Journal of Microbiology (2013) Vol. 51, No. 2, pp. 174–182
DOI 10.1007/s12275-013-2545-7

Cloning, Annotation and Expression Analysis of Mycoparasitism-Related Genes in *Trichoderma harzianum 88*[§]

Lin Yao[1,2], Qian Yang[1*], Jinzhu Song[1], Chong Tan[2], Changhong Guo[3], Li Wang[2,3], Lianhai Qu[1], and Yun Wang[1]

[1]*Department of Life Science and Engineering, Institute of Technology, Harbin 150001, P. R. China*
[2]*School of Municipal and Environmental Engineering, Harbin Institute of Technology, Harbin 150090, P. R. China*
[3]*Key Laboratory of Molecular and Cytogenetics, Heilongjiang Province, Department of Biology, Harbin Normal University, Harbin 150025, P. R. China*

(Received October 8, 2012 / Accepted November 22, 2012)

***Trichoderma harzianum 88*, a filamentous soil fungus, is an effective biocontrol agent against several plant pathogens. High-throughput sequencing was used here to study the mycoparasitism mechanisms of *T. harzianum 88*. Plate confrontation tests of *T. harzianum 88* against plant pathogens were conducted, and a cDNA library was constructed from *T. harzianum 88* mycelia in the presence of plant pathogen cell walls. Randomly selected transcripts from the cDNA library were compared with eukaryotic plant and fungal genomes. Of the 1,386 transcripts sequenced, the most abundant Gene Ontology (GO) classification group was "physiological process". Differential expression of 19 genes was confirmed by real-time RT-PCR at different mycoparasitism stages against plant pathogens. Gene expression analysis revealed the transcription of various genes involved in mycoparasitism of *T. harzianum 88*. Our study provides helpful insights into the mechanisms of *T. harzianum 88*-plant pathogen interactions.**

Keywords: *Trichoderma harzianum 88*, cDNA library, expressed sequence tags, plant pathogens, real-time RT-PCR, gene ontology

Introduction

Trichoderma species are opportunistic, avirulent plant symbionts, which also can be parasites of other fungi (Druzhinina *et al*., 2011). Among them, *Trichoderma harzianum* has been used as an effective biocontrol agent to antagonise phytopathogens by different mechanisms, such as competition for environmental resources of nutrients and light (Restuccia *et al*., 2006), recognising signals from the host fungus to induce transcription of mycoparasitism-related genes (Lin *et al*., 2012), enzyme production and secretion (Almeida *et al*., 2007; Yang *et al*., 2009), as well as inducing systemic resistance in plants (Alizadeh *et al*., 2011; Bae *et al*., 2011; Morán-Diez *et al*., 2012) and other as yet undefined mechanisms (Yang *et al*., 2012).

The reports above suggest that *T. harzianum* strains are excellent biocontrol agents against plant pathogenic fungi, and their molecular biocontrol mechanisms deserve further study. Expressed sequence tags (ESTs) have been generated from the transcriptome of *Trichoderma* spp. under various mycoparasitic and simulated nutrient stress conditions. This strategy is an efficient and economical approach for large-scale gene discovery, exploration of gene regulatory patterns and identification of differentially regulated genes (Adams *et al*., 1991; Bouck and Vision, 2007; Zhang *et al*., 2009; Reithner *et al*., 2011). Liu and Yang (2005) first identified genes from *T. harzianum 88* involved in protein processing and secretion. A total of 3,298 ESTs from cDNA libraries (T88-hzm) were sequenced, corresponding to 1,740 unique transcripts. The results provided a preliminary indication of gene expression in mycelium. Vizcaíno *et al.* (2006) later constructed several cDNA libraries of *T. harzianum* CECT 2413 (T34) under different induction conditions and obtained a collection of 8,710 ESTs (3,478 unique sequences). Many transcripts encoding proteins associated with *Trichoderma*-host (fungus or plant) interactions were obtained with *T. harzianum* strains. A comparison of the ESTs from the T88-hzm and T34 libraries via the Blast program revealed an overlap of 21.6% similar sequences between them (Vizcaíno *et al*., 2006). It is unclear why the similarity was found to be low between these two cDNA libraries. Liu *et al.* (2010) also found that only 31.2% of unique ESTs from the *Trichoderma asperellum* T4 library were present in the *T. asperellum* T53 library (Vizcaíno *et al*., 2007) under different culture conditions. However, the gene expression of *T. harzianum 88* in the presence of cell walls from plant pathogens is still lacking. Therefore, more information on *T. harzianum 88* is needed to more accurately characterise its functional processes at the genetic level.

In the present study, the ability of *T. harzianum 88* to inhibit the infectivity of three pathogens, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Fusarium oxysporum*, was investigated. A cDNA library from mycelium of *T. harzianum 88* in the presence of cell walls from the three plant pathogens was constructed to obtain a more comprehensive understanding of its mycoparasitic mechanisms. Randomly selected transcripts were sequenced from the library, and expression analysis by real-time RT-PCR was performed to

*For correspondence. E-mail: qianyangcn@126.com; Tel.: +86-451-8640 2652; Fax: +86-451-86402952
[§]Supplemental material for this article may be found at http://www.springer.com/content/120956.

identify changes in gene expression patterns during mycoparasitic interactions with the distinct plant-pathogenic fungi. Our study contributes useful genetic information on a biocontrol agent and provides a foundation for further studies into the mycoparasitic mechanisms of *T. harzianum 88* at the molecular level.

Materials and Methods

Fungal strains

The *T. harzianum* strain 88 (CGMCC 5403; GenBank No. of ITS: JN579713) and the three strains of fungal plant pathogens, *R. solani* (ACCC 36246), *S. sclerotiorum* (ACCC 36462), and *F. oxysporum* (ACCC 36966), were stored at the Microbial Genetic Engineering Laboratory, Harbin Institute of Technology (China).

Direct confrontation assays

Plate confrontation assays were carried out on potato glucose agar (PGA) plates. Mycelial plugs (0.5-cm diameter) of *T. harzianum* and the corresponding confrontation partner (*R. solani*, *S. sclerotiorum* or *F. oxysporum*; 0.5-cm diameter) were placed onto the agar plates, overlaid with cellophane and inoculated at a distance of 3 cm in total darkness at 28°C. Control plates, covered with cellophane, were inoculated with each of the three plant pathogens. The overgrowth of *T. harzianum 88* mycelia was observed after coming into contact with *R. solani*, *S. sclerotiorum* or *F. oxysporum* (Cortés *et al*., 1998).

