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EXPRESSION OF PHENAZINE BIOSYNTHETIC GENES DURING THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS OF *GLOMUS INTRARADICES*

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ABSTRACT

To explore the molecular mechanisms that prevail during the establishment of the arbuscular mycorrhiza symbiosis involving the genus *Glomus*, we transcriptionally analysed spores of *Glomus intraradices* BE3 during early hyphal growth. Among 458 transcripts initially identified as being expressed at presymbiotic stages, 20% of sequences had homology to previously characterized eukaryotic genes, 30% were homologous to fungal coding sequences, and 9% showed homology to previously characterized bacterial genes. Among them, *GintPbr*1a encodes a homolog to Phenazine Biosynthesis Regulator (Pbr) of *Burkholderia cenocepacia*, an pleiotropic regulatory protein that activates phenazine production through transcriptional activation of the protein D isochorismatase biosynthetic enzyme phzD (Ramos *et al*., 2010). Whereas *GintPbr*1a *i*s expressed during the presymbiotic phase, the *G. intraradices* BE3 homolog of *phzD* (B*GintphzD*) is transcriptionally active at the time of the establishment of the arbuscular mycorrhizal symbiosis. DNA from isolated bacterial cultures found in spores of *G. intraradices* BE3 confirmed that both B*GintPbr*1a and B*GintphzD* are present in the genome of its potential endosymbionts. Taken together, our results indicate that spores of *G. intraradices* BE3 express bacterial phenazine biosynthetic genes at the onset of the fungal-plant symbiotic interaction.

Key words: mycorrhizal fungi, *Glomus intradices*, phenazine, biosynthesis.

INTRODUCTION

The arbuscular mycorrhiza (AM) is a complex and intimate association of organisms formed by fungi of the *Glomeromycota* phylum and different taxonomic groups of plants, including 80% of the terrestrial flora (Schüssler et al., 2001; Smith and Read, 1997). After the consistent discovery of endocellular bacteria within mycorrhizal fungi (Macdonald M.R. and Chandler R.M. 1981; Bianciotto *et al*., 1996), AM has been considered to be the result of a tripartite symbiosis (Bonfante, 2003). The absence of these bacteria from fungal spore, results in important changes in fungal

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presymbiotic growth and sporal morphology, suggesting that they are important for mycellium germination and possibly ecological fitness (Lumini *et al*., 2007); however, their main role during the establishment and maintenance of the AM symbiosis remains unknown. Studies attempting to elucidate their physiological role have been hindered by a lack of protocols allowing their cultivation *in vitro* (Bianciotto *et al*., 2004; Bonfante and Anca, 2009), a difficulty that could be related to the impossibility of reproducing essential conditions of the fungal cytoplasmic milieu necessary for their survival (Jargeat *et al.*, 2004).

Some understanding of the role played by non-AM bacterial endosymbionts has emerged from studies of plant pathogenic fungi belonging to the genus *Rhizopus*, in which the antimitotic polyketide rhizoxin - responsible for causing seedling blight in rice – is biosynthesized by bacteria of the genus *Burkholderia* (Partida-Martínez and Hertweck, 2005). *Rhizopus microsporus* does not form sporangia and spores in the absence of its endosymbionts, indicating that *Burkholderia* produce metabolic factors that are essential for the fungal life cycle (Partida-Martínez *et al*., 2007). In addition to confirming the potential for a large genetic diversity intrinsic to *Burkholderia* and perhaps other families of endosymbiotic bacteria (Komatsu *et al*., 2003), the recent elucidation of a *Burkholderia rhizoxinica* genome revealed an evolutionary tendency not only towards specialized uptake of fungal metabolites, but also to a bacterial-dependent provision of putative phytotoxins and virus-related factors that could promote nutritional uptake from decaying plants (Lackner *et al.*, 2011). The phenotypic diversity of endosymbionts is also reflected by their capacity for producing a large variety metabolites involved in antagonistic interactions such as phenazines, a large family of heterocyclic nitrogen antibiotical compunds produced by different bacteria such as *Burkholderia*, *Streptomyces* and *Pseudomonas* ssp., but not animals or plants (Thomashow and Weller, 1988; Komatsu *et al*., 2003; Delaney *et al*., 2001; Blankenfeldt *et al*., 2004; Laursen and Nielsen, 2004; Fitzpatrick, 2009).

In contrast to non-AM endocellular bacteria, the genetic diversity, metabolic potential, and physiological contribution of AM endosymbionts remains largely unexplored. Endocellular bacteria have been reported in several *Glomeromycota* species that include *Glomus versiforme*, *Acaulospora laevis*, and *Gigaspora margarita* (MacDonald and Chandler, 1981; Bianciotto, 2000; Naumann, 2010), as well as the ectomycorrhizal basidiomycete *Laccaria bicolor* for which a complete genome is available (Bertaux *et al*., 2003; Martin *et al*., 2008). In several *Gigaspora* species, rod-shaped Gram negative endosymbiotic bacteria were demonstrated to be present in spores, germ tubes, and hyphae, but not arbuscules (Bianciotto *et al*., 1996). A genomic library of *G. margarita* had a partial representation of the genome of its bacterial endosymbionts (van Buuren *et al*., 1999), and although subsequent studies suggested that some sequences could have resulted from contamination with foreign bacterial DNA (Jargeat *et al*. 2004), a gene encoding a putative phosphate transporter, and a gene involved in cell colonization by entero-invasive pathogenic bacteria were unequivocally confirmed as being derived from genetic material contained in the spores *G. margarita* (Ruiz-Lozano and Bonfante, 1999; Anca *et al*., 2009), demonstrating the presence of bacterial DNA within the fungus.

Here we analyze a sample of cDNA clones generated from mRNA present in *Glomus intraradices* BE3 prior to the establishment of the AM symbiosis, identifying numerous transcripts with homology to eukaryotic or prokaryotic genes. We show that a gene with homology to the bacterial pleiotopic regulator *Phenazine Biosynthesis Regulator* (*Pbr*) is expressed within fungal cells at presymbiotic stages, and present in genomic DNA samples extracted from bacterial isolates cultivated from *Glomus intraradices* BE3 spores. We also show that a homolog of the bacterial phenazine biosynthetic gene *phzD* - also present in the genome of bacterial isolates from *Glomus intraradices* BE3 spores - is expressed at the time

of the establishment of the fungal-plant symbiosis. Our results indicate that phenazine biosynthetic genes are active in *G. intraradices* BE3, opening possibilities for studying its function and regulatory mechanisms during the AM symbiosis.

MATERIALS AND METHODS

Mycorrhizal material

Spores of *Glomus intraradices* strains BE2 and BE3 were obtained from the *in vitro* collection of Departamento de Biotecnología y Bioingeniería CINVESTAV Zacatenco, and recovered following chelation with sodium citrate as described in Doner and Bécard (1991). Spores of *Gigaspora margarita* BE2 were isolated from potted plant trap cultures with *Sorghum* sp. and *Lolium* sp., following the method of tween-sucrose flotation (Gerdemann and Nicholson, 1963).

cDNA library construction and sequencing

Spores of *G. intraradices* BE3 were germinated in the presence of Ri T-DNA transformed roots of carrot. No physical interaction was allowed by insertion of a cellophane membrane between the fungus and plant tissues. Germinated spores were recovered with insulin syringes (Beckton-Dickinson) under a stereo-microscope and immediately frozen in liquid nitrogen. Total RNA was obtained using the RNAeasy mini kit (Qiagen) and treated with RNase-free DNase I according to manufacturer's instructions (Invitrogen). Total RNA was quantified by NanoDrop® ND-1000 (Spectrophotometer Termo Scientific), and PCR-tested for integrity using primers r18S and f18S (sequence available in Supplementary Materials).

Total RNA concentration was adjusted to 450 ng and used for cDNA synthesis using a CDS III/3' PCR primer [5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30-(A,G,C)-(A,G,C,T)-3'] and the SMART kit (Clonetech). Double-stranded cDNA was purified and cloned into pCR 2.1 TOPO (Invitrogen). A random sample of 960 cDNA clones was sequenced using M13 forward and reverse primers and the Terminador Bigdye kit (ABI). Resulting sequences were assembled and filtered using parameters of minimum overlap 25, maximum gap 2, minimum overlap identity 80%, and maximum ambiguity 4 (Drummond et al., 2010. Geneious v 5.0); sequences representing unique genes, were compared to entries of the NCBI database using the non-redundant BLASTX algorithm (Worley *et al*,. 1995).

