combined with differential screening to collect low-abundant messengers and to enrich for genes with differential expression just prior to, and at early and late stages of, mycoparasitism. To validate their upregulation, selected genes were evaluated through quantitative reverse transcription (QRT)-PCR analysis of biological replicates. This study provides an insight into the transcriptomes that drive the mycoparasitic process in *S. elegans*.

Materials and methods

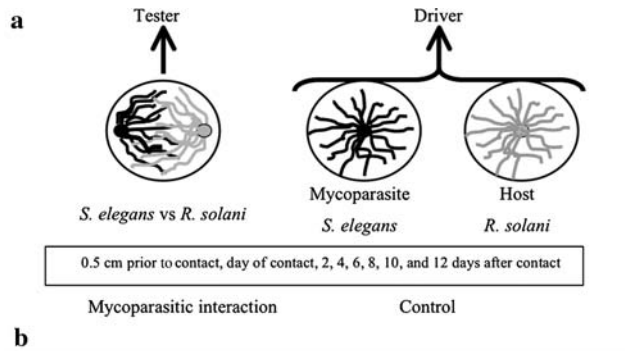
Fungal strains and plate confrontation assays

Starter cultures of *S. elegans* (Pidoplichko) W. Gams (anamorph; ATCC 188825) and the *R. solani* Kuhn AG-3 (ATCC 10183), the anamorph of *Thanatephorus cucumeris* (A.B. Frank) Donk, were revived on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) at 24°C for 7 and 5 days, respectively. Agar plugs (6 mm) from starter cultures of *S. elegans* and *R. solani* were placed on fresh PDA in different Petri plates (100 mm), and allowed to grow for 5 days.

Plate confrontation assays were carried out in the absence of light on culture plates containing minimal synthetic medium (Tweddell et al. 1995) supplemented with 1% agar (MSMA; Gellan Gum, Kelco, San Diego, USA) and covered with a permeable cellophane membrane (500 PUT; UCB, North Augusta, USA). Agar plugs (6 mm) from the respective fungi were placed on the surface of the cellophane membrane and separated by 6 cm from each other. This set-up allowed the mycoparasite and its host to grow toward and contact each other, and eventually interact with each other. For the purposes of this study, the zone where contact occurs is referred to as the zone of interaction. In addition, the use of the membrane in this set-up facilitated the removal of the fungi from the plate for subsequent RNA analysis. Total RNA from triplicate plates was extracted from (i) hyphae of both fungi when they were separated by 0.5 cm prior to contact, (ii) a 5-cm strip of both fungi at the zone of interaction every 48 h from the day of contact (day 0) until 12 days, and (iii) hyphae of pure cultures of *S. elegans* and *R. solani* grown alone, without interaction, on MSMA and harvested at the same time periods (control). The mycelia from all treatments and time periods were flash frozen in liquid nitrogen as they were harvested and stored at −80°C.

Isolation of genomic DNA and RNA

Genomic DNA (gDNA) from *S. elegans* and *R. solani* was extracted as described previously (Morissette et al. 2003). DNA quality and concentration were checked using 1% 1× TAE agarose gel and an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). Frozen mycelia, harvested from the dual interaction experiment, were ground to a fine powder in liquid nitrogen using a mortar and pestle. Isolation of total RNA and verification of quality and integrity was conducted as previously described (Morissette et al. 2006).



b

SSH denominations		cDNA content	Organisms
ST	Subtracted tester	cDNAs unique to the tester	*S. elegans* and *R. solani* in interaction during mycoparasitism
UT	Unsubtracted tester	All the tester cDNAs	*S. elegans* and *R. solani* in interaction during mycoparasitism
UD	Unsubtracted driver	All the driver cDNAs	*S. elegans* and *R. solani* not in interaction, growing on separate petri dishes

Fig. 1 Scheme for the isolation of RNA from mycoparasitic interaction between the mycoparasite *S. elegans* and its host *R. solani* (tester), and from control condition where both fungi grew in different petri plates (driver). (**a**) RNAs were used for the construction of a suppression subtractive hybridization (SSH) cDNA library to isolate ESTs unique to mycoparasitic interactions (tester). (**b**) Designation of SSH cDNA populations used in the differential screening analyses

Suppression subtractive hybridization

One microgram of RNA from time points of pre- and post-contact interaction were pooled (Fig. 1), precipitated, and resuspended to a final concentration of 1 μg/μl of RNA mixture. Tester and driver cDNA were synthesized from 1 μg of RNA using the SMART™ cDNA synthesis kit (BD Biosciences-Clontech, Palo Alto, CA). SSH (Diatchenko et al. 1996) was performed between the cDNA populations of the tester (T: zone of interaction between *S. elegans* and *R. solani*) and the driver (D: *S. elegans* and *R. solani* growing on different Petri plates, control; Fig. 1a). The SSH procedure was performed with the PCR Select cDNA Subtraction kit (BD Biosciences-Clontech) following the manufacturer's recommendations. The subtraction efficiency was monitored using primers encoding the Ascomycete histone 4, H4-1a (5′-GCTATCCGCCGT CTCGCT-3′) and H4-1b (5′-GGTACGGCCCTGGCG CTT-3; Glass and Donaldson 1995). According to the protocol, the difference in the number of cycles required for equal amplification of the corresponding product in the subtracted and unsubtracted samples reflects the efficiency of the subtraction. Histone 4 should amplify at 5–15 PCR cycles later in the subtracted cDNA compared to the unsubtracted tester.