Construction of a mycelium cDNA library (T88-wmaa)

T. harzianum 88 was primarily cultured on potato glucose (PG) medium for 24 h at 28°C and 150 rpm^{-1}. Thereafter, the biomass was harvested, rinsed twice with sterile distilled water, transferred into minimal medium [2.8 g/L $(NH_4)_2SO_4$, 0.6 g/L Urea, 0.0032 g/L $CoC_{l2}{\cdot}6H_2O$, 0.6 g/L $CaC_{l2}{\cdot}2H_2O$, 4 g/L KH_2PO_4, 0.0028 g/L $ZnSO_4{\cdot}H_2O$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 0.2 g/L $MgSO_4$] with 15 g/L of cell wall extract from *R. solani*, *S. sclerotiorum*, and *F. oxysporum* (1:1:1) and incubated for a further 36 h at 28°C and 150 rpm^{-1}. Treatment with cell walls of *R. solani*, *S. sclerotiorum*, and *F. oxysporum* (1:1:1) were performed according to the method of Cortés *et al.* (1998). Total RNA was extracted using Trizol reagent (Invitrogen, USA) from 0.5 g *T. harzianum 88* mycelium, and mRNA was isolated using oligo (dT)-cellulose column chromatography and the Oligotex mRNA Kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA library was constructed as previously reported (Liu and Yang, 2005), and clones were sequenced with a T3 primer from the 5′ end using a MegaBase1000 DNA sequencer (Amersham Biosciences, UK).

EST analyses of the *T. harzianum 88* library

Raw sequence files were imported to the Unix server and transformed into the FASTA format. The sequences were assembled using Phred-phrap-consed (Ewing and Green, 1998; Gordon, 2003) with default parameters after removal of vector and low-quality (< 100 bp) sequences containing more than 5% ambiguous bases. All unigenes (consensus sequences of assembled clusters, including contigs and singlets) were compared against nucleic acid sequences from the genomes of *Trichoderma reesei*, *Trichoderma virens*, and *Trichoderma atroviride* obtained from the Joint Genome Institute and the NCBI non-redundant (nt/nr) database, as well as with other collections of ESTs from *T. harzianum* via Blastn/Blastx algorithms (Altschul *et al*., 1990) with an E-value < 10^{-10} indicating significant sequence similarity.

Annotations were based on the Gene Ontology (GO) terms (Camon *et al*., 2005). The unigene set of EST contigs and singlets were annotated using the program Blast2GO (Götz *et al*., 2008) with the E-value <10^{-5} significance level. Blast2GO assigns the GO terms based on the BLAST definitions. The T88-wmaa and T88-hzm cDNA libraries were further analysed by comparing the GO classifications of each transcript between these two libraries. Signal peptide cleavage sites for unique sequences were predicted using neural network modules of SignalP 4.0 (Petersen *et al*., 2011).

Differential expression of mycoparasitism-related genes

Real-time RT-PCR was used to evaluate the relative changes in gene expression from *T. harzianum 88* during confrontation with the fungal pathogens *R. solani*, *S. sclerotiorum* or *F. oxysporum*. Plate confrontations of *T. harzianum 88* against *R. solani*, *S. sclerotiorum* or *F. oxysporum* were proposed as described by Carsolio *et al.* (1994). Agar plus cut form-growing colonies of each fungi were placed, on opposite sides, in 9-cm plates containing MM with 2% agar plus 2% glucose and covered with sterile cellophane sheets. *T. harzianum 88* mycelia were collected when they were touching at 3, 6, 12, 24, and 36 h; this was repeated three times. At these stages, only *Trichoderma* mycelia 0.5 cm away from the original inoculums and without spores were collected. Control plates were inoculated with *T. harzianum 88*, which was used to challenge itself, and *T. harzianum 88* mycelia were collected when they touched each other (3, 6, 12, 24, and 36 h). The fungi were allowed to grow at 28°C in the dark. Nineteen pairs of primers were selected for real-time RT-PCR analysis (Supplementary data Table S1).

Total RNA was extracted from the mycelium and digested with DNase I (TaRaKa, Japan) (Steiger *et al*., 2010). Total RNA (4 μg) from each pooled sample was reverse transcribed into cDNA in the presence of oligo (dT) in a total volume of 25 μl. The synthesised cDNA was diluted with 25 μl of RNase-free ddH_2O and used as the template for real-time RT-PCR. For avoiding the amplification of host fragments, three pairs of primers were designed according to ITS DNA from *R. solani*, *S. sclerotiorum* or *F. oxysporum*, respectively, and no fragments were amplified from cDNA templates. Beta tubulin (DY762540) transcripts were used as endogenous references for real-time RT-PCR. The primer set for beta tubulin was designed (Supplementary data Table S1), and real-time RT-PCR reactions were performed using the SYBR® Premix Ex TaqTMII (TaRaKa) on an ABI Model 7500 HT sequence detector (ABI, Harbin Institute of Technology, China). Reagent mixes were prepared by combining 12.5 ml of SYBR® Premix Ex Taq™ II (2×) with 2.5 μl cDNA, 2 μl of a mixture of forward and reverse primers and 8 μl dis-

tilled water per reaction. The 25-µl reactions were carried out with programmed thermal cycling conditions consisting of 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 40 sec at the appropriate annealing temperature for each real-time RT-PCR primer set. Three technical replicates of each of three biological replicates were used for calibrator normalised relative quantification analysis. Data from the threshold cycles were analysed according to the 2 (ΔΔ C (T)) methods (Livak and Schmittgen, 2001) in *PKS* gene expression.

Analysis of the student t-test was used to determine whether *pksT* genes had a statistically significant effect on the survival of *T. harzianum 88*, which was confrontation with three fungal plant pathogens and non-confrontation with three fungal plant pathogens. The significance level of the student t-test was set at $P<0.05$, and if $P<0.01$, it was noted.

Results

Direct confrontation assays

T. harzianum 88 grew considerably faster on PGA than did the *Fusarium* pathogen under the same conditions. When grown in the same plates, wild-type *T. harzianum 88* produced a zone of lysis and sporulated in the *R. solani*, *S. sclerotiorum* or *F. oxysporum* mycelia (Fig. 1). It has been demonstrated that the *in vitro* confrontation assay is a useful and reliable method for identifying the mycoparasitism potential of *Trichoderma* species (Hermosa *et al.*, 2000). In dual cultures, the first contact between *T. harzianum 88* and *R. solani*, *S. sclerotiorum* or *F. oxysporum* occurred after 24-, 26.5- or 26-h inoculations, respectively. After 96 h, *T. harzianum 88* completely overgrew the host and sporulated. There was a visible interaction zone between *R. solani* and *T. harzianum 88* at 96 h (Fig. 1A), while *S. sclerotiorum* (Fig. 1C) and *F. oxysporum* (Fig. 1B) were fully covered with mycelia and spores from *T. harzianum 88* at 96 h.

EST sequence determination and analysis

A directional cDNA library (T88-wmaa) from mycelia of *T. harzianum 88* was constructed. The primary titre of the cDNA library was approximately 1.2×10^6 pfu/ml with an insertion efficiency of 91%. After removing the vector sequence, poor quality sequences and sequences shorter than 100 bases, the final number of ESTs was 1,386 with an average length of 448 bp. These ESTs were submitted to GenBank under the accession numbers of DY761370-DY762921 as shown in Supplementary data Table S2. The 1,386 ESTs were clustered into 181 multi-sequence contigs (Supplementary data Table S3), with 734 singlets yielding an estimate of 915 unique sequences. Of the 181 contigs, 94.4% (171 contigs) consisted of only two or three ESTs and 0.52% (1 contig) contained 15 ESTs (Supplementary data Table S4). The high percentage of low-redundancy sequences reflected the complexity of the cDNA library and indicated a relatively good representation of the library.

