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ORIGINAL PAPER

Identification of differentially expressed genes of the fungus *Hydnangium* sp. during the pre-symbiotic phase of the ectomycorrhizal association with *Eucalyptus grandis*

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Abstract The pre-symbiotic phase that precedes physical contact between symbionts is a crucial phase in determining their compatibility, allowing the formation of the ectomycorrhiza. A subtractive cDNA library representing the differentially expressed genes of the fungus *Hydnangium* sp. in the pre-symbiotic phase was constructed using fungal mycelia obtained through the *in vitro* mycorrhization technique. The fungus was cultured in the presence of *Eucalyptus grandis* roots, but with no contact between the hyphae and the root system of the host plant. Genes that code for proteins related to carbohydrate, amino acid, and energy metabolisms, transcription, and protein synthesis, cellular communication, signal transduction, stress response, transposons, and proteins related to the biogenesis of cell components were identified among the 131 expressed sequence tags. Expression of the genes that code for acetyl-CoA acetyltransferase, pyruvate dehydrogenase, ATP synthase, a voltage-dependent protein from the selective ion channel, and hydrophobin was evaluated by the RT-qPCR technique, confirming the activation of these genes in this phase of the association.

Keywords Ectomycorrhiza · *Hydnangium* sp. · Pre-symbiotic phase · Gene expression · Suppression subtractive hybridization

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Introduction

Fungi in the ectomycorrhizal genus *Hydnangium* are Basidiomycetes belonging to the family Hydnangiaceae and are closely related to *Laccaria*; 70 *Hydnangium* species with specificity to *Eucalyptus* have been described (Malajczuk et al. 1982; Malajczuck and Hartney 1986; http://www.indexfungorum.org/Names/Names.asp). *Hydnagium* is widespread in Australia, New Zealand, Portugal, Spain, and USA (Malajczuk et al. 1982; Chu-Chou and Grace 1983; http://zipcodezoo.com/) and in 2004 was first reported in Brazil associated with commercial plantations of *Eucalyptus grandis* in Minas Gerais state. The *Hydnangium* sp. found in Brazil forms a red basidiocarp that may be hypogeous or sub-epigeous. Hyphae isolated from these sporocarps grow rapidly *in vitro* and display clamp connections and hyaline septa and produce rhizomorphs (Campos 2004).

Ectomycorrhiza formation may be divided into four stages: pre-infection, colonization, differentiation, and functioning. In the pre-infection stage, also called pre-symbiotic, plant roots and fungi exchange signals in the soil in order to determine symbiotic compatibility (Martin et al. 2007). After mutual recognition, the roots and the fungal mycelium start to grow and establish physical contact. Finally, the hyphae gradually grow, and the fungal mycelium undergoes differentiation inside the root cortex, forming the functional mycorrhiza (Smith and Read 1997; Tagu et al. 2002; Martin et al. 2007).

In contrast to some plant–microorganism interactions where the nature of the signal molecules and the process of perception and transduction are well known, more investigation is needed regarding the ectomycorrhizal association

(Menotta et al. 2004; Martin et al. 2007). Studies aiming to identify the genes related to the events that occur during the pre-symbiotic phase have been conducted (Podila et al. 2002; Krüger et al. 2004; Menotta et al. 2004; Zaretsky et al. 2006; Frettinger et al. 2007; Acioli-Santos et al. 2008; Heller et al. 2008). Detecting these events is crucial to understanding the establishment of the ectomycorrhizal association.

Physiological and morphological changes observed during ectomycorrhizal development are concurrent to changes in gene expression for both partners, starting before any physical contact (Voiblet et al. 2001; Duplessis et al. 2005; Le Quéré et al. 2005). The establishment of ectomycorrhizal symbiosis is activated by signals produced by both partners. These signals lead to morphological changes and a complex development of other specific structures in the plant and in the fungus (Martin et al. 2007). Among the diverse techniques used to evaluate gene expression, suppression subtractive hybridization allows obtains transcripts that are only expressed in one situation through the comparison of two mRNA populations (Diatchenko et al. 1996). This technique has been successfully used in the identification of differentially expressed genes in the pre-symbiotic phase of the ectomycorrhizal associations in other systems, such as *Laccaria bicolor* and *Pinus resinosa*, *Piloderma croceum* and *Quercus robur*, and *Tuber borchii* and *Tilia americana* (Podila et al. 2002; Krüger et al. 2004; Menotta et al. 2004).

The objectives of our study were to construct a cDNA library through the suppression subtractive hybridization technique representing the differentially expressed genes of *Hydnangium* sp. in the pre-symbiotic phase of association with *E. grandis*, identify genes expressed in this phase of the association, and improve the understanding of the molecular mechanisms involved in the ectomycorrhizal association.