The cDNA inserts were cloned using the TOPO TA Cloning kit (pCR 4® vector; Invitrogen, Carlsbad, CA, USA). The resulting clones were cultured on LB ampicillin (50 μg/ml) plates. A total of 2,471 clones were individually collected after 24 h, transferred to 700 μl liquid LB ampicillin 2-ml tubes overnight growth at 37°C. These cultures were used to establish stocks (40% glycerol stored at −80°C) and to amplify the inserts by PCR.

Differential screening

Differential screening was performed according to the manufacturer's protocol in the PCR-Select Differential Screening kit (BD Biosciences Clontech), and microarrays were used instead of nylon-based arrays. Inserts were amplified by PCR using SSH nested primers (BD Biosciences-Clontech) and 2 μl as template with cycling as follows: 30 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 1.5 min, followed by an extension cycle at 72°C for 10 min. Single PCR products were purified using a vacuum manifold and Multiscreen FB-plate (Millipore, Nepean, ON, Canada). Purified products (2 μg) were transferred to 96-well V-plates (Corning), lyophilized, resuspended in 50% DMSO and 50% Ultra pure water to give a final concentration of 0.2 μg/μl, and arrayed on glass slides with a Virtek printer (Chipwriter Pro SDD2, Virtek, Ontario, Canada). A total of 2,166 ESTs were spotted in triplicate on Corning GAPS II slides (Corning).

Hybridization

A total of eight hybridizations using the SSH generated probes (ST, UT, UD; Fig. 1b) were carried out including two technical replicates of the ST/UD and the ST/UT hybridizations and their respective dye-swaps. Probes were labeled directly using the BioPrime labeling kit (Invitrogen) and Cy3/Cy5 dyes (Amersham Biosciences Piscataway, NJ, USA).

Twenty microliter of pre-hybridization buffer (5× SSC, 0.1% SDS, 0.05% BSA) were deposited on the surface of the spotted slides and incubated in Hybridization Chambers (Corning) for 1 h at 42°C. Hybridization was carried out in a total volume of 20 μl consisting of 1/3 volume of Dig Easy Hyb (Hoffmann-La Roche Ltd, Mississauga, ON, Canada) and dye Cy3- and Cy5-labeled probes (5 pmol from cDNA). Probes were placed onto the center of the arrays and immediately incubated in the hybridization chambers at 42°C in a water bath for 17 h. Following hybridization, the slides were transferred in successive 1× SSC, 0.2% SDS baths with gentle shaking, then incubated for 5 min at room temperature in 0.1× SSC and dried.

Data analysis

Glass slides were scanned using the ScanArray Express HT (Perkin Elmer, Vaudreuil-Dorion, QC, Canada). Separate images were acquired for separate fluorochromes at a resolution of 10 μm per pixel. Data analysis was performed with ScanArray Express software (Perkin Elmer). After segmentation, local background correction and global normalization using LOWESS (locally weighted polynomial regression; Cleveland 1979). Spots with fluorescence signal intensity less than 400 pixels, with a signal-to-noise ratio of lower than 2, or replicate spots with a standard deviation of Cy5/Cy3 ratio greater than 2 were discarded. The median intensities of replicate spots were $\log_2$ transformed and UT/UD ratio was calculated based on equations developed by van den Berg et al. (2004).

$$\text{UT/UD} = \text{antilog}(\text{ER1} - \text{ER2}) \text{ in base } 2 \quad (1)$$

where

$$\text{ER1} = 1/2[(\log_2 \text{Cy3 ST/Cy5 UD}) - (\log_2 \text{Cy3 UD/Cy5 ST})]$$

$$\text{ER2} = 1/2\,[(\log_2 \text{Cy3 ST/Cy5 UT}) - (\log_2 \text{Cy3 UT/Cy5 ST})]$$

ER1 and ER2 are enrichment ratios of ST/UD and ST/UT, respectively, compiled from slides hybridized with ST and UD, and ST and UT, respectively.

Sequence analysis and data handling

Single pass sequencing of 261 upregulated cDNA clones was performed at the Genome Quebec Innovation Center (McGill University, Montreal, QC, Canada) using the M13 universal primer. Sequence analysis was carried out with Chromas 2.3 (http://www.technelysium.com.au/chromas.html), CAP3 (Huang and Madan 1999), the data mining tools on NCBI (http://www.ncbi.nlm.nih.gov/Tools/), InterProScan (Zdobnov and Apweiler 2001), and Biology WorkBench 3.2 (http://www.workbench.sdsc.edu/). Sequences were then analyzed by Standard BLAST (n and x) (Altschul et al. 1997) and PSI-Blast (Schäffer et al. 2001) on sequences from NCBI nr database. The functional significance of upregulated cDNAs was determined based on similarity with previously characterized sequences in the NCBI Genbank nr database (release 159.0). Comparison was carried out using BlastN, BlastX, and PSI-Blast. Matches with an *E* value of less than 10^{-20} were taken to be significant. Matches with an *E* value of 10^{-6} to 10^{-20} over more than 50% of the sequence length, and matches with an *E* value of more than 10^{-6} that share a functional domain were also considered significant. For simplicity of discussion, all cDNAs were classified into one of 13 functional categories (Table 2) based on functional characteristics of the significant blast hits. Cases with multiple possible categories were resolved in favor of categories with potential roles in fungal pathogenesis. The presence of a signal peptide or transmembrane domains was determined as the consensus of predicted protein sequence analysis with Phobius (Käll et al. 2004), TmPred (Hofmann and Stoffel 1993), and DAS (Cserzo et al. 1997).