The BLASTn/ BLASTx algorithm (Altschul *et al.*, 1990) was used to identify ESTs with sequence similarities to the nucleic acid sequences obtained from the genomic sequence datasets of *T. reesei*, *T. virens*, and *T. atroviride* (Kubicek *et al.*, 2011) or those from the NCBI nt/nr databases. An E-value of $<10^{-10}$ was considered indicative of significant sequence similarity (Supplementary data Table S5). In these comparisons, it is important to note that the *T. harzianum* sequences did not represent the complete genome.

Comparison with *T. harzianum* collections of ESTs

The BLASTn algorithm (Altschul *et al.*, 1990) was used to determine the presence of similar sequences in the publicly available collections of *T. harzianum* ESTs. At the E-value $<10^{-10}$ level, a higher percentage of similarity at 56.8% was found in the *T. harzianum* T34 library related to biocontrol, while 27.76% of similar sequences were found from the T88-hzm library (Vizcaíno *et al.*, 2006).

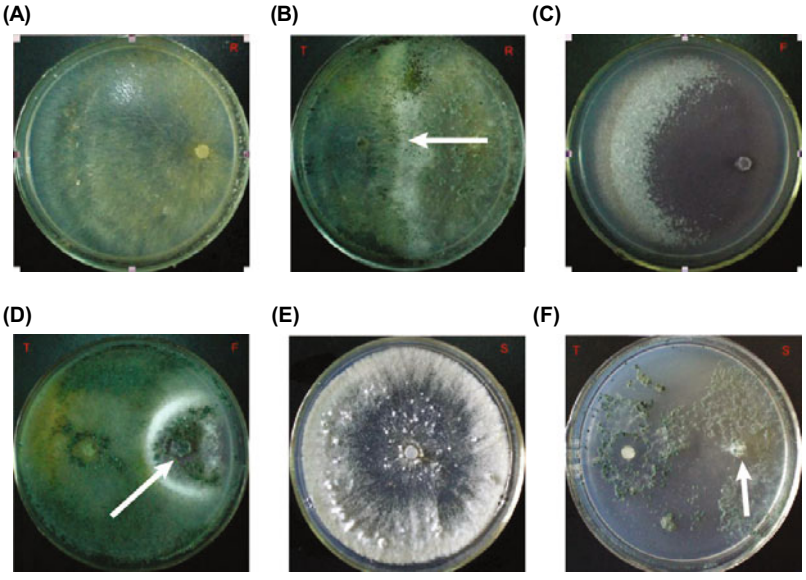

Fig. 1. Plate confrontation test of *T. harzianum 88* against three phytopathogens on PDA medium. (A) *R. solani* control. (C) *F. oxysporum* control. (E) *S. sclerotiorum* control. (B) Interaction zone between *R. solani* and *T. harzianum 88*. (D) Sporulation by *T. harzianum 88* on *F. oxysporum* (purple colony). (F) Sporulation by *T. harzianum 88* on *S. sclerotiorum* (white colony). Arrows indicate the mycoparasitic activity towards the plant pathogen.

Table 1. GO functional assignments for the T88-wmaa and T88-hzm libraries

GO term	GO ID	T88-wmaa	T88-hzm
Cellular component (10)			
Cell	GO:0005576	9 (0.9%)	431 (20.1%)
Extracellular	GO:0005623	1 (0.1%)	91 (4.2%)
Molecular function (149)			
Catalytic activity	GO:0003824	62 (41.6%)	286 (13.3%)
Enzyme regulator activity	GO:0030234	9 (6%)	6 (0.3%)
Transporter activity	GO:0005215	19 (12.8%)	104 (4.9%)
Transcription regulator activity	GO:0030528	2 (1.3%)	23 (1.1%)
Translation regulator activity	GO:0045182	3 (2%)	23 (1.1%)
Structural molecule activity	GO:0005198	7 (4.7%)	51 (2.4%)
Binding	GO:0005488	43 (28.85%)	148 (6.9%)
Signal transducer activity	GO:0004871	6 (4.02%)	27 (1.3%)
Biological process (238)			
Physiological process	GO:0007582	179 (75.2%)	422 (19.6%)
Regulation of biological process	GO:0050789	14 (5.9%)	0
Development	GO:0032502	4 (1.7%)	83 (3.9%)
Cellular process	GO:0009987	38 (16%)	235 (11%)
Cellular component organization or biogenesis	GO:0071840	1 (0.4%)	0
Behavior	GO:0007610	2 (0.84%)	0

Functional annotation and analysis

The unigene distribution was analysed using the Blast2GO program (Götz *et al.*, 2008), yielding percentages of unigene sequences with assigned GO terms falling into each of the main GO categories. For this purpose, 100% represented the total number of unigene sequences from each of the libraries that possessed an assigned GO term in each of the three GO organising principles of cell component, molecular function and biological process (see Table 1, GO functional assignments for the T88-wmaa and T88-hzm libraries). However, it should be noted that the individual percentages do not add up to 100% because many deduced proteins can have more than one assigned GO function (Vizcaíno *et al.*, 2006). Unigene sequences from the T88-wmaa library exhibited similarity to a broad range of genes, including those encoding proteins associated with ubiquitous metabolic pathways, structural proteins and components of transcriptional and translation machinery. There were 10 unigene sequences associated with a cell component, and 149 unigenes were involved in molecular functions, including 62 unigenes associated with catalytic activity and 43 unigenes encoding binding proteins and the remaining genes encoding proteins involved in transporter activity, enzyme regulator activity, structural molecule activity and signal transducer activity. Therefore, unigenes with annotation functions were associated with physiological, developmental and cellular processes.

Expression analysis of mycoparasitism-related genes

To verify the expression profiles of mycoparasitism-associated genes obtained by *in silico* EST data analysis, quantitative real-time RT-PCR (RT-qPCR) was performed for 19 selected genes. Nineteen genes associated with mycoparasitic processes were selected and are shown in the Supplementary data Table S6.

We focussed the validation mainly on the mycoparasitism-associated genes because they represent primary candidates, which reflected an integrated mycoparasitic mechanism operating during mycelium genes. All genes were selected so that (1) different expression patterns and/or (2) different functional pathways were represented.