Fungal spore cultivation

Spores of *G. intraradices* BE3 were recovered from petri dishes divided by 10 mM sodium acetate. Spore disinfection was performed with 0.05% Tween 20, 2% chloramine T twice for 5 minutes, followed by 3 rinses in sterile distilled water. Samples were subsequently stored in a 100 ppm of gentamicin, 200 ppm streptomycin solution at 4°C (Bécard and Piché, 1992). For spore bursting and bacterial isolation, approximately 500 spores/plate were inoculated in minimal mineral media (M) medium (Fortin *et al*., 2002), in the absence of sucrose and following a pH gradient from 3 to 10 with increments of 0.5 units. Three replicates of each plate were incubated at 25°C until spore bursting and bacterial growth occurred. Successfully isolated bacterial colonies were transferred into two different culture media: a rich Bacto Nutrient Agar culture medium (Difco), and a poor M culture medium containing sucrose and potato dextrose agar (Bioxon), and subsequently cultivated in Bacto Nutrient broth culture for DNA extraction following Harwood and Cutting (1990). FD1 and RD1 primers were used for 16S rDNA PCR amplification (Weisburg *et al*., 1991) and PCR products were cloned into the pCR 2.1 TOPO vector and sequenced by Sanger. Sequence comparisons were conducted following conventional BLAST algorithms (Worley *et al*,. 1995). Closely related sequences were analyzed using Geneious 5.0 under a Tamura-Nei genetic distance model, (Drummond *et al*., 2010; Geneious v 5.0). New DNA sequences were deposited in the National Center for Biotechnology Information database (NCBI).

RT-PCR

Total RNA was extracted from growing hyphae using TRIzol (Invitrogen). Reactions were performed using One-Step RT-PCR kit (QIAGEN) and the annealing temperature was standardized for each pair of primers used (Supplementary Table 2). In all cases reverse-transcription reactions were performed with 3 biological replicates representing independent events, using 100 ng of total RNA and 50 pmoles/µl of each primer. PCR conditions were 1 minute at 94°C (denaturation), 30 seconds at 55-60°C (alignment), 1 min at 72°C (extension) for 28 cycles, followed by a final extension at 72°C for 5 minutes.

RESULTS

Transcripts with homology to eukaryotic or prokaryotic genes are expressed at pre-symbiotic stages of the *G. intraradices* BE3 life cycle

To conduct a transcriptional analysis of AM spore germination and early hyphal growth, we constructed a cDNA library using mRNA from an *in vitro* culture of *G. intraradices* BE3 at asymbiotic to presymbiotic stages, in the absence of physical interaction with plant tissues (D.G. León-Martínez, J-Ph. Vielle-Calzada, and V. Olalde-Portugal, unpublished results). An initial screen of the 10^4 col/µg titer colony collection resulted in the identification of 458 distinct open reading frames on the basis of 960 sequenced clones (Supplementary Table 1). A comparison of assembled coding sequences to publically available databases revealed that the corresponding genes encode for a large variety of proteins covering a wide spectrum of predicted molecular functions in eukaryotes. Whereas 20% of sequences had homology to previously characterized eukaryotic genes, 30% were homologous to fungal coding sequences, 9% showed high homology to previously characterized bacterial genes, and 41% did not show homology with reported genomic sequences. cDNA sequences with homology to bacterial genes encode proteins involved in housekeeping, secondary metabolism or signal transduction pathways, putative transcription factors, or proteins conferring resistance to antibiotics such as tetracycline (Table 1).

Table 1. Identification of sequences with homology to prokaryotic genes and expressed during pre-symbiotic stages of *Glomus intradices* BE3.

Length (pb)	PROTEIN	ORGANISM	Accession number	E-value
635	PBR	Burkholderia cenocepacia	ACJ54935	1.15e-11
647	hypothetical protein	Clostridium nexile	ZP_03289628	2.71e-31
813	hypothetical 16.9K protein	Salmonella typhimurium	JQ1541	8.72e-40
269	hypothetical protein	Azorhizobium caulinodans	YP_001526574	5.88e+00
437	hypothetical protein	Bacteroides uniformis	ZP_02071990	9.91e+00
439	helix-hairpin-helix DNA-binding motif-containinG protein	Methylobacterium sp.	YP_001772034	2.77e-02
230	oxidoreductase aldo/keto reductase family1	Vibrio coralliilyticus	ZP_05886239	2.84e-02
235	NADH pyrophosphatase	Vibrio splendidus	ZP_00991846	2.64e+00
288	GGDEF domain protein	Desulfovibrio magneticus	YP_002953232	7.77e+00
421	hypothetical protein	Salmonella enterica subsp. arizonae serovar 62:z4,z23	YP_001571636	2.03e+00
538	Tetracycline resistance protein	Bacillus cereus	ZP_04234556	8.92e-01
528	putative nitrogen metabolite repression regulator	Rhizobium leguminosarum bv. viciae	YP_766861	5.57e-08
262	arginine biosynthesis bifunctional protein ArgJ	Clostridium papyrosolvens	ZP_05498017	6.74e-04
335	hypothetical protein BDI	Parabacteroides distasonis	YP0130449_03	6.89e-01
220	oxidoreductase aldo/keto reductase family	Vibrio coralliilyticus	ZP_05886239	5.38e-09
292	aldo/keto reductase	Azorhizobium caulinodans	YP_001527069	5.36e-01
642	sulfotransferase	Methanocaldococcus vulcanius	ZP_05303437	1.87e+00
220	hypothetical protein	Bacteroides fragilis	YP_212901	5.58e-07
314	mobilization (Mob)/recombination (Pre) protein	Listeria monocytogenes	AAA93293	7.79e+00
694	hypothetical protein CYB_1972	Synechococcus sp.	YP_478184	2.28e-05
302	high-affinity zinc transporter periplasmic component	Actinobacillus succinogenes	YP_001345069	1.40e-01

Supplementary table 1.

NAME	accession number	Lenth	PROTEIN	ORGANIMS	E value	IDENTITY
1_A01	ZP_03289628	647	hypothetical protein	Clostridium nexile DSM	2.71e-31	96%
1_A03		203	No significant similarity found			
1_A04	XP_001274737	625	cytidine and deoxycytidylate deaminase zinc-binding domain protein	Aspergillus clavatus	1.63e-38	57%
1_A08		445	No significant similarity found			
1_A09	JQ1541	813	hypothetical 16.9K protein	Salmonella typhimurium	8.72e-40	98%
1_B01	CAP69667	222	glutaredoxin 1	Glomus intraradices	1.84e-09	96%
1_B02	XP_960148	576	hypothetical protein	Neurospora crassa	4.41e-34	45%
1_B03	ACJ54935	635	PBR	Burkholderia cenocepacia	1.92e-18	92%
1_B07	XP_001025277	357	hypothetical protein	Tetrahymena thermophila	4.48e+00	35%
1_B11		498	No significant similarity found			
1_C02	ZP_02071990	437	hypothetical protein	Bacteroides uniformis	9.91e+00	40%
1_C03	BAD38920	321	lactate dehydrogenase A	Rhizopus oryzae	3.13e-09	55%
1_C04	XP_001772713	815	predicted protein	Physcomitrella patens subsp. patens	2.38e-45	52%
1_C07	ACT97501	483	putative transposase	uncultured organism	1.01e-23	96%
1_C09	AAX11701	858	chitin deacetylase	Rhizopus stolonifer	1.31e-12	43%
1_D03	XP_001014731	536	Protein kinase domain containing protein	Tetrahymena thermophila	5.62e-03	32%
1_D04		386	No significant similarity found			
1_D07	XP_002196907	178	Fanconi anemia, complementation group L	Taeniopygia guttata	9.89e+00	25%
1_D09	NP_001082494	274	RAD23 homolog B	Xenopus laevis	1.83e-01	48%
1_D11		320	No significant similarity found			
1_E01	EDK40915	347	hypothetical protein	Pichia guilliermondii	1.08e-09	80%
1_E03		308	No significant similarity found			
1_E04		289	No significant similarity found			
1_E05		495	No significant similarity found			
1_E07	YP_001526574	269	hypothetical protein	Azorhizobium caulinodans	5.88e+00	51%
1_E09		201	No significant similarity found			
1_E10	XP_002397490.1	376	hypothetical protein	Moniliophthora perniciosa	7.19e-14	52%
1_F01	XP_505403	448	YALI0F14223p	Yarrowia lipolytica	2.21e-23	61%
1_F03	EDV08954	420	E3 ubiquitin-protein ligase RSP5	Saccharomyces cerevisiae	9.76e-11	62%
1_F08		139	No significant similarity found			
1_F10		308	No significant similarity found			
1_G01		405	No significant similarity found			
1_G07	YP_003118655	432	ATPase associated with various cellular activities AAA_5	Catenulispora acidiphila	3.42e+00	57%
1_G09		193	No significant similarity found			
1_G10	XP_567997	557	ubiquinol-cytochrome C reductase complex core protein 2 precursor	Cryptococcus neoformans var. neoformans	4.18e-07	46%
1_H01	XP_001503071	259	similar to CfOLF3 isoform 1	Equus caballus	7.76e+00	43%
1_H02		332	No significant similarity found			

1_H04	XP_002175734	267	predicted protein	Schizosaccharomyces japonicus	7.65e+00	50%
1_H08		104	No significant similarity found			