Primer design and PCR analysis

In order to determine the fungal origin of the cDNA sequences, primer pairs were designed for the 94 unique ESTs using Primer 3 (Rozen and Skaletsky 2000) and tested in conventional PCR against *S. elegans* and *R. solani* total RNA and gDNA. Primer sets (Table 1) flanking 12 selected target genes were further tested in QRT-PCR on cDNA from the eight different time points of interaction in order to validate the upregulation of the genes. Genes were selected based on their putative functional classification and relevance in pathogenicity.

All conventional PCR reactions were performed in an Applied Biosystems 9600 (Foster City, CA, USA) with primers synthesized by AlphaDNA (Montreal, QC, Canada). PCR products were all resolved on agarose gels (1%, 1× TAE) with Gene Ruler^{TM} 100 bp DNA Ladder (Invitrogen), stained with ethidium bromide and pictures were recorded by a gel print 2000i documentation system (BIOCAN Scientific, Mississauga, ON, Canada). Amplification was performed in a 27 μl PCR reactions containing 1× PCR buffer (Invitrogen), 200 μmol dNTPs, 200 μmol primers, 1.5 mmol $MgCl_2$, and 1U Taq polymerase (Invitrogen). The conditions were 10 min at 94°C (hot start), 35 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min, and then an extension cycle at 72°C for 10 min.

QRT-PCR assays were conducted on 12 target genes (Table 1). Five hundred nanogram of total RNA/each time point of the interaction and for control treatment (*S. elegans* grown alone) was reverse transcribed into cDNA using RT Quantitech kit (QIAGEN) and each cDNA was diluted to 1/20th. Three biological replicates and two technical replicates were performed for each template and a negative control was included in each run. QRT-PCR was conducted in Mx3000 (Stratagene, Cedar Creek, USA) with SYBR Green master mix (Stratagene) following the manufacturer's recommendations. Amplification was performed in a 22 μl reaction mixture containing the following concentrations: 125 nmol of each primer, 1× SYBR Green master mix, 30 nmol of reference dye ROX, and 2 μl of cDNA template. The amplification conditions were 95°C for 10 min (hot start), followed by different number of cycles and annealing temperatures (Table 1) and

Table 1 List of primers used in QRT-PCR assays

Target gene	Accession number	QRT-PCR conditions	Sequence (5′–3′)	Tm (°C)[a]	Amplicon size (bp)
Ankyrin repeat protein	DW520683	A	TACTCTCAACACTCAGGACCGCTT	72	138
			TCACATGGACTCATCGTTGTCGCAT	72	
Mixed functions	EU008742	B	GAGAACAGCAGTCTTCATTTC	62	111
			CTAAGTATAGCACCAGAGGCA	60	
Transcription factor	DW520684	C	CGACCTTGTATAGCGTGCGAAGTT	72	118
			TTCTACAATGCTAGGCCCTTTGCG	72	
No match	DW520692	D	CCCTCTTGTTGCCCTTTCCTTTGT	72	140
			ACGTTGGTGAGACCTAGCATCGAG	74	
Yop	DW520685	A	TGACTTACTGGGTCGTCTTTGCCT	72	111
			AGAGCCACAGCAGGAAGATGAACT	72	
Mog	EU008745	C	TCGATCAGGATGGTTTCACCAGCA	72	129
			TGATATCGGTGCCAACCATGTCCT	72	
MFS hexose transporter	EU008753	C	CCAGATTGCCTTTGTCTGGCTGTT	72	119
			TGATCATCATACCCTTGGCACGGA	72	
Cytochrome P450	DW520689	A	AGATGCGAGTGGCGCAAGTTCTTT	72	137
			TTCGCAGCGACTCGAGAACCATTA	72	
Calmodulin	EU008747	C	CGGCAGAGATGAAACCGTTGTTGT	72	120
			TTGACTTCCCAGAGTTCCTGACCA	72	
Ribosomal protein L10	DQ369806	A	TATCCCATGTCGCGATTCAACCGT	72	133
			TCAGCTGCTCATACTCGTTGGAGA	72	
Mixed functions	EU008756	D	AATACGCCATACAGCCGAGAGACA	72	110
			TCAAGACGCCCGATTGGTTCTGAT	72	
Mixed functions	DW52087	E	TCTACACCTTGGACTCCATCTC	66	416
			GAGCCTCGAGTGTTTCTGATTC	66	

[a] Tm is provided as Tm = 2× (A + T) + 4× (C + G)

A = 53 cycles at 95°C for 30 s, annealing at 64°C for 1 min

B = 50 cycles at 95°C for 30 s, annealing at 58°C for 1 min

C = 40 cycles at 95°C for 30 s, annealing at 61°C for 1 min

D = 40 cycles at 95°C for 30 s, annealing at 64°C for 1 min

E = 45 cycles at 95°C for 30 s, annealing at 61°C for 1 min

then an extension at 72°C for 10 s. The fluorescence reading was measured at 72°C at the end of the elongation cycles, except for clone 1144 (DW520879) which was at 80°C for 11 s. Following amplification, a melting curve was generated by programming the thermocycler to reach 95°C (60 s), 55°C (30 s) (2.5°C/s) and 95°C (0 s) (0.1°C/s). Data generated by QRT-PCR were estimated using Stratagene analysis software.

Relative expression ratios of the target genes during interaction of *S. elegans* with *R. solani* versus *S. elegans* alone (control) was normalized against a housekeeping gene (HKG) that showed minimal variation across treatments. Primer sets, Bt2a and Bt2b, H3-1a and H3-1b, and H4-1a and H4-1b (Glass and Donaldson 1995) were designed to amplify a β-tubulin-encoding gene segment of 260 bp in length, a histone-3 encoding genes segment of 390 bp, and a histone 4-encoding gene segment of 161 bp in length from Ascomycetes, respectively. The statistical software tool Bestkeeper (Pfaffl et al. 2004; http://www.wzw.tum.de/gene-quantification/bestkeeper.html) was applied to select the best HKG that exhibited minimal variation across treatments. The expression levels of histone 4-encoding gene had the lowest variation with a standard variation (SD) less than 1 and a coefficient of variation (CV) of 2.32. Because of this low variation in expression, histone 4 can be considered stable and was chosen as the appropriate HKG.