Quantitative RT-PCR analyses to determine nineteen genes and beta-tubulin mRNA levels were performed using treated and untreated *T. harzianum 88* mRNA extracts and the 2 (ΔΔ C (T)) methods. The data showed normalised relative gene expression at the different stages of the growth of *T. harzianum 88* (Table 2). PCR efficiency (E) of the 19 genes based on qRT-PCR is shown in Table 3. Differences in gene expression levels were compared in *T. harzianum 88* with and without confrontation by three fungal plant pathogens and without confrontation by fungal plant pathogens. The time course of the expression of 15 of the 19 mycoparasitism-related genes responding to confrontation against *S. sclerotiorum*, *R. solani* or *F. oxysporum* are shown in Fig. 2. They are the statistically significant ($P<0.05$). The remaining four of the 19 genes were not induced by plant pathogens, as shown in Table 2.

As illustrated in Figs. 2A, 2B, and 2C, a subset of six cell wall hydrolase genes among the 19 mycoparasitism-associated genes tested were exclusively expressed at different induced times, indicative of their roles in fungus cell wall degradation. Among them, two genes namely *DY761788* and *CK909606* encoding chitinases showed maximum expression induced by *S. sclerotiorum* at 1.18-fold (Fig. 2A) and 1.72-fold induced by *F. oxysporum* (Fig. 2C), respectively. In addition, two genes (*DY761493*, *DY762862*) coding for glucanases were induced approximately 3-fold by plant pathogens. Another gene (*CK907860*) encoding β-1, 4-glucosidase also showed a detectable, statistically significant ($P<0.05$) increase in expression with the maximum level of 1.126-fold induced by *R. solani* (Fig. 2B). However, a gene (*DY761933*) encoding 1, 3-β-glucan synthase was not induced by confrontation with

Table 2. Expression profiles of the 19 genes based on qRT-PCR

GenBank accession no.	mRNA expression (fold induction relative to β tubulin normalized)					
	Induced by *S. sclerotiorum*		Induce by *R. solani*		Induced by *F. oxysporum*	
	$2^{-\Delta\Delta Ct}$± SE[a]	*P* value	$2^{-\Delta\Delta Ct}$±SE	*P* value	$2^{-\Delta\Delta Ct}$±SE	*P* value
DY761788	1.068048±0.16735	0.008974646	0.399696±0.001016	0.0637	0.707306±0.0694519	0.0417428
	0.823243±0.07944	0.016207	0.200047±0.042021	0.002737	0.799806±0.0346423	0.0259121
	0.563286±0.012367	0.025153144	0.345019±0.0593458	0.002982	0.794335±0.029445	0.0454879
	0.936732±0.018694	0.031225	0.532559±0.0316683	0.000101	0.654953±0.023336	0.010593
	0.5546±0.139316	0.0466745	0.54876±0.0749271	0.03889	0.666897±0.062949	0.025771
CK909606	0.586854±0.060355	0.01815	0.946302±0.016736	0.02504	1.279703±0.478525	0.0145201
B	0.314336±0.059675	0.04833	0.524212±0.0351852	0.003968	1.714456±0.55977	0.024016
	0.65784±0.034469	0.004643	0.505594±0.0241292	0.000482	1.554306±0.172374	0.023101
	0.813157±0.217827	0.0149	0.461443±0.020592	0.004812	1.081272±0.00991	0.0272059
	0.342027±0.020075	0.027706	0.516967±0.028899	0.017983	1.724915±0.22378	0.002877
DY761933	0.465834±0.185869	0.208	0.542288±0.04570289	0.0788	0.923189±0.0472251	0.445064
C	0.087104±0.0041516	0.618	1.203671±0.05168653	0.09563	1.005293±0.232143	0.824369
	0.497909±0.028109	0.23416	1.316117±0.2691014	0.42673	1.3526±0.13994	0.07509
	1.425503±0.048414	0.5803	0.767713±0.04917468	0.275	1.163089±0.14912	0.06114
	1.193738±0.24177	0.158646	0.597239±0.07207278	0.0956	0.674314±0.040082	0.341
DY761493	2.532606±0.026	0.0857	0.399696±0.0191849	0.000967	0.112861±0.0648462	0.0199
D	2.952896±0.47899	0.000143	0.200047±0.060252	0.040702	0.576671±0.018989	0.047638
	1.780001±0.494527	0.041803	0.345019±0.051276	0.044255	0.144354±0.016888	0.009084
	1.787862±0.31206	0.006266	0.532559±0.0432783	0.006131	0.484323±0.035743	0.022323
	1.094225±0.392933	0.000738	0.54876±0.010058	0.000446	0.179431±0.04496	0.001411
DY762862	0.196749±0.18364	0.00483	0.132568±0.08456	0.040418	0.205708±0.0312457	0.000494
E	0.229798±0.00994	0.0018	0.61419±0.0421598	0.016302	0.173017±0.007504	0.017915
	0.478251±0.0150626	0.009338	0.22306±0.065602	0.04088	0.690916±0.045685	0.003089
	2.230749±0.161141	0.00693	0.219205±0.00011	0.003162	0.753287±0.081515	0.0165185
	0.596525±0.038307	0.035151	2.560647±0.14403	0.000172	0.897411±0.088364	0.0691092
CK907860	0.59428±0.06608	0.000645	1.126398±0.00257	0.042623	0.80736±0.01739	0.0398968
F	1.077821±0.02953	0.043345	0.499562±0.030927	0.021049	0.412167±0.0664905	0.001075
	0.731878±0.0460682	0.043825	0.77833±0.042406	0.0102818	0.352852±0.034033	0.017111
	1.183896±0.124086	0.016648	1.094766±0.42549	0.0459242	0.872623±0.061547	0.0468693
	0.448698±0.0395391	0.005944	0.623964±0.0236602	0.008174	0.521558±0.099988	0.0116072
DY762667	0.532464±0.034551	0.4275	2.624169±0.14358	0.0909	1.103881±0.459085	0.0431281
G	0.924922±0.0335101	0.327497	0.786151±0.066172	0.0722	1.045427±0.107653	0.496412
	1.696438±0.02098	0.205	0.651722±0.0125707	0.43	0.921438±0.017594	0.239614
	2.53336±0.57759	0.2972	0.660697±0.0353296	0.0709	0.914973±0.02811	0.084823
	1.482186±0.071459	0.12483	0.700824±0.038574	0.33238	0.453199±0.053163	0.03016
DY762710	0.23841±0.039513	0.033697	0.823212±0.0175493	0.01714	0.83300±0.0192044	0.03099
H	1.149193±0.05722	0.0422	1.326996±0.15711	0.000557	1.59494±0.20715	0.028774
	0.643985±0.16527	0.047515	2.417113±0.078559	0.00054	1.08784±0.09538	0.004486
	0.620924±0.0123581	0.005046	2.0203±0.49611	0.000675	1.15473±0.0319	0.0176422
	0.64282±0.10159	0.002339	0.81646±0.03473	0.001412	2.30492±0.62959	0.002175
DY761413	1.90001±0.084463	0.002592	0.531444±0.040538	0.018565	0.53714±0.110881	0.0181
I	0.541±0.03644	0.0361	0.387038±0.0374207	0.000115	0.886796±0.036048	0.042719
	2.107593±0.093725	0.016883	0.748805±0.028449	0.035924	0.67118±0.08785	0.014765
	1.857981±0.119092	0.044606	0.734961±0.07147	0.001239	1.57853±0.17879	0.010629
	0.610447±0.0218623	0.037984	1.004666±0.65085	0.0477635	0.529867±0.019037	0.003921
DY762883	0.694223±0.04152	0.0323	1.532025±0.23938	0.000261	0.662402±0.0282423	0.023965
J	0.95109±0.13405	0.027551	0.416607±0.00921	0.000203	1.359802±0.605582	0.0173301
	0.788791±0.00074	0.028087	0.841002±0.04244	0.000911	1.19393±0.136128	0.015928
	0.640281±0.0262973	0.00126	0.491731±9.27E-05	0.000154	1.153252±0.098087	0.09061
	0.514751±0.02682	0.04279	0.483675±0.07126	0.00014	1.810707±0.42433	0.001211
CK908648	0.126972±0.0188533	0.09002	1.389521±0.045441	0.18381	0.89±0.0202031	0.359332
K	0.581887±0.060263	0.175	0.776366±0.0209864	0.05489	1.262959±0.561005	0.292616
	1.875869±0.42005	0.1911	0.56315±0.035121	0.058577	1.996132±0.092403	0.083017
	0.719454±0.058568	0.06809	0.445826±0.048523	0.1237	1.560565±0.06387	0.054329
	0.050522±0.0024918	0.3587	2.022026±0.248582	0.12925	0.48809±0.070134	0.124672