Material and methods

Microorganism and culture conditions

Basidiocarp of *Hydnangium* was collected under commercial plantation of *E. grandis* in November, 2003 in the Minas Gerais State, Brazil (Campos 2004). The specimen was deposited in the herbarium of Federal University of Viçosa (Herbarium VIC) with the accession number VIC 31272. The fungal cultures were maintained in Petri dishes containing modified Melin Norkrans (MMN) medium (Marx 1969) at 28°C and transferred to new culture media every 30 days.

In vitro interactions between *Hydnangium* sp. and *E. grandis*

E. grandis seeds (0.4–0.7 mm in diameter) were immersed for 1 min in 70% ethanol, rinsed with sterilized water, superficially disinfected with 20% hydrogen peroxide for 6 min, and rinsed again three times with sterilized water. After superficial disinfection, the seeds were transferred to dishes containing 30 mL of synthesis medium (Burgess et al. 1996), covered with cellophane membrane, and incubated at 28°C for 3 days. After germination, five seedlings were transferred to a growth chamber at 25°C and 16 h of light at an intensity of 80 μmol $m^{-2}s^{-1}$ photons. After 15 days, the roots of the seedlings were covered with cellophane membrane and inoculated with 10-mm agar disks containing mycelium removed from the borders of *Hydnangium* sp. colonies cultured in MMN medium for 7 days at 28°C. Petri dishes with fungal isolates and no seedlings were maintained in the same conditions. The dishes remained in the growing chamber for 15 days, and afterward, the mycelium was collected and stored at −80°C. Therefore, two treatments were established: fungus cultured in the presence of the plant (tester sample) and fungus cultured in the absence of plant (driver sample).

Construction of the suppressive subtractive library

Total RNA extraction of fungal mycelium cultured in both treatments (tester and driver samples) was performed using Trizol® reagent (Invitrogen, São Paulo, SP, Brazil), according to the manufacturer's instructions. Messenger RNA was isolated using the PolyATtract® mRNA Isolation Systems Kit (Promega, Madison, WI, USA).

Suppressive subtractive hybridization was performed using the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. Double-stranded cDNA was obtained from the mRNA of the fungal mycelia using the SMART PCR cDNA Synthesis Kit (Clontech Laboratories, Palo Alto, CA, USA), according to the manufacturer's instructions. The cDNA was digested with *Rsa* I. In two separate ligations, tester cDNA was ligated to adapters 1 and 2. In the first hybridization, an excess of driver cDNA was hybridized at 68°C for 8 h with each tester cDNA. In the second hybridization, reactions 1 and 2 were hybridized together in the presence of fresh driver cDNA at 68°C overnight. The subtractive product was amplified by PCR using oligonucleotides that were complementary to adapters 1 and 2. PCR was performed according to the following parameters: 75°C for 5 min and 27 cycles at 94°C for 30 s, 66°C for 30 s, and 72°C for 1.5 min. Then, a nested PCR was performed as follows: 12 cycles at 94°C for 30 s, 66°C for 30 s, and 72°C for 1.5 min. The final PCR product was identified as differentially expressed cDNA and corresponded to the gene population differently expressed in the *Hydnangium* fungus in the presence of the *E. grandis*.

The amplified cDNA fragments from the suppressive subtractive hybridization of the *Hydnangium* sp. mycelium

in the pre-symbiotic phase were cloned in the pGEM-T Easy Vector (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Ultracompetent DH5α *Escherichia coli* cells were transformed with the ligation reaction according to Inoue et al. (1990). The recombinant bacterial clones cultured in Luria–Bertani medium (Sambrook et al. 1989) containing ampicillin (50 μg/mL) and isopropyl β-D-thiogalactoside (X-Gal; Promega, Madison, WI, USA) were selected.

Plasmid DNA of the bacterial clones was extracted and sequenced using the M13F (5′-GTTTTCCCAGTCACGAC-3′) oligonucleotide through the Sanger method (Sanger et al. 1977), using the MegaBase 1000 DNA Analysis System sequencer (Molecular Dynamics and Life Science, Sunnyvale, CA, USA).

Subtractive library sequence annotations

The obtained sequences that represent the subtractive library were submitted to electropherogram quality analysis (http://asparagin.cenargen.embrapa.br/phph/) and the VecScreen program (http://www.ncbi.nlm.nih.gov). It was considered sequences with Phred value higher than 20. After this procedure, the Cap3 program (http://deepc2.psi.iastate.edu/aat/cap/cap.html) was used to group and setup the contiguous sequences. These sequences were compared to the sequences deposited in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) using the BLASTX algorithm (Altschul et al. 1997). Only the alignments between the amino acid sequences deduced from the subtractive library nucleotides and database amino acids with $e<10^{-4}$ were considered.

The MIPS database (http://mips.gsf.de/projects/funcat) was used to define the functional categories of the evaluated sequences.

Subtractive hybridization efficiency test

The subtracted and unsubtracted cDNAs were diluted to the same final concentration. PCR reaction was performed using 1 μL of the diluted cDNA preparations, 1× Go Taq Flexi Promega Buffer (Promega, Madison, WI, USA), 2 mM of $MgCl_2$, 200 μM of each triphosphate deoxynucleoside, 0.4 μM of each oligonucleotide, and 1 U of Go Taq Flexi Promega (Promega, Madison, WI, USA).