The relative expression ratios of the 12 target genes during interaction of *S. elegans* with *R. solani* versus *S. elegans* alone (control), normalized with histone 4, were calculated at different time points using two technical and three biological replicates. Quantification was based on an

Table 2 Potentially upregulated cDNAs following the mycoparasitic interaction between *S. elegans* and *R. solani*

Accession number	Blast organism[a]	Blast accession number	*E*-value[b]	% Coverage[c]	Putative function	CD[d]	ψ-blast[e]	IPS[f]	Number of clones	SP/TM[g]	UT/UD ratio[h]	Organism
Functional classification												
Toxin and toxin metabolism												
EU008749	*Chaetomium globosum*	XP_001227833.1	2E-42	74.40	Carboxylesterase	✔	✔	✔	2		1.32	*S. elegans*
DW520689[i]	***Phaeosphaeria nodorum***	**EAT85329.1**	**9E-29**	**96.6**	**Cytochrome P450 monooxygenase**	✔	✔	✔	1		1.23	*S. elegans*
EU008753	***Gibberella zeae***	**XP_385528.1**	**2E-86**	**81.4**	**MFS hexose transporter**	✔	✔	✔	4	SP-TM5	1.51	*S. elegans*
DW520859	*Gibberella zeae*	XP_383019.1	8E-36	87.5	*O*-methyltransferase		✔		1	SP	1.23	*S. elegans*
Pathogenic processes												
EU008743	*Aspergillus nidulans*	XP_660880.1	1E-09	50.1	Oxidoreductase				8	TM1	3.48	*S. elegans*
EU008755	*Aspergillus nidulans*	XP_664718.1	2E-21	50.4	Ferric-chelate reductase	✔	✔		3	SP-TM3	1.50	*S. elegans*
DW520732	*Coprinopsis cinerea*	EAU80911.1	1E-21	76.2	Ferric reductase		✔		1		1.20	*S. elegans*
DW520713	*Magnaporthe grisea*	XP_366698.1	7E-10	70.7	Ctr copper transporter	✔	✔	✔	1	SP-TM	1.37	*S. elegans*
EU008747	***Gibberella zeae***	**XP_382067.1**	**1E-79**	**66.9**	**Calmodulin (CaM)**	✔	✔	✔	2		1.29	*S. elegans*
DW520712	*Gibberella zeae*	XP_385556.1	1E-49	98.8	Cysteine protease	✔	✔	✔	1		1.27	*S. elegans*
DW520683	***Aspergillus oryzae***	**BAE55555.1**	**4E-09**	**68.4**	**Ankyrin repeat domain protein**		✔		1		1.55	*S. elegans*
DW520694	*Neurospora crassa*	XP_963887.1	2E-12	48.9	Alcohol oxidase		✔		1		1.26	*S. elegans*
DW520684	***Magnaporthe grisea***	**XP_366804.1**	**2E-18**	**60.0**	**Xylanolytic transcriptional activator**		✔		1		1.32	*S. elegans*
Stress response												
DW520723	*Magnaporthe grisea*	XP_363893.1	7E-32	64.8	Glycogen phosphorylase	✔	✔	✔	1		1.24	*S. elegans*
Apoptosis												
DW520735	*Gibberella zeae*	XP_387345.1	5E-83	99.2	Chromosome segregation protein	✔	✔	✔	1		1.22	*S. elegans*
DW520861	*Cryptococcus neoformans*	XP_572523.1	5E-50	86.9	Proteasome subunit alpha type 3	✔	✔	✔	1		1.41	*R. solani*
Transport												
DW520862	*Coprinopsis cinerea*	EAU82748.1	2E-63	87.0	Purine permease	✔	✔	✔	1		1.21	*R. solani*
DW520685	***Gibberella zeae***	**XP_387595.1**	**1E-65**	**55.7**	**YOP1**				1		1.39	*S. elegans*
EU008745	***Gibberella zeae***	**XP_388124.1**	**4E-87**	**66.9**	**Mog**	✔	✔	✔	2		1.79	*S. elegans*
Vitamin metabolism												
EU008744	*Coprinopsis cinerea*	EAU87803.1	9E-32	81.4	Pyridoxal reductase AKR8	✔	✔	✔	11		1.79	*R. solani*
EU008757	*Coprinopsis cinerea*	EAU87803.1	4E-31	85.4	Pyridoxal reductase AKR8	✔	✔	✔	8		1.62	*R. solani*