Table 2. Continued

GenBank accession no.	mRNA expression (fold induction relative to β tubulin normalized)					
	Induced by *S. sclerotiorum*		Induce by *R. solani*		Induced by *F. oxysporum*	
	$2^{-\Delta\Delta Ct}$± SE[a]	*P* value	$2^{-\Delta\Delta Ct}$±SE	*P* value	$2^{-\Delta\Delta Ct}$±SE	*P* value
DY762016	0.93629±0.1336	0.548337	0.650836±0.0131264	0.023398	1.203835±0.335772	0.551889
L	0.433904±0.07255	0.001146	0.767296±0.0540673	0.0216364	0.86171±0.025709	0.159108
	1.190731±0.17634	0.521509	0.889674±0.0328829	0.0189034	0.946128±0.020705	0.706641
	1.996976±0.0147	0.000428	1.123189±0.56593	0.0499778	1.243755±0.24579	0.047542
	0.759526±0.097	0.031552	0.79144±0.048136	0.013785	0.703698±0.038759	0.093916
CK434092	0.900813±0.045508	0.0451326	0.787845±0.03795	0.014663	1.087449±0.41847	0.58979
M	0.909923±0.020432	0.0285864	0.293325±0.0546599	0.00363	0.6591±0.039366	0.000269
	0.357548±0.03114	0.00382	0.415925±0.015017	0.003146	0.948205±0.07708	0.019829
	0.673933±0.0190319	0.007924	0.567176±0.0147805	0.00121	0.864288±0.017262	0.004282
	0.35918±0.046847	0.000635	0.260131±0.085474	0.00356	0.304053±0.00489	0.00327
DY762836	0.363594±0.0559027	0.000157	0.520744±0.0346276	0.002029	1.522365±0.212003	0.003305
N	0.583177±0.027926	0.038649	0.321974±0.0244626	0.000628	1.280106±0.055822	0.036059
	1.358921±0.53261	0.026049	0.612556±0.019694	0.00339	0.82622±0.016794	0.04733
	0.577037±0.029086	0.042539	0.385773±0.031134	0.000488	1.59818±0.28472	0.043713
	0.068031±0.00748444	0.000562	0.137245±0.0546128	0.000765	1.55115±0.58109	0.0152197
CK907343	0.435927±0.0157505	0.013075	0.813104±0.069947	0.0139179	1.631702±0.14376	0.00423
O	0.698897±0.031168	0.007253	0.970962±0.0104763	0.0404086	0.790021±0.099607	0.002364
	1.141534±0.41696	0.0225949	0.9276±0.027801	0.0335237	1.033701±0.112758	0.0324829
	0.570423±0.0624407	0.042423	0.645941±0.049355	0.049609	1.920163±0.00892	0.000804
	0.629139±0.0127869	0.000227	0.556094±0.0780629	0.040362	1.2913±0.44349	0.000445
DY762875	0.695198±0.030869	0.0164437	0.9334±0.0280854	0.0264171	1.616163±0.655435	0.0138116
P	3.711706±0.08729	0.000285	0.595043±0.0485791	0.017478	1.106238±0.035299	0.089062
	3.947487±0.07815	0.014714	0.914861±0.0175745	0.03108	1.265033±0.028202	0.04281
	4.931953±0.28686	0.00273	0.658678±0.0484197	0.026552	1.368324±0.038643	0.074508
	0.890109±0.0147061	0.0240642	0.475248±0.0327708	0.00176	0.911576±0.057841	0.0387984
DY762463	0.388514±0.0100472	0.0408	0.360585±0.063465	0.007398	0.390216±0.0346343	0.0218
Q	0.439282±0.0291705	0.02494	0.1427±0.007545	0.00782	0.530254±0.0670293	0.004735
	1.364181±0.044102	0.0148	0.308612±0.039755	0.039532	0.567479±0.019611	0.013938
	0.801499±0.022531	0.0118	0.371489±0.0145864	0.001275	0.682563±0.04145	0.003291
	0.375676±0.0250476	0.0267	0.93896±0.050009	0.027508	0.15159±0.015799	0.00254
DY762900	0.320556±0.026277	0.009039	0.4456±0.06387	0.010888	1.61616±0.276668	0.0498954
R	0.66494±0.0271203	0.022493	0.085641±0.00770398	0.000905	1.259568±0.550127	0.0355512
	0.404369±0.32268	0.046015	0.339416±0.05223	0.000582	1.265033±0.06543	0.043928
	0.969844±0.01678	0.0472976	0.5131±0.0239027	0.000195	1.368324±0.01138	0.019831
	0.333822±0.020796	0.00474	0.546615±0.0210455	0.0002	1.029846±0.69767	0.023981
DY762807	0.452646±0.014785	0.00804	0.265425±0.07368	0.004989	1.310611±0.403492	0.0125793
S	2.577999±0.08049	0.00478	0.493356±0.0324939	0.000152	0.649216±0.0316176	0.009501
	1.482331±0.44991	0.041274	0.623164±0.018003	0.013758	0.276895±0.09073	0.044387
	3.528988±0.35692	0.00633	0.444708±0.046978	0.034949	0.94963±0.07634	0.0481143
	1.65829±0.11692	0.007522	0.650153±0.022568	0.020174	0.451348±0.025957	0.049163

[a], Standard Error.

fungi (P>0.05) (Table 2).