1_H09	EEY22609	617	phenylalanyl-tRNA synthetase beta chain	Verticillium albo-atrum	6.15e-51	69%
1_H10	XP_742497	373	hypothetical protein	Plasmodium chabaudi chabaudi	2.03e+00	38%
1_H11		216	No significant similarity found			
102C	XP_757950	525	hypothetical protein	Ustilago maydis	1.88e+00	56%
103C	XP_001831594	411	hypothetical protein	Coprinopsis cinerea okayama	3.76e-07	33%
106C	>XP_955949	480	hypothetical protein	Neurospora crassa	8.63e-04	25%
111C	XP_001243817	818	hypothetical protein	Coccidioides immitis	1.62e-09	33%
12C	XP_759155	413	hypothetical protein	Ustilago maydis	3.50e-29	67%
1C		381	No significant similarity found			
2_A01		151	No significant similarity found			
2_A02		192	No significant similarity found			
2_A03	3IVS-A	348	Chain A, Homocitrate Synthase Lys4	Schizosaccharomyces Pombe	1.01e-15	65%
2_A05	XP_002002034	674	GI14236	Drosophila mojavensis	4.60e+00	29%
2_A09	XP_755579	208	acetylornithine aminotransferase	Aspergillus fumigatus	7.37e-03	80%
2_A10	XP_002473933	279	predicted protein	Postia placenta	4.02e-01	59%
2_A11	XP_001756365	124	predicted protein	Physcomitrella patens subsp. patens	1.00e-15	100%
2_B03		153	No significant similarity found			
2_B05	XP_001348635	314	unknown function	Plasmodium falciparum	9.23e-01	27%
2_B07		153	No significant similarity found			
2_C01	XP_001933239	489	conserved hypothetical protein	Pyrenophora tritici-repentis	6.46e-23	39%
2_C03	XP_002110664	372	alpha tubulin	Trichoplax adhaerens	4.19e-38	93%
2_C04		152	No significant similarity found			
2_C05	XP_002623540	194	60S ribosomal protein L17	Ajellomyces dermatitidis	2.40e-17	69%
2_C07	XP_001553545	515	hypothetical protein	Botryotinia fuckeliana	1.00e-24	58%
2_C09	EEU35087	480	hypothetical protein	Nectria haematococca	1.29e-07	47%
2_C10	ACT65758	494	F-ATPase beta subunit	Glomus sp.	5.75e-48	86%
2_D05		304	No significant similarity found			
2_D07	XP_776311	132	hypothetical protein	Cryptococcus neoformans var. neoformans	2.53e-03	91%
2_E01	XP_001232602	572	similar to GRB2-associated binding protein 1 isoform 1	Gallus gallus	3.64e-01	28%
2_E04		299	No significant similarity found			
2_E11		264	No significant similarity found			
2_F02		526	No significant similarity found			
2_F04	NP_713536	412	glutathione S-transferase	Leptospira interrogans serovar Lai str. 56601	1.48e-13	58%
2_F05	ABX65441	533	glutathione S-transferase	Chimonanthus praecox	1.30e-20	43%
2_F07	CAQ19259	151	Ste12-like transcription factor	Glomus intraradices	8.01e-21	100%
2_F10	XP_001096324	436	similar to NmrA-like family domain containing 1 isoform 2	Macaca mulatta	4.30e-03	55%
2_G06	XP_784605	211	similar to LRP16 protein|ref	Strongylocentrotus purpuratus	5.81e-08	55%
2_G08		286	No significant similarity found			

2_H01	XP_757156	206	hypothetical protein	Ustilago maydis	4.09e-65	65%
2_H05		229	No significant similarity found			
2_H06	XP_001888362	256	predicted protein	Laccaria bicolor	1.51e-19	77%
2_H10	XP_001877241	164	predicted protein	Laccaria bicolor	8.22e-02,	43%

28C		346	No significant similarity found			
2c	CAF94125	610	unnamed protein product	Tetraodon nigroviridis	1.95e-01	30%
2G04		214	No significant similarity found			
3_A01	XP_002550148	448	conserved hypothetical protein	Candida tropicalis	1.50e-03	43%
3_A02	EEQ30863	866	conserved hypothetical protein	Microsporum canis	1.41e-14	25%
3_A03		199	No significant similarity found			
3_A07	XP_569615	424	transmembrane protein	Cryptococcus neoformans var. neoformans	3.33e-19	57%
3_A08	AAY62524	823	glutamine synthase	Glomus intraradices	1.74e-51	99%
3_A09		439	helix-hairpin-helix DNA-binding motif-containing protein	Methylobacterium sp.	2.77e-02	37%
3_A10	XP_368109	157	cytochrome c subunit Vb, putative	Magnaporthe grisea	4.38e-11	56%
3_A12	XP_758506	279	hypothetical protein UM02359.1	Ustilago maydis	5.20e-33	88%
3_B02	XP_001494445	495	similar to methionyl aminopeptidase 2 isoform 2	Equus caballus	1.19e-45	80%
3_B08	XP_001837205	672	hypothetical protein	Coprinopsis cinerea	3.17e-17	59%
3_B11	CAX64354	327	unknown function	Plasmodium falciparum	7.64e+00,	30%
3_B12	XP_001876984	749	predicted protein	Laccaria bicolor	1.78e-09	29%
3_C01		152	No significant similarity found			
3_C02	ZP_05886239	230	oxidoreductase aldo/keto reductase family1	Vibrio coralliilyticus	2.84e-02	61%
3_C04	XP_001750808	142	hypothetical protein	Monosiga brevicollis	2.96e-04	73%
3_C05		163	No significant similarity found			
3_C07		261	No significant similarity found			
3_C09	A55092	150	catalase	Zea mays	2.12e-05	100%
3_C10	ZP_00991846	235	NADH pyrophosphatase	Vibrio splendidus	2.64e+00	41%
3_C11	XP_001522405	389	hypothetical protein	Magnaporthe grisea	1.37e-25	52%
3_C12	YP_002953232	288	GGDEF domain protein	Desulfovibrio magneticus	7.77e+00	36%
3_D03	ABM90641	796	cytochrome P450 aromatase B	Cynoglossus semilaevis	4.06e-02	21%
3_D10	XP_002479808	628	ATP synthase subunit E, putative	Talaromyces stipitatus	3.24e-26	43%
3_D12		125	No significant similarity found			
3_E03	XP_002489230	356	hypothetical protein	Sorghum bicolor	1.07e-09	73%
3_E07	XP_716749	312	potential NADH-dependent flavin oxidoreductase	Candida albicans	4.57e+00	31%
3_E09	XP_001262247	206	elastinolytic metalloproteinase Mep	Neosartorya fischeri	4.62e-13	54%
3_E10		470	No significant similarity found			
3_E12		114	No significant similarity found			
3_F02	BAA13797	279	unnamed protein product	Schizosaccharomyces pombe	1.47e-19	83%
3_F06	AAN52148	308	70 kDa heat shock protein 3	Rhizopus stolonifer	9.78e-40	91%
3_F07		227	No significant similarity found			
3_F09	YP_001571636	421	hypothetical protein	Salmonella enterica	2.03e+00	41%

3_F10	AAZ23511	297	cytochrome oxidase subunit II	Aspidiotus nerii	3.12e-01	53%
3_F11		251	No significant similarity found			
3_G02	XP_002171726	571	chaperonin-containing T-complex theta subunit Cct8	Schizosaccharomyces japonicus	1.29e-22	54%
3_G06	XP_757093	108	hypothetical protein	Ustilago maydis	9.18e-09	75%
3_G09		213	No significant similarity found			
3_G11	CAA23888	247	unnamed protein product	Escherichia coli	2.20e-15	69%

3_H03	YP_831133	544	conserved hypothetical protein	Arthrobacter sp.	2.44e-01	41%
3_H04	EER18749	777	Tubulin alpha chain, putative	Perkinsus marinus	5.96e-11	100%
3_H05		297	No significant similarity found			
3_H06		261	No significant similarity found			
3_H07	XP_001817829	283	hypothetical protein	Aspergillus oryzae	4.28e-06	46%
39C		289	No significant similarity found			
4_A01		303	No significant similarity found			
4_A03		241	No significant similarity found			
4_A04		291	No significant similarity found			
4_A08		169	No significant similarity found			
4_A10		303	No significant similarity found			
4_B02	ABQ09367	439	NADH dehydrogenase subunit 3	Crocodylus siamensis	8.92e-01	24%
4_B08		136	No significant similarity found			
4_B09	XP_773060	309	hypothetical protein	Cryptococcus neoformans var. neoformans	4.56e-21	68%
4_B10		125	No significant similarity found			
4_B11	NP_001122340	488	Arf GTPase activating protein 10	Ciona intestinalis	4.37e-03	30%
4_B12		196	No significant similarity found			
4_C01		119	No significant similarity found			
4_C03	XP_001785944	384	predicted protein	Physcomitrella patens subsp. patens	1.34e-04	61%
4_C04	EDV08774	391	vacuolar protease B	Saccharomyces cerevisiae	1.38e-01	54%
4_C08	XP_002627157	322	zinc metalloprotease	Ajellomyces dermatitidis	3.71e-02	26%
4_C09	XP_664835	184	LacOPZ-alpha peptide from pUC9	Cryptosporidium hominis	8.08e-05	100%
4_C10		391	No significant similarity found			
4_C11	XP_663670	426	hypothetical protein	Aspergillus nidulans	3.00e-20	59%
4_D04	>ACA30301	296	putative senescence-associated protein	Cupressus sempervirens	7.38e-35	86%
4_D05		649	No significant similarity found			
4_D10	XP_451954	445	unnamed protein product	Kluyveromyces lactis	9.75e-03	57%
4_D11		342	No significant similarity found			
4_DO9	XP_001247759	209	hypothetical protein	Coccidioides immitis	5.83e-08	45%
4_E06		481	No significant similarity found			
4_E10	YP_605634	590	aldo/keto reductase	Deinococcus geothermalis	1.67e-31	64%
4_E11		387	No significant similarity found			
4_F03	XP_001910788	438	unnamed protein product	Podospora anserina	2.26e-12	29%