Table 2 continued

Accession number	Blast organism[a]	Blast accession number	*E*-value[b]	% Coverage[c]	Putative function	CD[d]	ψ-blast[e]	IPS[f]	Number of clones	SP/TM[g]	UT/UD ratio[h]	Organism
Respiration chain												
DW520714	*Ustilago maydis*	XP_758855.1	3E-44	61.2	Cytochrome c	✔	✔	✔	1		1.26	*R. solani*
DW520715	*Ustilago hordei*	ABC42035.1	9E-26	57.5	Cytochrome oxidase subunit 3	✔	✔	✔	1		1.24	*R. solani*
DQ369819	*Coprinopsis cinerea*	EAU92441.1	3E-84	69.1	40S ribosomal protein S9 (S7)	✔	✔	✔	2		1.27	*R. solani*
DW520739	*Hypocrea jecorina*	NP_570150.1	1E-10	42.6	NADH-ubiquinone oxidoreductase chain 3		✔		1	SP-TM	1.21	*S. elegans*
Replication, transcription, and DNA repair												
DW520716	*Gibberella zeae*	XP_386492.1	8E-62	100.0	DNA ligase	✔	✔	✔	1		1.19	*S. elegans*
DW520724	*Neurospora crassa*	XP_959442.1	1E-47	55.4	Histone H2A	✔	✔	✔	1		1.27	*S. elegans*
Translation												
DQ369842	*Magnaporthe grisea*	XP_370398.1	1E-56	82.0	60S ribosomal protein L12	✔	✔	✔	1		1.24	*S. elegans*
Transduction												
DW520710	*Coprinopsis cinerea*	EAU83485.1	3E-03	72.5	MAP kinase	✔	✔	✔	1		1.24	ND[j]
Protein degradation												
DW520873	*Eremothecium gossypii*	FAA00316.1	4E-99	98.9	Polyubiquitin	✔	✔	✔	2		1.33	Both
Ribosomal protein												
DQ369823	*Gibberella zeae*	XP_390909.1	6E-73	56.9	40S ribosomal protein S23	✔	✔	✔	2		1.35	*S. elegans*
DW520885	*Metarhizium anisopliae*	AY094071.1	0E+00	94.2	28S ribosomal RNA gene				1		1.54	Both
DQ369806	***Gibberella zeae***	**XP_390422.1**	**5E-38**	**64.0**	**60S ribosomal protein L10**	✔	✔	✔	2		1.62	*S. elegans*
DQ369801	*Neurospora crassa*	CAB99177.2	4E-20	70.3	Related to ribosomal protein MRP49		✔	✔	1		1.28	*S. elegans*
Lipid metabolism												
DW520718	*Coprinopsis cinerea*	EAU91391.1	2E-23	99.8	Esterase/lipase		✔		1		1.52	*R. solani*
Others												
DW520717	*Aspergillus clavatus*	XP_001273321.1	2E-98	99.7	Dynamin family protein	✔	✔	✔	1		1.24	*S. elegans*
DW520721	*Neurospora crassa*	XP_958320.1	1E-54	58.0	Glucose-6-phosphate 1-dehydrogenase	✔	✔	✔	1		1.21	*S. elegans*
DW520727	*Chaetomium globosum*	XP_001224433.1	3E-27	80.9	Integral membrane protein		✔		1	SP-TM	1.25	*S. elegans*
DW520730	*Coprinopsis cinerea*	EAU91834.1	2E-37	96.8	Histidine acid phosphatase		✔	✔	1		1.69	*R. solani*

Table 2 continued

Accession number	Blast organism[a]	Blast accession number	*E*-value[b]	% Coverage[c]	Putative function	CD[d]	ψ-blast[e]	IPS[f]	Number of clones	SP/TM[g]	UT/UD ratio[h]	Organism
Unclassified												
Mitochondrial RNA												
DQ369844	*Hypocrea jecorina*	AF447590.1	2E-25	39.4	Small subunit ribosomal				11	SP	1.63	*S. elegans*
DQ369809	*Hypocrea jecorina*	AF447590.1	5E-65	69.8	Large subunit ribosomal				4	SP	1.43	*S. elegans*
DQ369834	*Fusarium oxysporum*	AY874423.1	4E-155	56.2	Large subunit ribosomal				3	SP-TM	2.02	*S. elegans*
DQ369841	*Lecanicillium muscarium*	AF487277.1	8E-63	51.4	Large subunit ribosomal				3	SP	1.63	*S. elegans*
DQ369815	*Cordyceps capitata*	AB027340.1	1E-96	80.3	Small subunit ribosomal				16		1.45	*S. elegans*
DQ369800	*Fusarium oxysporum*	AY945289.1	9E-80	93.4	Small subunit ribosomal				3		3.81	*S. elegans*
Hypothetical proteins												
DW520725	*Chaetomium globosum*	XP_001224408.1	1E-18	79.1	Hypothetical protein				1		1.26	*S. elegans*
DW520726	*Aspergillus fumigatus*	XP_753167.1	3E-16	81.7	Hypothetical protein				1		1.22	ND
DW520860	*Chaetomium globosum*	XP_001219459.1	1E-31	76.8	Predicted protein				1		1.22	*S. elegans*
EU008746	*Gibberella zeae*	XP_386306.1	3E-63	77.1	Hypothetical protein				2	SP	1.31	*S. elegans*
EU008756	***Pyrenophora teres***	**AM269883.2**	**8E-36**	**12.0**	**Hypothetical protein**				3		1.54	*S. elegans*
No significant homology (no match)												
DQ369839	No match								2		1.56	*S. elegans*
DQ369848	No match								5	SP-TM	1.59	*S. elegans*
DW520692	**No match**								1		1.30	*S. elegans*
EU008754	No match								1	SP	1.29	*S. elegans*
DW520729	No match								2	SP	1.32	*S. elegans*
DW520731	No match								2	SP-TM	2.88	*S. elegans*
DW520733	No match								1		1.22	*R. solani*
EU008748	No match								2		1.24	*S. elegans*
DW520746	No match								1		1.22	*S. elegans*
EU008741	No match								1	SP-TM2	1.43	*S. elegans*
DW520756	No match								1	SP-TM	1.52	*R. solani*
DW520776	No match								1	SP	1.20	*S. elegans*
EU008752	No match								1	TM1	1.29	*S. elegans*
EU008739	No match								1	SP	1.33	*S. elegans*
DW520786	No match								1		1.28	ND
DW520790	No match								2	SP	1.30	*S. elegans*
DW520793	No match								1	SP	1.20	*S. elegans*
DW520795	No match								1		1.22	*S. elegans*
DW520797	No match								1	SP	1.23	*S. elegans*