For the activation of the mycoparasitic response, a model of different signalling pathways responding to multiple signals from the host can be assumed. The expression of two genes (*DY762710*, *DY761413*) encoding molecules belonging to the protein kinase C family was significantly, highly induced by confrontation with *S. sclerotiorum*, *R. solani* or *F. oxysporum* (P<0.05) (Fig. 2); whereas, other genes (*DY762667* and *DY762883*) encoding Mitogen activated protein kinases (MAPKs) or Catalases, respectively, were not induced by plant pathogens (P>0.05) (Table 2).

Pathogenicity proteins are expressed under stress conditions. Analysis of the expression levels of four genes (*DY762016*, *CK434092*, *DY762836*, and *CK907343*) involved in toxin production, such as the biosynthesis of ergosterol, Bleomycin or Polyketide, showed that they were induced by plant pathogens (P<0.05) (Fig. 2), indicating a participation in anti-pathogenic metabolism. The expression of one gene (*CK908648*) did not show a difference in response to confrontation with fungi (P>0.05) (Table 2).

Proteases take part in the breakdown process of host cell walls and act as proteolytic inactivators of pathogen-derived enzymes. In the present study, two genes (*DY762875*, *DY762463*) coding for proteases were identified in *T. harzianum*

Table 3. PCR efficiency (E) for real-time RT-PCR

GenBank Acc:	Standard curves	Correlation coefficient (R^2)	Efficiency (E) (%)
DY762540	Y = -3.3302x + 39.144	0.9932	99.6%
DY761788	Y = -3.3837x + 33.714	0.9933	97.4%
CK909606	Y = -3.3342x + 33.694	0.9935	99.4%
DY761933	Y = -3.4015x + 34.104	0.9996	96.7%
DY761493	Y = -3.3677x + 33.383	0.9903	98.1%
DY762862	Y = -3.3916x + 36.285	0.9908	97.1%
CK907860	Y = -3.4125x + 35.717	0.9905	96.3%
DY762667	Y = -3.3721x + 35.42	0.9983	97.9%
DY762710	Y = -3.4385x + 37.892	0.9903	95.3%
DY761413	Y = -3.3412x + 36.904	0.9921	99.2%
DY762883	Y = -3.4868x + 33.177	0.9916	93.5%
CK908648	Y = -3.5147x + 38.599	0.9961	92.5%
DY762016	Y = -3.4939x + 37.226	0.996	93.2%
CK434092	Y = -3.3776x + 37.804	0.9919	97.7%
DY762836	Y = -3.3906x + 35.425	0.9946	97.2%
CK907343	Y = -3.4758x + 34.049	0.998	93.9%
DY762875	Y = -3.3103x + 36.052	0.9901	100.4%
DY762463	Y = -3.3501x + 34.15	0.9916	98.8%
DY762900	Y = -3.3576x + 38.305	0.9909	98.5%
DY762807	Y = -3.5028x + 35.907	0.999	92.9%

88, and their expression levels significantly increased in response to different fungi ($P<0.05$) (Fig. 2). For example, the expression of *DY762875* showed a peak at 4.93-fold with the highest induction ratio at 24 h by *S. sclerotiorum* (Fig. 2A). The enhanced protease activities may play a role in plant pathogen resistance.

Hydrophobins are involved in cell surface hydrophobicity, adhesion and virulence, and constitute the protective spore coat structure known as the rodlet layer (Zhang *et al.*, 2011). In this study, one gene (*DY762900*) encoding hydrophobins expressed in response to confrontation with phytopathogens showed a detectable increase with the maximum level reached at 1.61-fold (Fig. 2C). Its enhanced activities may help *T. harzainum 88* to avoid toxic, lytic enzymes and/or adherence to the hydrophobic surfaces of fungi.

Proteinaceous elicitor SM1 can initiate plant defense responses and systemic resistance (Buensanteai *et al.*, 2010). The expression analysis showed that an EST (DY762807) encoding elicitor protein SM1 was highly increased with a maximal 3.52-fold expression ratio by confrontation with *S. sclerotiorum* (Fig. 2A). This suggests a prominent role for elicitor protein SM1 in initiating the defense responses against fungi.

However, each gene generally displayed varied expression patterns when confronted with different fungal pathogens, suggesting that *T. harzianum* generates specific response patterns to different challenges.

Discussion

In this study, a cDNA library was constructed using mycelia cultivated in SM medium with pathogenic fungal cell wall