4_F08		211	No significant similarity found			
4_F09		234	No significant similarity found			
4_G01	XP_001829026	135	hypothetical protein	Coprinopsis cinerea	4.31e-03	89%
4_G03	BAG97315	617	unnamed protein product	Oryza sativa	1.69e-08	42%
4_G04	XP_001299590	402	hypothetical protein	Trichomonas vaginalis	1.07e-01	44%
4_G11		339	No significant similarity found			
4_H03		234	No significant similarity found			
4_H04	ZP_04234556	538	Tetracycline resistance protein	Bacillus cereus	8.92e-01	24%

4_H05		169	No significant similarity found			
4_H06	XP_001829795	639	hypothetical protein	Coprinopsis cinerea	8.45e-38	50%
4_H08	XP_664835	184	LacOPZ-alpha peptide from pUC9	Cryptosporidium hominis	8.08e-05	100%
4_H09		273	No significant similarity found			
4_H11	ZP_03041609	326	protein of unknown function	Geobacillus sp.	6.71e-04	36%
4_H12		232	No significant similarity found			
41C	NP_069807	473	bile acid-inducible operon protein F (baiF-2)	Archaeoglobus fulgidus	6.93e-01	39%
42c	ABK60177	285	putative reverse transcriptase	Zingiber officinale	4.12e-17	100%
4C	XP_002473987	433	hypothetical protein	Postia placenta	6.22e-10	30%
4H02	YP_766861	528	putative nitrogen metabolite repression regulator	Rhizobium leguminosarum bv. viciae	5.57e-08	30%
5_A_01		168	No significant similarity found			
5_A02		101	No significant similarity found			
5_A04	ZP_05498017	262	arginine biosynthesis bifunctional protein ArgJ	Clostridium papyrosolvens	6.74e-04	64%
5_A06		446	No significant similarity found			
5_A12		415	No significant similarity found			
5_B01	XP_001765628	455	Arl1-family small GTPase	Physcomitrella patens subsp. patens	8.38e-23	59%
5_B03		161	No significant similarity found			
5_B04		127	No significant similarity found			
5_B06	ACO05908	274	cAMP dependent protein kinase regulatory subunit	Mucor circinelloides	4.77e-10	53%
5_B08		201	No significant similarity found			
5_B09	Q59296	173	Catalase	Campylobacter jejuni	1.39e-09	93%
5_B11	ACN10109	220	Ubiquitin	Salmo salar	1.52e-03	91%
5_C02	XP_001215724	474	cerevisin precursor	Aspergillus terreus	3.88e-12	67%
5_C03		275	No significant similarity found			
5_C04		289	No significant similarity found			
5_C06	XP_001670982	777	Hypothetical protein CBG19959	Caenorhabditis briggsae	4.26e-09	48%
5_C07	EEQ35453	578	ZZ type zinc finger domain-containing protein	Microsporum canis	1.43e-08	30%
5_C09	AAF23219	153	putative long-chain-fatty-acid--CoA ligase	Arabidopsis thaliana	3.04e-12	66%
5_C10	XP_001595662	437	hypothetical protein	Sclerotinia sclerotiorum	1.09e-06,	60%
5_C11		538	No significant similarity found			
5_D02		338	No significant similarity found			
5_D04		397	No significant similarity found			

5_D06	XP_001633643	595	predicted proteingb	Nematostella vectensis	1.12e-51	57%
5_D07		294	No significant similarity found			
5_E02		338	No significant similarity found			
5_E08		139	No significant similarity found			
5_E09		208	No significant similarity found			
5_F01		222	No significant similarity found			
5_F07	ABX71761	659	glutamine synthetase	Glomus intraradices	5.26e-56	100%
5_F09	XP_001670407	260	Hypothetical protein	Caenorhabditis briggsae	7.02e-01	39%
5_G01	XP_001731737	368	hypothetical protein	Malassezia globosa	7.30e-03	78%
5_G02	XP_844631	249	amino acid transporter 10	Trypanosoma brucei	4.43e+00	40%

5_G03	XP_786368	393	similar to beclin 1	Strongylocentrotus purpuratus	2.12e-05	52%
5_G07		156	No significant similarity found			
5_G08	XP_002396446	130	hypothetical protein	Moniliophthora perniciosa	6.24e-10	65%
5_G11	YP_001304493	335	hypothetical protein	Parabacteroides distasonis	6.89e-01,	32%
5_H01		336	No significant similarity found			
5_H02		204	No significant similarity found			
5_H03		354	No significant similarity found			
5_H05	XP_001877132	181	predicted protein	Laccaria bicolor	2.97e-15	69%
5_H06	XP_001229682	718	hypothetical protein	Chaetomium globosum	4.42e-31	47%
5_H07	XP_001023614	296	hypothetical protein	Tetrahymena thermophila	4.52e+00	44%
5_H09		136	No significant similarity found			
56c	AAD37832	433	metallothionein-like protein	Jasus edwardsii	4.77e-02	27%
57C	XP_001910694	467	unnamed protein product	Podospora anserina	4.23e-06	53%
6__D01		124	No significant similarity found			
6_A01		214	No significant similarity found			
6_A02		253	No significant similarity found			
6_A04		500	No significant similarity found			
6_A05		596	No significant similarity found			
6_A08		290	No significant similarity found			
6_A09		124	No significant similarity found			
6_A10		237	No significant similarity found			
6_B01		160	No significant similarity found			
6_B03	EEQ29167	565	hscarg protein	Microsporum canis	1.43e-02	43%
6_B05	XP_772638	499	hypothetical protein	Cryptococcus neoformans var. neoformans	4.14e-01,	42%
6_B11	YP_015994	337	F0F1 ATP synthase subunit alpha	Mycoplasma mobile	9.91e+00	36%
6_C06		247	No significant similarity found			
6_C07		169	No significant similarity found			
6_C12		404	No significant similarity found			
6_D05		249	No significant similarity found			

6_D06		258	No significant similarity found			
6_D08	XP_001663040	457	hypothetical protein	Aedes aegypti	1.13e-03	30%
6_D09	XP_001835135	572	40S ribosomal protein S14	Coprinopsis cinerea	8.30e-46,	76%
6_D11	XP_757560	105	hypothetical protein	Ustilago maydis	7.07e-09	82%
6_E01		273	No significant similarity found			
6_E06		120	No significant similarity found			
6_E07	XP_001384486	425	hypothetical protein	Pichia stipitis	2.64e-16	41%
6_F02	XP_002593599	332	hypothetical protein	Branchiostoma floridae	2.78e-26	92%
6_F04		498	No significant similarity found			
6_F05		152	No significant similarity found			
6_F07	XP_759316	299	hypothetical protein	Ustilago maydis	4.48e-16	68%
6_F09	YP_046169	230	protein secretion efflux system ABC transporter ATP-binding/membrane protein	Acinetobacter sp.	2.65e+00	40%

6_G01	XP_001588748	385	hypothetical protein	Sclerotinia sclerotiorum	3.90e-12	45%
6_G03		252	No significant similarity found			
6_G10		136	No significant similarity found			
6_H02		249	No significant similarity found			
6_H06	XP_001833710	497	hypothetical protein	Coprinopsis cinerea	2.26e-44	84%
6_H07	AAD00455	135	heat shock protein 70	Pneumocystis carinii f. sp. carinii	2.21e-07	72%
6_H08	XP_001215073	144	phosphoenolpyruvate carboxykinase	Aspergillus terreus	4.91e-02	83%
6_H09		273	No significant similarity found			
6_H12	EEH16996	430	ketol-acid reductoisomerase	Paracoccidioides brasiliensis	5.39e-46	68%
62c	P20015	459	Full=Proteinase T; Flags: Precursor		9.78e-32	53%
63C	XP_001261930	225	AMP deaminase, putative	Neosartorya fischeri	7.77e+00	51%
7_A02		198	No significant similarity found			
7_A04	XP_571997	275	bud site selection-related protein	Cryptococcus neoformans var. neoformans	2.87e-15	63%
7_A07		147	No significant similarity found			
7_A09		136	No significant similarity found			
7_A11	AAR11779	129	cyclophilin A	Chlamys farreri	8.13e-18	95%
7_A12	ACT82769	181	polyubiquitin	Nicotiana tabacum	2.51e-06	96%
7_B03	ZP_05091409	720	hypothetical protein	Carboxydibrachium pacificum	4.07e+00	34%
7_B05	ZP_03396463	686	hypothetical protein	Pseudomonas syringae pv. tomato	4.80e+00	27%
7_B07	XP_001989813	160	GH18591	Drosophila grimshawi	1.41e-01	36%
7_B08		138	No significant similarity found			
7_B09		112	No significant similarity found			
7_B10		161	No significant similarity found			
7_C01	XP_001832262	291	hypothetical protein	Coprinopsis cinerea	5.73e-03,	66%
7_C06		134	No significant similarity found			
7_C07	ABX52130	434	olfactory receptor 604 (predicted)	Papio anubis	4.47e+00	37%
7_C11		197	No significant similarity found			