Table 2 continued

Accession number	Blast organism[a]	Blast accession number	*E*-value[b]	% Coverage[c]	Putative function	CD[d]	ψ-blast[e]	IPS[f]	Number of clones	SP/TM[g]	UT/UD ratio[h]	Organism
DW520799	No match								1	SP	1.22	*S. elegans*
DW520800	No match								1		1.60	*R. solani*
DW520804	No match								1	SP	1.28	*S. elegans*
EU008750	No match								1	SP-TM1	1.23	*R. solani*
DW520811	No match								1		1.21	ND
DW520812	No match								1		1.30	ND
DW520813	No match								1		1.29	*R. solani*
DW520814	No match								1		1.21	ND
DW520820	No match								1	TM	1.34	*S. elegans*
DW520822	No match								1		1.23	ND
DW520827	No match								1		1.21	*S. elegans*
DW520832	No match								1	SP	1.30	*S. elegans*
DW520835	No match								11	SP	1.91	*S. elegans*
DW520837	No match								1	SP-TM	1.38	*S. elegans*
DW520839	No match								1		1.20	*S. elegans*
DW520844	No match								1		1.23	*R. solani*
DW520855	No match								1		1.26	*S. elegans*
DW520857	No match								2		1.46	*S. elegans*
EU0088751	No match								1		1.52	ND
DW520879	**No match**								6		1.41	*S. elegans*
EU008742	**No match**								1		1.63	*S. elegans*
DW520884	No match								50		2.97	*S. elegans*
DW520886	No match								22		1.83	*S. elegans*
EU008740	No match								1	TM1	1.37	*S. elegans*
DW520889	No match								1	SP	1.37	*S. elegans*

[a] Highest scoring blast hit

[b] *E*-value threshold lower than 10^{-20}, or between 10^{-6} and 10^{-20} when % > 50%, or higher than 10^{-6} when % > 50% and a conserved domain was present

[c] Percentage coverage of the query by the blast match

[d] Conserved domain determined by ψ-blast

[e] *E*-value threshold lower than 10^{-20}

[f] Motif and domain search by InterPro Scan

[g] SP, signal peptide; TM, transmembrane region(s) and number of transmembrane domains based on the consensus among Phobius, TMPred and DAS programs

[h] UT/UD = antilog (ER1 – ER2) in the base 2. Calculation was estimated based on the equation developed by van der Berg et al. (2004)

[i] Accession numbers highlighted in bold represent genes whose expression was quantified using real-time QRT-PCR

[j] Not detected

amplicon generated using the different gene-specific primer pairs (Table 1). Data generated by QRT-PCR were analyzed using Stratagene analysis software. Data from technical replicates were averaged before normalization. QRT-PCR data were calculated as a normalized relative expression of gene using the equations developed by Pfaffl (2001) and Liu and Saint (2002), based on crossing point (CP) and efficiency obtained for the HKG histone-4 and the different target gene amplifications. The relative expression of each target gene was tested for significance between treatments by an analysis of variance (ANOVA) using the software SAS (SAS Institute Inc., Cary, NC, USA, version 8.2). Comparison between means at each time point was made using least significant differences (LSD) at $P < 0.05$.

Results

Creation of a subtracted cDNA library and differential screening

An SSH library was constructed with RNA isolated at several sequential time points during the mycoparasitic interaction between *S. elegans* and its host *R. solani*. These time points were chosen to cover all stages of mycoparasitism and investigate expressed genes both before and during the interaction. In order to restrict analysis to the mycoparasitic process and defense reaction of the host, subtraction was performed between pooled mycoparasitic interaction cDNAs (tester) and pooled cDNAs of each of the interacting partners alone (driver) (Fig. 1). The efficiency of the SSH was confirmed by the successful removal (i.e., reduction in the levels) of histone-4 in the subtracted tester (ST). Histone-4 was amplified 5 PCR cycles later (corresponding to 20-fold cDNA enrichment) in the subtracted library (data not shown).

A total of 2,166 clones, containing single inserts that ranged from 200 to 1,300 bp, were spotted in triplicate on microarray glass slides and screened with combinations of SSH generated probes. An examination of the UT/UD ratios indicated that 1,016 clones out of the 2,166 (47%) may be upregulated in the tester compared to the driver (ratio >1), while the rest of the clones (1,150, 53%) had escaped subtraction (ratio <1), being as abundant in the driver as in the tester (data not shown).

Sequence analysis and annotation of expressed genes

Among the 1,016 clones potentially representing differential gene expression, 256 showing a UT/UD ratio higher than 1.20, and five additional clones having a UT/UD ratio of 1.20 or lower were picked randomly giving a total of