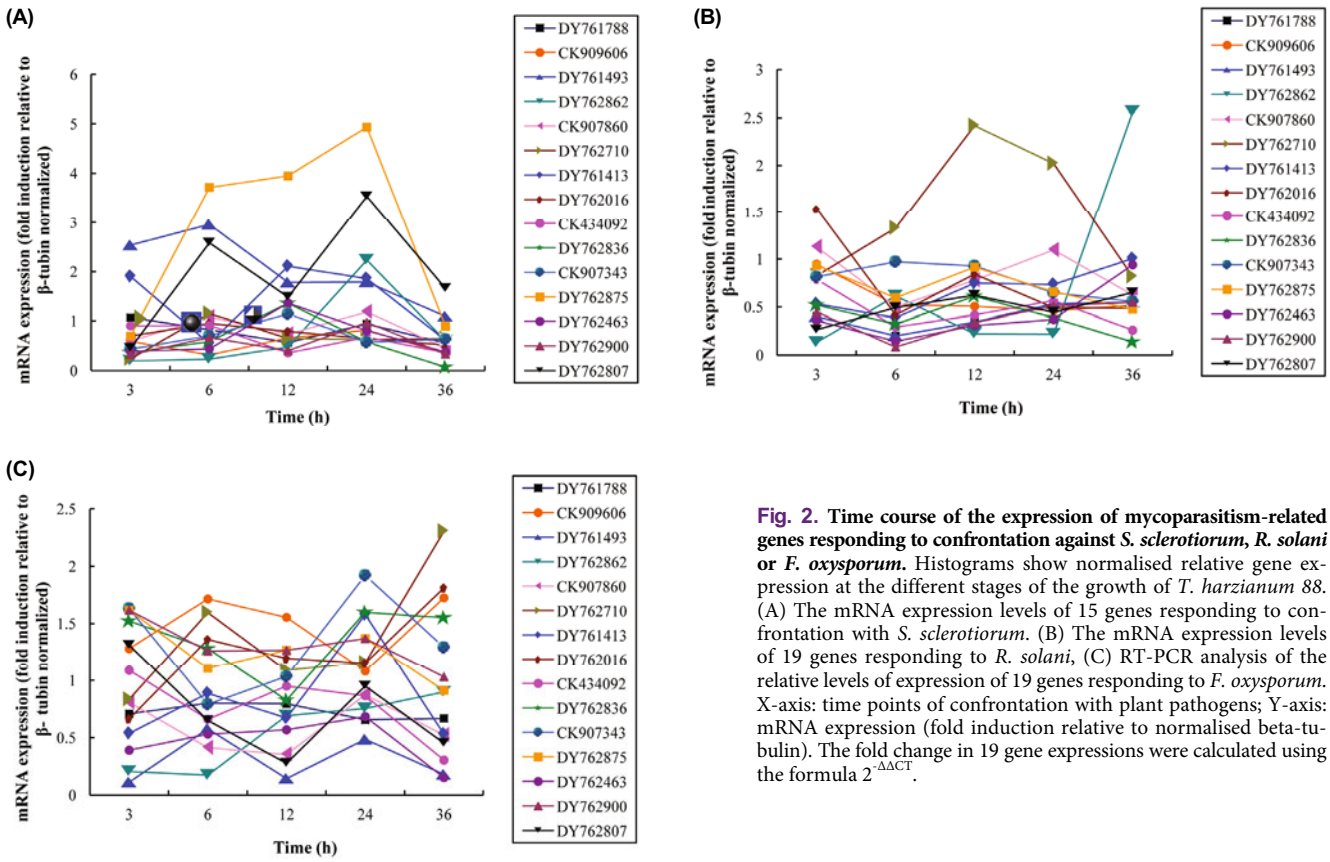

Fig. 2. Time course of the expression of mycoparasitism-related genes responding to confrontation against *S. sclerotiorum*, *R. solani* or *F. oxysporum*. Histograms show normalised relative gene expression at the different stages of the growth of *T. harzianum 88*. (A) The mRNA expression levels of 15 genes responding to confrontation with *S. sclerotiorum*. (B) The mRNA expression levels of 19 genes responding to *R. solani*, (C) RT-PCR analysis of the relative levels of expression of 19 genes responding to *F. oxysporum*. X-axis: time points of confrontation with plant pathogens; Y-axis: mRNA expression (fold induction relative to normalised beta-tubulin). The fold change in 19 gene expressions were calculated using the formula $2^{-\Delta\Delta CT}$.

extracts from *R. solani*, *S. sclerotiorum*, and *F. oxysporum* (1:1:1).

Of the 915 unique sequences from *T. harzianum*, 862 unique sequences showed significant similarities to genes in other *Trichoderma* spp.; five unigenes did not exhibit significant similarity to any genes in the *Trichoderma* genomes but did to other sequences in the NCBI nt database. Meanwhile, 48 unigenes did not show significant similarity to any genes in the genomic databases of *Trichoderma* spp. or in the NCBI nt database. The annotations of unigene sequences are shown in the Supplementary data Table S5.

Since the library was neither normalised nor subtracted, the number of clones derived from a unique gene could approximately reflect the expression level of genes induced by the presence of cell wall extracts from the three plant pathogenic fungi in the mycelium stage. Therefore, a high frequency of a specific cDNA sequence among ESTs may indicate a higher expression level of the corresponding gene. Some contigs consisted of up to six or more ESTs together in the original library (additional data are given in Supplementary data Table S4). The results illustrated the functional diversity of these highly expressed unigene sequences with apparently no particular functional category dominating the analysis.

We found that 56.8% of the unique sequences had similar sequences in the *T. harzianum* T34 library related to biocontrol, while only 27.76% matched those from the T88-hzm library. Vizcaíno *et al.* (2006) had found that only 21.6% of the unique sequences from the *T. harzianum* T34 library had similar sequences to the related biocontrol transcript collection obtained from the T88-wmaa library (Liu and Yang, 2005). As for the differences in the proportion of similar sequences among the three comparable EST collections from *T. harzianum*, it should be noted that the lowest percentages of similar sequences were found in libraries obtained in very different growth conditions, such as stress-related growth conditions (Vizcaíno *et al.*, 2006) or carbon starvation (Liu and Yang, 2005), than the ones used in our study.

In the investigation of the molecular mycoparasitic mechanism of *T. harzianum 88*, we compared ESTs between two libraries for which the results showed that the percentage of ESTs assigned GO terms was 13.9% in the T88-wmaa library (Table 1), which was lower than the 26.7% in the T88-hzm library (Liu and Yang, 2005) using the same automated annotation method. There were some other obvious differences between these two libraries as well. For example, the GO items of catalytic activity, enzyme regulator activity, transporter activity, binding, signal transducer activity, physiological process, regulation of biological process, cellular component organisation or biogenesis, behaviour and cellular process were higher in the T88-wmaa library than in the T88-hzm library (Table 1), indicating that these pathways may be enhanced in the presence of cell wall extracts from *R. solani*, *S. sclerotiorum*, and *F. oxysporum* (1:1:1).

T. harzianum 88 is the source of a number of secreted proteins produced for various industrial applications. To identify potential secreted proteins encoded by the identified unigenes, SignalP 4.0 (Petersen *et al.*, 2011) was used to search for predicted proteins with a signal peptide. Sixty of the predicted proteins were found to possess a putative signal peptide (Supplementary data Table S7). Seven of them, including Contig27, Contig34, Contig39, Contig175, Contig176, and Contig178, share significant homology with known proteins in the NCBI nt database (*E*-value<0.01) using the BLASTx program. The expressions of those ESTs did not show induction by *T. harzainum 88* confrontation with phytopathogens ($P>0.05$).

Other genes encoding secreted proteins, such as cell wall-degrading enzymes including chitinases (encoded by *DY761788* or *CK909606*), 1, 3-β-glucan synthase (encoded by *DY761933*), glucanase (encoded by *DY761493* or *DY762862*), β-1, 4-glucosidase (encoded by *CK907860*), proteases (encoded by *DY762875* or *DY762463*), hydrophobins (ecoded by *DY762900*), and proteinaceous elicitor SM1 (encoded by *DY762807*) *et al.*, were not acquired fragments of signal peptide sequences because ESTs from this cDNA library were randomly selected. All of them were challenged by phytopathogens ($P<0.05$).

In conclusion, our results demonstrated that *T. harzianum 88* is highly resistant to phytopathogens. The cDNA library generated from the mycelium stage and corresponding EST collections will, therefore, be important resources for further investigations to gain a better understanding of the molecular events that occur during mycelium development of *T. harzianum 88*.

Acknowledgements

This work was supported by a grant from the Key Science and Technology Program of Heilongjiang Province, China (No. GA08B101).

References

Adams, M.D., Kelley, J.M., Gocayne, J.D., Dubnick, M., Polymeropoulos, M.H., Xiao, H., Merril, C.R., Wu, A., Olde, B., Moreno, R.F., and *et al.* 1991. Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* **252**, 1651–1656.

Alizadeh, F., Abdullah, S.N., Khodavandi, A., Abdullah, F., Yusuf, U.K., and Chong, P.P. 2011. Differential expression of oil palm pathology genes during interactions with *Ganoderma boninense* and *Trichoderma harzianum*. *J. Plant Physiol.* **168**, 1106–1113.

Almeida, F.B., Cerqueira, F.M., Silva Rdo, N., Ulhoa, C.J., and Lima, A.L. 2007. Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evaluation of coiling and hydrolytic enzyme production. *Biotechnol. Lett.* **29**, 1189–1193.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Bae, H., Roberts, D.P., Lim, H.S., Strem, M.D., Park, S.C., Ryu, C.M., Melnick, R.L., and Bailey, B.A. 2011. Endophytic *Trichoderma* isolates from tropical environments delay disease onset and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms. *Mol. Plant. Microbe Interact.* **24**, 336–351.