7_D01	AAM90675	767	heat shock protein Hsp90	Achlya ambisexualis	1.33e-63	57%
7_D03		239	No significant similarity found			
7_D04	XP_001381397	124	similar to Napsin A aspartic peptidase, partial	Monodelphis domestica	4.65e-13	80%
7_D05	XP_001912404	262	unnamed protein product |emb|CAP59885.1	Podospora anserina	4.10e-01	60%
7_D08	XP_680306	203	hypothetical protein	Plasmodium berghei	7.68e+00	35%
7_D11		369	No significant similarity found			
7_E01		222	No significant similarity found			
7_E03	XP_002430829	277	scabrous protein, putative	Pediculus humanus corporis	4.46e+00,	36%
7_E04	XP_001834700	401	predicted protein	Coprinopsis cinerea	9.75e-03	51%
7_E05	XP_001263957	598	mating-type protein MAT1-2	Neosartorya fischeri	4.54e+00	46%
7_E07		245	No significant similarity found			
7_E08	XP_001223491	561	hypothetical protein	Chaetomium globosum	8.85e-02	30%
7_E09		387	No significant similarity found			
7_E10	XP_676548	225	hypothetical protein	Plasmodium berghei	5.95e+00	38%

7_E11	XP_002585310	279	predicted protein	Uncinocarpus reesii	2.20e-07	42%
7_F03		155	No significant similarity found			
7_F04	XP_001321831	485	hypothetical protein	Trichomonas vaginalis	1.56e+00	29%
7_F10	AAU43212	687	actin	Phalansterium solitarium	7.22e-04	96%
7_F11	XP_001983483	266	GH15554	Drosophila grimshawi	7.71e+00	30%
7_F12	EEY18608	786	C2H2 finger domain containing protein FlbC	Verticillium albo-atrum	4.98e-21	72%
7_G01	XP_001822192	423	hypothetical protein	Aspergillus oryzae	3.69e-10,	47%
7_G03	YP_002720507	560	hypothetical hypothetical protein	Brachyspira hyodysenteriae	2.04e-01	33%
7_G04	XP_367364	517	glycogen synthase	Magnaporthe grisea	7.83e-25	51%
7_G05		201	No significant similarity found			
7_G06	NP_001016124	209	interferon-related developmental regulator 1	Xenopus (Silurana) tropicalis	3.31e-03	34%
7_G07	XP_002542314	242	calcium/calmodulin-dependent protein kinase	Uncinocarpus reesii	7.62e-08	57%
7_G10	XP_002155447	672	similar to zonadhesin	Hydra magnipapillata	4.42e-19	52%
7_H06	XP_001637380	577	predicted protein	Nematostella vectensis	1.21e-15	65%
7_H12	XP_001729946	684	hypothetical protein MGL_2932	Malassezia globosa	1.56e-03	53%
75	NP_730591	845	skuld, isoform E	Drosophila melanogaster	1.44e+00	27%
77C	XP_001829157	338	predicted protein lk	Coprinopsis cinerea	2.43e-14	50%
8_A02		373	No significant similarity found			
8_A03	Q59296	173	Catalase	Campylobacter jejuni	1.39e-09	93%
8_A06	XP_002611728	407	hypothetical protein	Branchiostoma floridae	2.36e-25	53%
8_A08	XP_970073	456	similar to serine/threonine protein kinase	Tribolium castaneum	4.45e+00	34%
8_A09		156	No significant similarity found			
8_A11		330	No significant similarity found			
8_B01		222	No significant similarity found			
8_B05	EEU38190	184	hypothetical protein	Nectria haematococca	1.68e-02	39%

8_B07		305	No significant similarity found			
8_B08		321	No significant similarity found			
8_B10		395	No significant similarity found			
8_B11		245	No significant similarity found			
8_C03	XP_002047433	338	GJ13437	Drosophila virilis	4.45e+00	36%
8_C07	XP_001428970	620	hypothetical protein	Paramecium tetraurelia	1.72e+00	29%
8_C10		430	No significant similarity found			
8_C12	ZP_02612185	301	putative membrane protein	Clostridium botulinum	7.66e+00	32%
8_D01		298	No significant similarity found			
8_D02	XP_002158150	811	similar to uncharacterized hypothalamus protein HT010, partial	Hydra magnipapillata	2.47e-02	30%
8_D06	XP_001878372	842	predicted protein	Laccaria bicolor	1.75e-43	68%
8_D08	>XP_759384	263	40S ribosomal protein S20	Ustilago maydis	1.35e-12,	70%
8_D09	XP_570849	202	endopeptidase	Cryptococcus neoformans var. neoformans	1.76e-20	94%
8_D10		123	No significant similarity found			
8_E_04	XP_002283214	527	glutathione S-transferase 3	Vitis vinifera	1.64e-20	41%
8_E05	AAN35142	189	alpha-tubulin	Nowakowskiella elegans	9.17e-09	100%

8_E06		241	No significant similarity found			
8_E12		201	No significant similarity found			
8_F07	XP_001945715	647	similar to Glycerol-3-phosphate dehydrogenase	Acyrthosiphon pisum	1.34e-56,	82%
8_F08	ABM92196	387	tubulin, alpha 3	synthetic construct	4.12e-38	92%
8_F09	CAR65717	236	DEHA2D19008p	Debaryomyces hansenii	5.89e+00	33%
8_F10		380	No significant similarity found			
8_F12		389	No significant similarity found			
8_G07	ZP_01730719	536	hypothetical protein CY0110_06274	Cyanothece sp.	4.66e-20	56%
8_G08	YP_002355004	248	Glutathione S-transferase domain protein	Thauera sp.	5.98e-13	66%
8_H02	XP_001877094	413	predicted protein	Laccaria bicolor	1.43e-14	40%
8_H04		129	No significant similarity found			
8_H09	XP_001525198	272	hexokinase	Lodderomyces elongisporus	5.85e-08,	40%
8_H11	XP_745554	477	hypothetical protein	Plasmodium chabaudi chabaudi	8.97e-01	38%
88C	XP_001008343	553	hypothetical protein	Tetrahymena thermophila	1.67e+00	45%
89C	XP_001879447	473	predicted protein	Laccaria bicolor	5.66e-11	34%
9__E03	XP_385327	615	hypothetical protein	Gibberella zeae	2.14e-03	31%
9_A09	XP_001728741	576	hypothetical protein	Malassezia globosa	9.19e-24	68%
9_B05	NP_595707	213	transcription factor Atf21	Schizosaccharomyces pombe	5.07e-04,	42%
9_B09		102	No significant similarity found			
9_B11	ABB90955	420	elongation factor 1-alpha	Glomus intraradices	9.98e-48	99%
9_C01		266	No significant similarity found			
9_C06	ACU18400	153	unknown	Glycine max	6.36e-02	56%
9_C09	XP_761073	446	hypothetical protein	Ustilago maydis	3.34e-11	58%

9_D01	NP_001116984	460	rab11 GTPase homolog SUrab11p	Strongylocentrotus purpuratus	7.00e-38	90%
9_D03	XP_682393	169	hypothetical protein	Aspergillus nidulans	1,07E-01	85%
9_D06		229	No significant similarity found			
9_E01	CAB88663	259	argininosuccinate lyase	Agaricus bisporus	3.14e-08	81%
9_E07		162	No significant similarity found			
9_E09	XP_801913	468	hypothetical protein isoform 2	Strongylocentrotus purpuratus	3.55e-21	44%
9_F05	XP_002565512	450	Pc22g15960	Penicillium chrysogenum	2.30e-04,	51%
9_F06		292	No significant similarity found			
9_F09	ZP_04047349	297	predicted oxidoreductase similar to aryl-alcohol dehydrogenase	Brachyspira murdochii	3.00e-04	52%
9_G03	NP_001076115	372	inter-alpha (globulin) inhibitor H1	Oryctolagus cuniculus	2.41e-01,	44%
9_G05		292	No significant similarity found			
9_G08	EDP56321	247	P-type calcium ATPase, putative	Aspergillus fumigatus	7.55e-16,	54%
9_G09	XP_002152882	266	RING finger protein, putative	Penicillium marneffei	4.99e-07,	38%
9_G10	XP_001771315	288	predicted protein	Physcomitrella patens subsp. patens	1,66E-18	78%
9_G12	XP_002475016	324	predicted protein	Postia placenta	3.45e-08	48%
9_H04	XP_002416916	292	homoserine dehydrogenase	Candida dubliniensis	5.04e-23	62%
9_H07	XP_001877844	489	glyoxal oxidasegb	Laccaria bicolor	6.29e-02	33%
9_H09		328	No significant similarity found			