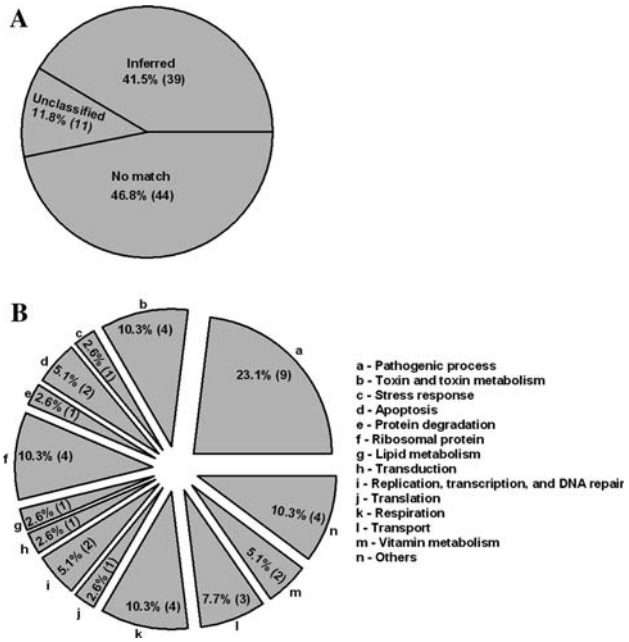


Fig. 2 Pie charts showing functional distribution of *S. elegans* and *R. solani* putative up-regulated genes. (**a**) 94 genes were categorized according to their putative function. (**b**) Putative functional classes for 39 genes. Percentages were calculated from the 94 potentially upregulated gene total (*a*) and from the 39 sequences with functional classes (*b*). *Note*: "no match" represents sequences that are not similar to any protein in the database, while "unclassified" represents sequences whose matching genes do not have a clear function. Numbers in brackets represent the number of genes

261 sequences. The sequences were analyzed using the program CAP3 in order to identify redundant clones. A total of 94 unique genes were identified of which 63 appeared only once, and 31 genes were represented by multiple clones at frequencies ranging from 2 to 50 (Table 2).

PCR analysis using specific primers showed that out of 94 unique sequences, 73.4% (69 ESTs) belong to the mycoparasite *S. elegans*, 16.0% (15 ESTs) belong to *R. solani*, 2.1% (2 ESTs) were amplified in both organisms, while 8.5% (8 ESTs) were not amplified (data not shown).

Thirty-nine sequences (41.5%) were placed in putative functional classes based on Blast similarity as described (Fig. 2; Table 2). Genes related to pathogenic processes formed the largest category corresponding to 23% of total unique sequences, followed by those belonging to toxin metabolism, respiration, ribosomal proteins, and others (Table 2, Fig. 2b). InterProScan and PSI-Blast analyses support the protein domain identity of 37 out of 39 inferred putative genes. No domains were assigned to the unclassified genes (Table 2); however, several of them have a signal peptide and transmembrane regions and so may represent secreted or membrane bound proteins (Fig. 2a; Table 2). The sequences with no match were considered as novel genes.

Table 3 Relative expression ratios of 12 genes using quantitative RT-PCR

Putative function	Accession number	Time point[a] (days)	UT/UD ratio[b]	QRT-PCR ratio[c]
Cytochrome P450	DW520689	2	1.23	5.1
		4		3.9
		6		12.6*
		12		6.2*
Calmodulin	EU008747	4	1.29	5.6*
		6		2.3
No match	DW520692	2	1.30	5.3
		4		2.0
		8		3.2
		12		19.0*
Transcription factor	DW520684	Pre-contact	1.32	2.9*
Yop	DW5200685	0	1.39	3.8
		4		2.6
MFS hexose transporter	EU008753	4	1.51	8.3*
		6		3.8*
Hypothetical protein	EU008756	0	1.54	3.5
Ankyrin repeat protein	DW520683	6	1.55	2.0
Ribosomal protein L10	DQ369806	0	1.62	2.0
		2		2.4
		4		3.2
No match	EU008742	0	1.63	4.7*
		6		5.1*
MOG	EU008745	2	1.79	3.0*
		4		2.6
		6		7.8*
No match	DW520879	12	1.41	2.7

[a] Days after interaction between *S. elegans* and *R. solani*. Day 0: day of contact; pre-contact: mycelium of the two fungi was distanced by 0.5 cm

[b] UT/UD = antilog (ER1 – ER2) in the base 2. Calculation was estimated based on the equation developed by van den Berg et al. (2004)

[c] Real-time QRT-PCR values were normalized against histone 4 and the relative expression ratios were calculated based on formulae developed by Pfaffl (2001). The ratio represents interaction of *S. elegans* with *R. solani* versus *S. elegans* alone. Ratios with values higher than 2.0 are shown. Asterisk (*) denotes target genes whose expression was significantly up-regulated during interaction ($P < 0.05$). Values represent the average of two technical and three biological replicates

Validation and QRT-PCR expression analysis of selected clones

To validate the potential list of candidate genes drawn from the array results and to confirm that these genes were differentially expressed at some time point either prior to or during the mycoparasitic interaction, QRT-PCR was performed for 12 selected genes on biological samples at different time points of the interaction. Compared to *S. elegans* alone (control), the relative expression of 7 out of 12 genes were significantly upregulated ($P < 0.05$) for at least one time point during *S. elegans–R. solani* interaction (Table 3).

Discussion

EST analysis reported in this study provides an efficient means of gene discovery in the mycoparasite *S. elegans* for which molecular and genetic information is not available. To our knowledge, this is the first study of gene analysis for a mycoparasite during direct interaction with its host over an extended period of mycoparasitism. Consistent with the findings of other fungal EST studies (Keon et al. 2005; Liu and Yang 2005), in the current study, nearly 46.8% of the translated cDNA sequences showed no similarity to known protein sequences, reflecting the lack of genetic information on fungi generally and more specifically on mycoparasites. It is therefore likely that some of the sequences represent newly discovered genes. However, it cannot be completely discounted that part of these EST sequences belongs to 5′ and 3′ untranslated regions. Overall the number of novel sequences found here suggests that mycoparasitism in *S. elegans* is likely to involve many more genes than so far described in this study.