Bouck, A. and Vision, T. 2007. The molecular ecologist's guide to expressed sequence tags. *Mol. Ecol.* **16**, 907–924.

Buensanteai, N., Mukherjee, P.K., Horwitz, B.A., Cheng, C., Dangott, L.J., and Kenerley, C.M. 2010. Expression and purification of biologically active *Trichoderma virens* proteinaceous elicitor

Sm1 in *Pichia pastoris*. *Protein Expr. Purif.* **72**, 131–138.

Camon, E.B., Barrell, D.G., Dimmer, E.C., Lee, V., Magrane, M., Maslen, J., Binns, D., and Apweiler, R. 2005. An evaluation of GO annotation retrieval for BioCreAtIvE and GOA. *BMC Bioinformatics* **1**, S17.

Carsolio, C., Gutie´rrez, A., Jime´nez, B., Van Montagu, M., and Herrera Estrella, A. 1994. Characterization of ech-42, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. *Proc. Natl. Acad. Sci. USA* **91**, 10903–10907.

Cortés, C., Gutierrez, A., Olmedo, V., Inbar, J., Chet. I., and Herrera-Estrella, A. 1998. The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. *Mol. Gen. Genet.* **260**, 218–225.

Druzhinina, I.S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B.A., Kenerley, C.M., Monte, E., Mukherjee, P.K., Zeilinger, S., and Grigoriev, I.V. 2011. *Trichoderma*: the genomics of opportunistic success. *Nat. Rev. Microbiol*. **9**, 749–759.

Ewing, B. and Green, P. 1998. Base-calling of automated sequencer traces using Phred. II. Error probabilities. *Genome Res*. **8**, 186–194.

Gordon, D. 2003. Viewing and Editing Assembled Sequences Using Consed, pp. 11.2.1–11.2.43. *In* Baxevanis, A.D. and Davison, D.B. (eds.), Curr. Protoc. Bioinformatics. John Wiley & Co., New York, USA.

Götz, S., García-Gómez, J.M., Terol, J., Williams, T.D., Nagaraj, S.H., Nueda, M.J., Robles, M., Talón, M., Dopazo, J., and Conesa, A. 2008. High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids. Res.* **36**, 3420–3435.

Hermosa, M.R., Grondona, I., Iturriaga, E.A., Diaz Minguez, J.M., Castro, C., Monte, E., and Garcia-Acha, I. 2000. Molecular characterization and identification of biocontrol isolates of *Trichoderma* spp. *Appl. Environ. Microbiol*. **66**, 1890–1898.

Kubicek, C.P., Herrera-Estrella, A., Seidl-Seiboth, V., Martinez, D.A., Druzhinina, I.S., Thon, M., Zeilinger, S., Casas-Flores, S., Horwitz, B.A., Mukherjee, P.K., and *et al.* 2011. Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. *Genome Biol.* **12**, R40.

Lin, Y.R., Lo, C.T., Liu, S.Y., and Peng, K.C. 2012. Involvement of pachybasin and emodin in self-regulation of *Trichoderma harzianum* mycoparasitic coiling. *J. Agric. Food. Chem*. **60**, 2123–2128.

Liu, P.G. and Yang, Q. 2005. Identification of genes with a biocontrol function in *Trichoderma harzianum* mycelium using the expressed sequence tag approach. *Res. Microbiol*. **156**, 416–423.

Liu, Z., Yang, X., Sun, D., Song, J., Chen, G., Juba, O., and Yang, Q. 2010. Expressed sequence tags-based identification of genes in a biocontrol strain *Trichoderma asperellum*. *Mol. Biol. Rep*. **37**, 3673–3681.

Livak, K. and Schmittgen, T. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25**, 402–408.

Morán-Diez, E., Rubio, B., Domínguez, S., Hermosa, R., Monte, E., and Nicolás C. 2012. Transcriptomic response of *Arabidopsis thaliana* after 24 h incubation with the biocontrol fungus *Trichoderma harzianum*. *J. Plant Physiol.* **169**, 614–620.

Petersen, T.N., Brunak, S., Heijne, G.V., and Nielsen, H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**, 785–786.

Reithner, B., Ibarra-Laclette, E., Mach, R.L., and Herrera-Estrella, A. 2011. Identification of mycoparasitism-related genes in *Trichoderma atroviride*. *Appl. Environ. Microbiol*. **77**, 4361–4370.

Restuccia, C., Giusino, F., Licciardello, F., Randazzo, C., Caggia, C., and Muratore, G. 2006. Biological control of peach fungal pathogens by commercial products and indigenous yeasts. *J. Food Prot.* **69**, 2465–2470.

Steiger, M.G., Mach, R.L., and Mach-Aigner, A.R. 2010. An accurate normalization strategy for RT-qPCR in *Hypocrea jecorina* (*Trichoderma reesei*). *J. Biotechnol*. **145**, 30–37.

Vizcaíno, J.A., González, F.J., Suárez, M.B., Redondo, J., Heinrich, J., Delgado-Jarana, J., Hermosa, R., Gutiérrez, S., Monte, E., Llobell, A., and Rey, M. 2006. Generation, annotation and analysis of ESTs from *Trichoderma harzianum* CECT 2413. *BMC. Genomics* **7**, 193.

Vizcaíno, J.A., Redondo, J., Suárez, M.B., Cardoza, R.E., Hermosa, R., González, F.J., Rey, M., and Monte, E. 2007. Generation, annotation, and analysis of ESTs from four different *Trichoderma* strains grown under conditions related to biocontrol. *Appl. Microbiol. Biotechnol*. **75**, 853–862.

Yang, C.A., Cheng, C.H., Lee, J.W., Lo, C.T., Liu, S.Y., and Peng, K.C. 2012. Monomeric L-amino acid oxidase-induced mitochondrial dysfunction in *Rhizoctonia solani* reveals a novel antagonistic mechanism of *Trichoderma harzianum* ETS 323. *J. Agric. Food Chem.* **60**, 2464–2471.

Yang, H.H., Yang, S.L., Peng, K.C., Lo, C.T., and Liu, S.Y. 2009. Induced proteome of *Trichoderma harzianum* by *Botrytis cinerea*. *Mycol. Res.* **113**, 924–932.

Zhang, S., Xia, Y.X., Kim, B., and Keyhani, N.O. 2011. Two hydrophobins are involved in fungal spore coat rodlet layer assembly and each play distinct roles in surface interactions, development and pathogenesis in the entomopathogenic fungus, *Beauveria bassiana*. *Mol. Microbiol*. **80**, 811–826.

Zhang, H.Y., Yang, Q., Wang, G., and Shang, F.D. 2009. Analysis of expressed sequence tags from *Chaetomium cupreum* grown under conditions associated with mycoparasitism. *Lett. Appl. Microbiol*. **48**, 275–280.