98C	XP_001592989	500	hypothetical protein	Sclerotinia sclerotiorum	1.42e-01	58%
9C	EEQ83856	210	hemolysin-III channel protein Izh2	Ajellomyces dermatitidis	1.33e-12,	62%
c_1		255	No significant similarity found			
c_10		150	No significant similarity found			
c_11		307	No significant similarity found			
c_12	XP_001645325	715	hypothetical protein	Vanderwaltozyma polyspora	2.76e-09	27%
c_13	YP_740739	287	heme maturase	Tetrahymena malaccensis	7.80e+00	33%
c_14		741	No significant similarity found			
c_15		120	No significant similarity found			
c_16		159	No significant similarity found			
c_17		138	No significant similarity found			
c_18	ACJ04669	458	glycoprotein	Iris yellow spot virus	5.79e+00,	70%
c_19	XP_757513	545	hypothetical protein UM01366.1	Ustilago maydis	3.70e-66	92%
c_2	XP_001729651	487	hypothetical protein	Malassezia globosa	1.73e-28	51%
c_20		196	No significant similarity found			
c_21	XP_001386405	588	hypothetical protein	Pichia stipitis	5.17e-07	31%
c_22		201	No significant similarity found			
c_23	AAW82439	508	guanine nucleotide binding protein beta subunit	Thanatephorus cucumeris	2.40e-63,	81%
c_24		249	No significant similarity found			
c_25	ZP_00143234	515	hypothetical protein	Fusobacterium nucleatum subsp. vincentii	1.35e+00	32%
c_26	NP_000773	339	cytochrome P450 family 24 subfamily A polypeptide 1 isoform 1 precursor	Homo sapiens	1.52e+00,	33%

c_27		274	No significant similarity found			
c_28		191	No significant similarity found			
c_29	XP_002499194	147	ZYRO0E06248p |emb|CAR30939.1| ZYRO0E06248p	Zygosaccharomyces rouxii	8.63e-07	65%
c_3	YP_1527069	292	aldo/keto reductase	Azorhizobium caulinodans	5.36e-01	40%
c_30	NP_690845	299	Mitochondrial protein of unknown function	Saccharomyces cerevisiae	1.40e-01	50%
c_31	XP_001544351	207	heat shock 70 kDa protein 7	Ajellomyces capsulatus	3.10e-01	92%
c_32		190	No significant similarity found			
c_33	XP_002031750	691	GM26172	Drosophila sechellia	3.75e+00,	30%
c_34		304	No significant similarity found			
c_36	XP_001765643	533	predicted protein	Physcomitrella patens subsp. patens	2.38e-22,	41%
c_37	XP_002618663	464	predicted protein	Clavispora lusitaniae	5.65e+00	50%
c_39	XP_001371508	238	hypothetical protein	Monodelphis domestica	1.39e-09	84%
c_4		224	No significant similarity found			
c_40	XP_002004468	477	GI19951	Drosophila mojavensis	7.59e+00	35%
c_41	XP_780136	286	similar to Im:7148063 protein	Strongylocentrotus purpuratus	9.18e-01	56%
c_42	XP_001606326	683	similar to GA19427-PA	Nasonia vitripennis	6.17e+00	45%
c_43		294	No significant similarity found			
c_44	XP_002045681	423	GM16395	Drosophila sechellia	1.39e-33	97%
c_45		467	No significant similarity found			
c_46		449	No significant similarity found			

c_47		502	No significant similarity found			
c_48		161	No significant similarity found			
c_49	ACT78499	537	cysteine/serine protease	Porcine reproductive and respiratory syndrome virus	9.87e+00	46%
c_5	ZP_05303437	642	sulfotransferase	Methanocaldococcus vulcanius	1.87e+00	29%
c_50		146	No significant similarity found			
c_51	YP_212901	220	hypothetical protein	Bacteroides fragilis	5.58e-07	55%
c_52	EEQ28156	177	alpha-centractin	Microsporum canis	4.01e-09	79%
c_53		176	No significant similarity found			
c_54	XP_001799670	372	hypothetical protein SNOG_09375	Phaeosphaeria nodorum	2.65e-16	64%
c_55	XP_759979	343	hypothetical protein UM03832.1	Ustilago maydis	4.55e+00	35%
c_56	XP_001470964	643	conserved hypothetical protein	Tetrahymena thermophila	4.92e-01,	30%
c_57		257	No significant similarity found			
c_58	XP_759578	260	hypothetical protein UM03431.1	Ustilago maydis	3.59e-13	55%
c_59	XP_001731772	228	hypothetical protein	Malassezia globosa	2.65e-16	98%
c_6		651	No significant similarity found			
c_60		212	No significant similarity found			
c_61		209	No significant similarity found			
c_62	AAA93293	314	mobilization (Mob)/recombination (Pre) protein	Listeria monocytogenes	7.79e+00,	30%

c_63	XP_002547626	300	hypothetical protein CTRG_01933	Candida tropicalis	3.77e-15,	52%
c_64	XP_002374655	452	CP2 transcription factor, putative	Aspergillus flavus	1.26e-10	37%
c_65	YP_001345069	302	high-affinity zinc transporter periplasmic component	Actinobacillus succinogenes	1.40e-01	30%
c_66		456	No significant similarity found			
c_67	XP_001881146	187	eu2.Lbscf0012g03030	Laccaria bicolor	1.09e-01	72%
c_68	XP_774968	212	hypothetical protein CNBF1320	Cryptococcus neoformans var. neoformans	1.10e-06	48%
c_69		547	No significant similarity found			
c_7		397	No significant similarity found			
c_70	YP_155013	498	glutathione S-transferase-like protein	Idiomarina loihiensis	4.57e-08	44%
c_71		136	No significant similarity found			
c_72		170	No significant similarity found			
c_73	ABJ98722	172	heat shock protein 71	Perna viridis	3.08e-17,	96%
c_74		136	No significant similarity found			
c_75	ACM89242	102	histone H3A	Meta ovalis	6.83e-12	100%
c_76		173	No significant similarity found			
c_77		195	No significant similarity found			
c_78		166	No significant similarity found			
c_79		143	No significant similarity found			
c_8	XP_758864	703	hypothetical protein UM02717.1	Ustilago maydis	1.10e-06	89%
c_80		468	No significant similarity found			
c_81	XP_570002	355	hypothetical protein	Cryptococcus neoformans var. neoformans	4.45e-24	68%
c_82	YP_003005810	811	Glutathione transferase	Dickeya zeae	3.20e-34	58%

c_83	XP_002514260	302	Galactose oxidase precursor, putative	Ricinus communis	6.92e-01	41%
c_84		138	No significant similarity found			
c_85	YP_478184	694	hypothetical protein	Synechococcus sp.	2.28e-05	49%
c_86	BAB09331	542	unnamed protein product	Arabidopsis thaliana	9.72e-03	46%
c_87	ACJ11224	474	vacuolar serine protease	Cladosporium cladosporioides	2.88e-07	77%
c_88		145	No significant similarity found			
c_9		349	No significant similarity found			

To validate the presence of some of these transcripts in *G. intraradices* BE3 and determine their temporal pattern of expression, we conducted reverse-transcriptase PCR (RT-PCR) in a random group of genes at different developmental phases during the establishment of the AM symbiosis (Figure 1 and Supplementary Table 2). These phases include fungal spores at the time of germination (asymbiotic phase), germinated spores during early hyphal growth (pre-symbiotic phase), initial penetration of fungal hyphae into plant roots prior to the appearance of arbuscular structures (symbiotic phase), and fully established AM symbiosis showing external hyphae with second generation spores and well developed arbuscular structures within plant roots (extradical phase). Most genes were expressed either before or at the time of spore germination, confirming that all of them are transcribed during the presymbiotic phase of hyphal growth. Whereas most transcripts could also be detected during the extradarical phase of the life cycle, only 2 out of 8 were expressed at symbiosis, suggesting that an important shift in gene expression distinguishes the pre-symbiotic and symbiotic phases. These results indicate that transcripts identified in our presymbiotic cDNA library are expressed in germinating spores of *G. intraradices* BE3.