In agreement with recent genomic approach-based studies on *Trichoderma harzianum* (Liu and Yang 2005) and *T. hamatum* (Carpenter et al. 2005), we identified several sequences (10.3%) corresponding to homologues of proteins required for synthesis and regulation of toxins (Table 2) such as aflatoxins and trichothecenes in several fungi. Cytochrome P450-type monooxygenase together with the MFS (major facilitator superfamily) hexose transporter, and the *O*-methyltransferase B are known to be involved in aflatoxin biosynthesis pathway in *Aspergillus parasiticus* and *Aspergillus flavus* (Bhatnagar et al. 2003;

Ehrlich et al. 1999). In the presence of *R. solani*, both cytochrome P450 and MFS hexose transporter encoding genes were significantly upregulated in *S. elegans* compared to when the mycoparasite was alone. However, whether *S. elegans* can produce mycotoxins remains to be determined.

Several upregulated genes were predicted to encode a variety of functions involved in pathogenic processes, including those required for pigment synthesis and melanization processes. The gene identified as (Ctr) copper transporter belongs to the high affinity SLC31 family that mediates copper uptake (Petris 2004). This family was shown to be involved in melanin synthesis and pathogenesis in fungi (Rees and Thiele 2004). Two different genes encoding ferric-chelate reductases are known to mediate iron uptake and acquisition during pathogen growth by limiting this ion to their hosts (Zarnowski and Woods 2005). This enzyme could be involved in providing nutrition for *S. elegans* from host cell components. Another gene identified as calmodulin is required for appressorial development in several plant pathogenic fungi such as *Colletotrichum trifolii* and *Magnoporthe grisea* (Warwar et al. 2000; Liu and Kolattukudy 1999). The mycoparasitic interaction between *S. elegans* and *R. solani* involves morphogenetic processes that result in the formation of specific structures including hyphal coils, and appressoria or penetration pegs after 3 days of hyphal interaction (Benyagoub et al. 1994). Thus, it is not surprising that the calmodulin gene was preferentially expressed 4 days after contact.

Nearly 8% of the genes were predicted to encode membrane proteins involved in intracellular transport processes of eukaryotic cells (Baker et al. 2001; Calero et al. 2001). These include genes homologous to Yop-1 and Mog1p proteins in *Gibberella zeae*. In yeasts, *mog1* encodes MOG1 protein that is essential for bidirectional nuclear protein import and export and membrane traffic (Baker et al. 2001). The overproduction of the membrane protein Yop-1, whose function in intracellular transport is similar to MOG1, negatively regulates cell growth leading to accumulation of internal cell membranes and a blockage in membrane traffic (Calero et al. 2001). During the interaction of *S. elegans* with an actively growing *R. solani* culture, conidial germination was substantially delayed by more than 30 h compared to that occurring in the absence of a host (Morissette et al. 2006). In this study, the upregulation of *Mog* could be an indicator of cell growth regulation in *S. elegans* at different stages of the interaction with *R. solani*, but further research is needed.

There is convincing evidence that ribosomal proteins have extraribosomal functions in eukaryotic cells (Wool 1996). Gene expression in prostate-cancer cell lines using SSH showed that several cDNAs encoding ribosomal proteins were preferentially upregulated, suggesting that these proteins play a role in pathogenicity (Vaarala et al. 1998). In our study, the preferential upregulation of different genes homologous to ribosomal proteins suggests that these genes may contribute to the mycoparasitic ability of *S. elegans*. Similarly in *T. hamatum* transcript levels of RPL36, gene encoding ribosomal proteins were substantially increased during the mycoparasitic interaction with *Botrytis cinerea* (Fekete et al. 2001). Furthermore, as a component of the genetic machinery that regulates the synthesis of ribosomal constituents, the increase in expression of these genes during mycoparasitism may be due to an increase in the synthesis of mycoparasitism-induced proteins. Whether they play a similar role during mycoparasitism needs to be investigated.

Interestingly, none of the queried sequences in this study were found to match those encoding cell wall-degrading enzymes (CWDEs). This finding greatly differs to what had been reported on EST and proteomic analyses of *Trichoderma* species (Grinyer et al. 2005; Liu and Yang 2005; Vizcaino et al. 2006). This could be due to the different growth conditions used. Our genomic-based strategy (SSH and differential screening) used construction of a subtracted cDNA library under conditions in which both the mycoparasite and the host were in direct contact with each other over an extended period of mycoparasitism. This finding differs greatly from *Trichoderma* cDNA libraries in which the mycoparasite was grown in the absence of a host (Liu and Yang 2005), on a carbon source or simulated mycoparasitism (Grinyer et al. 2005; Vizcaino et al. 2006) or in the presence of a host except that both partners were separated by a cellophane membrane (Vizcaino et al. 2006).

Notably, two genes matched pyridoxal reductase ARK8 from *Coprinopsis cinerea* that is involved in the production of vitamin B6, an essential element to the growth of several fungi (Morita et al. 2004). The presence of AKR8 could be either a response of *R. solani* in order to resist attack by *S. elegans*, or a response to elicitors produced by *S. elegans* to increase the production of vitamin B6 by its host.

Our results provide a first step toward the understanding of the mycoparasitic process of *S. elegans* during its interaction with *R. solani*. The exact roles of the genes identified in this study are still to be determined; however, sequence similarities with known genes had provided clues regarding some of the genes that were differentially upregulated during mycoparasitism. Seven genes, out of which two are novel, showed significant upregulation of their expression during interaction, suggesting that genes other than those encoding CWDEs could be a target to improve mycoparasitic activity and plant resistance. These genes present a broad range of functions reflecting the complexity of the genetic regulation during this process.

For the first time, putative defense-related genes from the host *R. solani* were isolated.

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