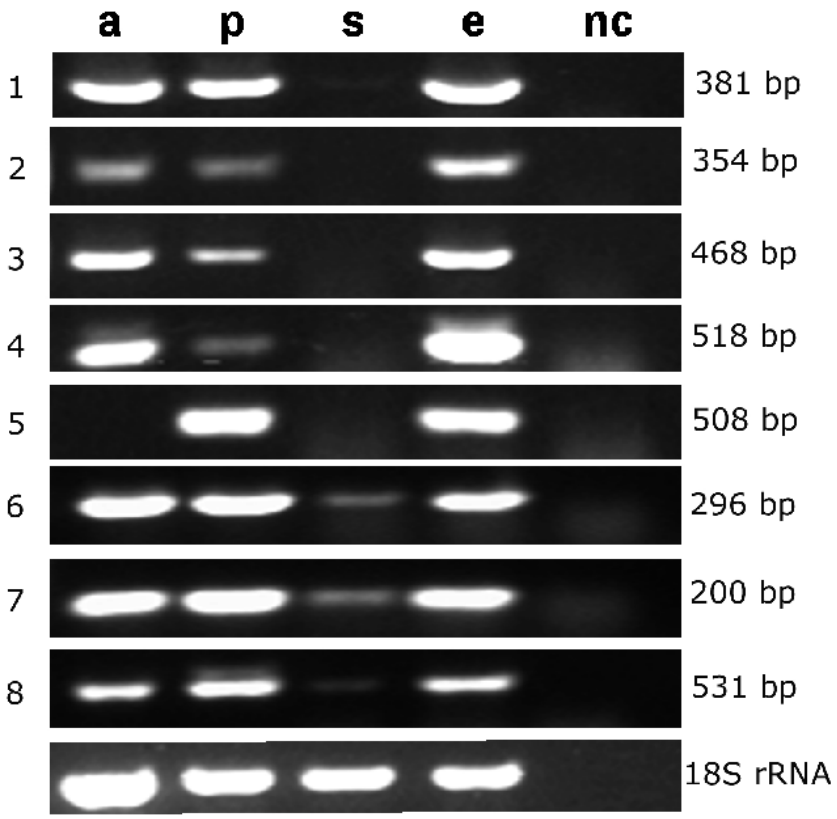

Figure 1. RT-PCR of genes expressed during the *G. intraradices* BE3 life cycle. cDNA amplification was performed during the asymbiotic (a), presymbiotic (p), symbiotic (s), and extraradical (e) phase of the fungal life cycle (see text for details), and (nc) negative control. (1) Hypothetical Protein homologue of *Cryptococcus neoformans* XP570866.1; (2) Hypothetical Protein homologue of *Schizosaccharomyces pombe* NP594031.1; (3) Hypothetical Protein homologue of *Caenorhabditis briggsae* CAE71635.1; (4) Hypothetical Protein homologue of *Coprinopsis cinerea* EAU86123.); (5) Hypothetical Protein homologue of *Botryotinia fuckeliana* XP001557861.1; (6) Hypothetical Protein homologue of *Coprinopsis cinerea* EAU88851.1; (7) Hypothetical protein with no significant homology; (8) Hypothetical Protein homologue of *Yarrowia lipolytica* XP502676.1.

Supplementary Table 2. Primer sequence, annealing temperatures, and predicted size of amplification products described in this study.

Primer name	Sequence	Temperature (°C)	Size (bp)	Reference
PBR42f	TACACTTTATGCTTCCGGCTC	60	136	This study
PBR42r	AGCACACTGGCGGCCGTTAC			
fBC2	ACACTTTATGCTTCCGGCTC	60	246	This study
rBC2	AGCATATGGGAGCCGATCAG			
18S fGi*	ACGACACCGGAGAGGGAGCC	60	197	This study
18S rGi*	CGGCTGCTGGCACCAGACTT			
phzfD*	TCGTTCATGCTGCCAGGTTG	60	152	This study
phzrD*	CTGCTGGTGCATGACATGCA			
rD1	AAGGAGGTGATCCAGCC	55	1500	Weisburg *et al*., 1991
FD1	AGAGTTTGATCCTGGCTCAG			
RCNN3c	CCAGTATCCATTCAAAATAC	59	381	This study
FCNN3c	GAAACTTGGCTTGAAGGTTG			
RSP44	TGATCGCTCGTCAGTTATC	59	354	This study
FSP44	CGGCAGACATGAATTGAAG			
RCB47	CTGCGAACCATGTCATCAG	59	468	This study
FCB47	GCAACAAATCAACTTCTCC			
RCCO60	CTTTATATTCAGTCTGCGC	59	518	This study
FCCO60	CGTTTGTTCAAACGGTTCC			
RBF79	GTAAGTGTAATGAGGAGAC	59	508	This study
FBF79	CATTGGAATGGCCATAAG			
RCCO55	GTCCTGGCCAATACATTTAC	55	296	This study
FCCO55	CCATCTTTTAGACTTCATC			
Rno71	GAGTATGTATTTATACCAC	55	200	This study
Fno71	CAAAGAGCTAAGCTAATAAG			
RYL129	CGACACCTAATTTCAAAGC	59	531	This study
FYL129	CGTTAACTAAGCAATGGA			

Expression of *GintPbr*1a, a gene encoding a protein homolog of Phenazine Biosynthesis Regulator (Pbr)

We identified a cDNA clone with high homology to *Phenazine Biosynthesis Regulator* (*Pbr*), a transcription regulator of the *Burkholderia cenocepacia* complex required for the expression of *phzD* and *phzF*, two genes encoding proteins with significant homology to *Pseudomonas choloroaphis* PhzD and PhzF and involved in phenazine biosynthesis. We named this sequence *GintPbr*1a (for *Glomus intraradices* Pbr protein 1a; Franken P. 2002). The cDNA includes a transcript sequence corresponding to a 57-amino acid residue containing a predicted helix-residue-helix motif that is commonly found on prokaryotic transcriptional regulators of *Burkholderia cenocepacia* (Figure 2A). At the amino acid level, both sequences only differ at Pbr amino acid positions 27, in which glycine (G) is replaced by alanine (A), and positions 29 to 32, in which an arginine-valine-valine (R-V-V) motif is replaced by threonine-isoleucine-glycine-isoleucine (T-I-G-I). The strong conservation of the amino acid sequence between Pbr and *GintPbr*1a suggested that both proteins could play equivalent biochemical roles, despite being present in bacteria with highly divergent biological habits.

To confirm the expression of *GintPbr*1a within cells of *G. intraradices* BE3 and determine its temporal pattern of activity, we conducted RT-PCR at previously described developmental phases during the establishment of the AM symbiosis. *GintPbr*1a was abundantly expressed at the presymbiotic phase and weakly at the extraradical phase, but not in the asymbiotic or symbiotic phase (Figure 2B), suggesting that its expression is mainly restricted to initial stages of hyphal growth, before the establishment of the AM symbiosis. These results validated the expression of the *Burkholderia Pbr* homolog in *G. intraradices* BE3, and indicated that its transcriptional activity occurs mainly during the presymbiotic phase of the fungal life cycle.

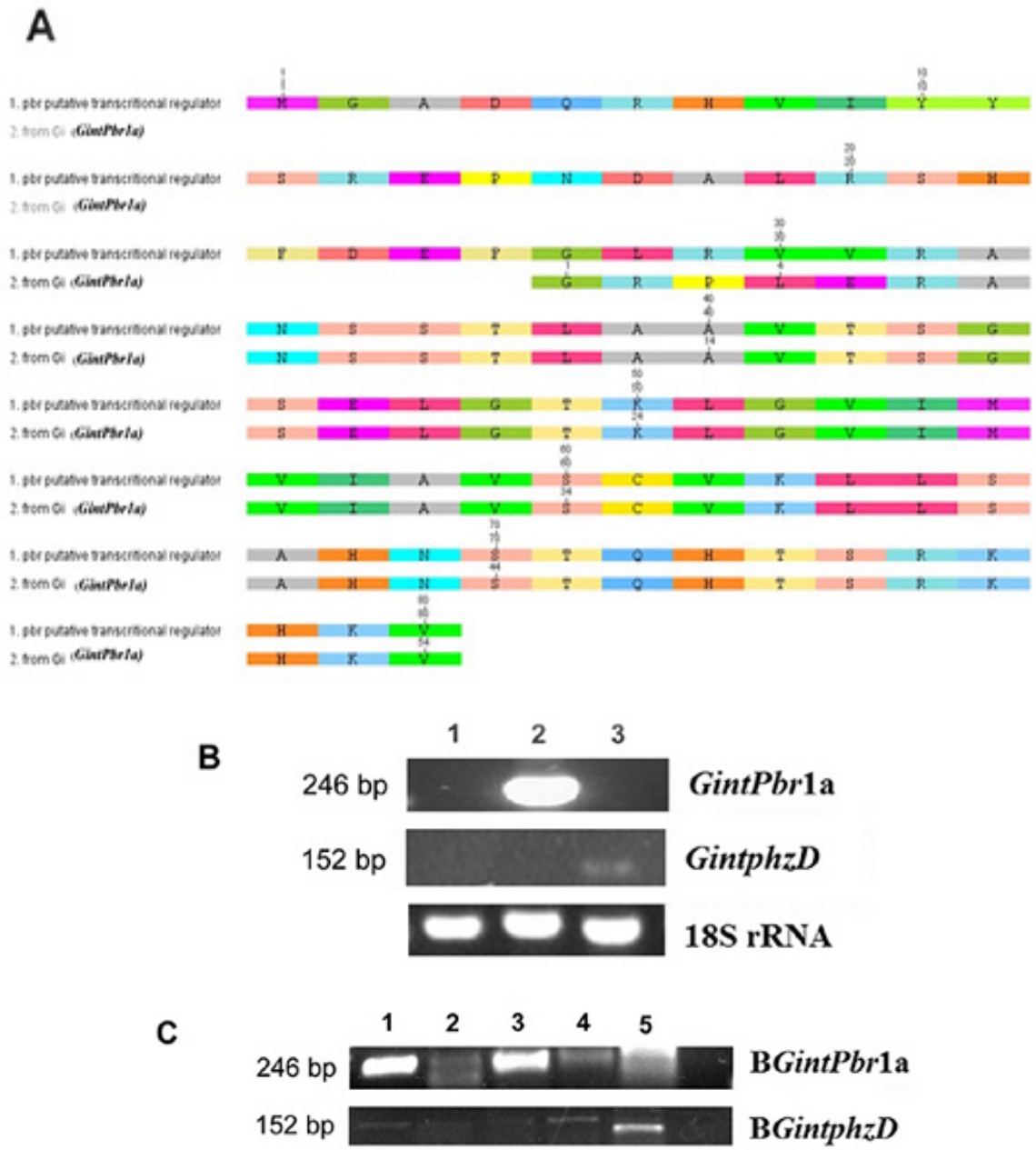

Figure 2. Expression of a phenazine biosynthetic pathway in endocellular bacteria of *Glomus intraradices* BE3. (A) Predicted amino acid alignment of *GintPbr*1a of *G. intraradices* BE3 and *Pbr* of *Burkholderia cenocepacia.* (B) RT-PCR expression of *GintPbr*1a and *BGintphzD* in *G. intraradices* BE3; cDNA amplification was performed during the asymbiotic (1), presymbiotic (2) and, symbiotic (3), phase of the fungal life cycle (see text for details). (C) Genomic PCR amplification of *BGintPbr*1 (for bacterial *GintPbr*1a) and *BGintphzD* (for bacterial *GintphzD*) in a bacteria isolated from spores of *G. intraradices* BE3. Whereas lane 1 represents the isolate BG1 from *G. intraradices* BE3, lanes 2 to 5 represent isolates BG3, BG4, BG7 and BG10 from *G.margarita* BE2, respectively.

Bacterial isolates from *in vitro* cultivated *G. intraradices* spores express genes involved in phenazine biosynthesis

The molecular nature and the pattern of expression of *GintPbr*1a suggested that the corresponding gene could have a bacterial origin within the AM fungus considering that the cDNA library was generated using polyadenylated primers that can potentially target prokaryotic transcripts present within spores and hyphae. To determine if the genome of a potential bacterial symbiont could contain a *GintPbr*1a gene, we isolated bacterial cultures originating from sterilized spore inocula of *G. intraradices* BE3, and of *Gigaspora margarita* BE2 under *in vitro* culture and variable pH conditions. Whereas one bacterial isolate could be recovered from *G. intraradices* BE3 (pH range from 4 to 7), four were recovered from *G. margarita* BE2 (pH range from 4 to 9). After the extraction of sufficient DNA from these isolates, and to attempt their taxonomic identification, we amplified and sequenced PCR products of approximately 1.5 kb corresponding to the conserved genomic DNA 16S ribosomal subunit (Table 2; Weisburg *et al*., 1991). Comparison to publicly available genomic databases revealed that the isolate recovered from *G. intraradices* BE3 corresponds to a previously described "uncultured bacteria" that we named BG1, whereas isolates from *G. margarita* BE2 correspond to presumed *Brevibacillus sp.* (BG10), *Paenibacillus sp.* (BG4), and two uncultured bacterium (BG3 and BG7), respectively.

To find if some of these bacteria could include a *GintPbr*1a copy in their genome, we attempted the amplification of a 246 bp fragment using genomic DNA from all 5 previously cultivated bacterial isolates. A PCR product of the correct size was only amplified from BG1, the bacterial isolate from *G. intraradices* BE3. (Figure 2C). PCR products derived from BG4 and BG10 were also amplified, but their molecular size did not correspond to the predicted *GintPbr*1a fragment, indicating that a different genomic version of a *Pbr*-like gene could be present in the genome of some *G. margarita* BE2 endosymbionts. We also conducted genomic PCR amplification with primers specific to *phzD*, a gene that is under transcriptional control of *Pbr* in *Burkholderia*. As expected, a PCR product was amplified in BG1 (Figure 2C), confirming that key regulatory enzymes of the phenzine biosynthetic pathway are encoded in the genome of bacteria isolated from in vitro cultures of *G. intraradices* BE3 spores. A PCR product was also derived from BG7 and BG10, confirming that isolates from *G. margarita* BE2 are also likely to express the same pathway. Finally, we conducted RT-PCR to determine if *G. intraradices* BE3 could express a homologue of *phzD,* a gene encoding a protein D isochorismatase phenazine biosynthetic enzyme involved in the pathway of pyocianin (Komatsu et al., 2003). As shown in Figure 2B, a gene encoding a *phzD* ortholog is expressed at the symbiotic phase, confirming that a phenazine transcriptional regulatory pathway is active in *G. intraradices* BE3.

Table 2. Identification of bacteria derived from spores of *G. intraradices* BE3 and *G. margarita* BE2.

Name	Lenth (pb)	Phylogenetic relationship and GenBank accession number	Isolated from	Similarity %	E-value	Identity %
BG10	1523	*Brevibacillus centrosporus* (AB112719.1)	*G. margarita*	97	0.0	99
BG4	1575	*Paenibacillus graminis* (AB428571.1)	*G. margarita*	98	0.0	98
BG3	1523	Uncultured bacterium (EU236261.1)	*G. margarita*	98	0.0	99
BG7	1524	Uncultured bacterium (EU560794.1)	*G. margarita*	98	0.0	96
BG1	1434	Uncultured bacterium GU223217.1	*G. intraradices*	100	0.0	99

DISCUSSION

To explore the molecular mechanisms that prevail during the establishment of the AM symbiosis involving the genus *Glomus*, we initiated a systematic global expression analysis of early phases of the *G. intraradices* life cycle. The large amount of expressed genes found at presymbiotic stages confirmed that fungal cells are active during early hyphal growth, and contain a wide diversity of transcripts with homology to eukaryotic and prokaryotic genes. While some of the identified transcripts with homology to prokaryotic genes (such as a sulfotransferase, a pyrophosphastase, and several reductases) could reflect global housekeeping functions related to the general metabolism, other expressed genes such as a G protein with a DNA binding domain, a high affinity Zn transporter, or a nitrogen metabolism negative regulator, could suggest that a cross-talk between trancriptionally active bacterial cells and fungal cells occurs early during the fungal life cycle. While a large group of hypothetical and unknown proteins requires further annotation and functional elucidation, additional prokaryotic expressed genes include those related to the biosynthesis of essential components such as amino acids, and those involved in the protection against potential pathogens or the production of antibiotics.

Phenazines are secondary metabolites of bacterial origin that have been implicated in the control of plant pathogens, contributing to the ecological fitness and pathogenicity of the producing strains. While the evolution and distribution of phenazine genes has revealed that they are mainly found in soil-dwelling or plant associated bacterial species (Mavrodi et al., 2010), their presence in the genome of AM fungal species had not been reported. The establishment of a pH gradient-based protocol allowed the isolation of bacterial colonies from *G. intraradices* BE3 spores. Although endocellular bacteria have not been reported in *G. intraradices*, this type of endosymbionts have been reported in other *Glomus* species (Naumann *et al*., 2010). The identification of a numerous transcripts with homology to eukaryotic genes, associated to the recovery of bacterial colonies from spores, suggests that *G. intraradices* BE3 indeed could contain bacterial cells within hyphae. While their taxonomic identification remains elusive, successful cultivation and DNA extraction confirmed that both *Pbr* and *phzD* homologs are present in genomic DNA extracted from *G. intraradices* spores and its associated bacterial isolates.

Our overall results indicate that a phenazine biosynthetic pathway is active during the *G. intraradices* BE3 life cycle.

They also suggest that this pathway is not exclusive of G. *intraradices*, but is also active in *G. margarita*. As in previous studies of the biochemical mechanisms that regulate phenazine biosynthesis in *Burholderia* and *Pseudomonas* (Laursen *et al*., 2004; Mavrodi *et al*., 2001; Parsons *et al*., 2004), *GintPbr*1a is transcriptionally active at developmental stages that precede the expression of *GintphzD*, a result in agreement with the role of *GintPbr*1a as a pleiotropic transcriptional regulator necessary for the activation of phenazine biosynthetic enzymes (Ramos *et al*., 2010), presuming that *GintPbr*1a transcripts precede the translation of the corresponding protein at subsequent developmental stages. Interestingly, initial expression of *GintphzD* occurs at the onset of the establishment of the fungal-plant symbiotic interaction, suggesting that phenazine production could play a role a later stages of the AM symbiosis establishment; however, a detailed molecular and biochemical analysis will be require to confirm the presence of endocellular bacteria in hyphal cells, as well as the presence of phenazines within roots before elucidating the physiological role of these molecules during AM symbiosis. Taken together, our results open new possibilities for using *G. intraradices* BE3 as a model system to study the molecular and biochemical mechanisms that allow the successful establishment of the tripartite AM symbiosis.

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