Based on the sequence database of *L. bicolor*, oligonucleotides (Table 1) were designed to amplify a region of the gene that codes β-tubulin of *Hydnangium* sp. The PCR product, with a DNA fragment of 206 bp, was purified and sequenced. This sequence was compared with sequences deposited in the databases of the NCBI (http://www.ncbi.nlm.nih.gov/) using the BLASTX algorithm (Altschul et al. 1997), which confirmed the similarity of the translated amino acids of this sequence to the amino acids of β-tubulin proteins of other organisms.

Table 1 Oligonucleotides used for gene expression evaluation and subtractive library validation of the pre-symbiotic interaction between *Hydnangium* sp. and *E. grandis* through RT-qPCR

Clone	Oligonucleotides	Sequence 5′–3′
D12SH1	D12SH1_L1	GTTGCACTCCCATCAACATC
	D12SH1_R1	GCACCTCTAGCATTTGAACG
A02aSH2	A02aSH2_L1	GACTCAACTGAGCTCCACGA
	A02aSH2_R1	CCCAACAATTCTTCCTTGGT
A05SH3	A05SH3_L1	AATGAGCTCCTGGATCAACA
	A05SH3_R1	GTTGTCGACCTCCTTGCC
D02SH3	D02SH3_L1	GTCGCGACCAATGACAGTAT
	D02SH3_R1	TTCCCTCCTTCCAGAGAAGA
D09SH3	D09SH3_L1	GATGTTGAAGACCGGTAGCA
	D09SH3_R1	GGGTCAAAGAGATTGGCATT
	BTUB_L1	AGGAGGCTGAAGGGACAGAC
	BTUB_R1	TCAAGAGGGTTCCCATACCA

The oligonucleotides BTUBHYD1 and BTUBHYD2 (Table 1) used for the efficiency test were designed from the gene sequence region that codes for β-tubulin in *Hydnangium* sp. and does not possess a site for the *Rsa*I enzyme. The amplification conditions were: 33 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min. Samples of 5 μL were taken at cycles 18, 23, 28, and 33; these samples were afterwards analyzed through 2% agarose gel electrophoresis containing ethidium bromide at a final concentration of 0.5 μg/mL.

Gene transcription analysis of the fungus *Hydnangium* sp. in the pre-symbiotic phase with *E. grandis* through RT-qPCR

The RT-qPCR technique was used to evaluate and compare gene expression in the fungus *Hydnangium* sp. cultured in the presence and in the absence of the plant.

The mRNAs isolated for the construction of the suppression subtractive library were used. Synthesis reactions of the first cDNA strand were performed in a total volume of 20 μL containing 200 ng of mRNA, 1× Improm II™ Reverse Transcriptase Buffer (Promega, Madison, WI, USA), 0.5 mM of each triphosphate deoxynucleoside, 500 ng of oligo dT, 20 U of ribonuclease RNAsin® inhibitor, and 10 U of Avian Myeloblastosis Vírus Reverse Transcriptase (Promega, Madison, WI, USA). The reaction was submitted to 42°C for 90 min.

Experiments regarding the relative quantification of the expression of mycorrhization-related genes were conducted in a 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA), according to the manufacturer's instructions. The

oligonucleotide sequences for RT-qPCR analysis were determined in the Real-Time PCR Primer Design program (https://www.genscript.com/ssl-bin/app/primer), based on the cDNA sequences of the suppression subtractive library constructed (Table 1).

Relative standard curve was the method chosen for amplification. The β-tubulin gene was used as an endogenous control. Therefore, the amplification of the target gene was normalized with the amplification of the endogenous control to correct amplification variations owing to the initial quantity of material present in the samples, processing conditions, and presence of PCR inhibitors. All samples were amplified in triplicate.

For the PCR, 1 μL of the first cDNA strand, 0.2 μM of each oligonucleotide, and SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) at a final concentration of 1× were used at a final reaction volume of 25 μL.

Results

The *in vitro* mycorrhization technique was efficient for obtaining mycelium from the fungus *Hydnangium* sp. during the pre-symbiotic phase of its interaction with *E. grandis* (Fig. 1), thus allowing the construction of the suppression subtractive library. *E. grandis* seedlings were separated from the mycelium of *Hydnangium* sp. by a sheet of cellophane membrane through the *in vitro* mycorrhization technique (Fig. 1), avoiding physical contact between the symbionts and allowing only the passage of diffusible compounds released by both, simulating the pre-symbiotic phase.

To assess the subtraction efficiency of the suppressive subtractive hybridization procedure, subtractive and unsubtractive cDNA were amplified using oligonucleotides that anneal in the gene that code for β-tubulin. We observed a reduced amplification of subtracted cDNA in relation to unsubtracted cDNAs (Fig. 2). Because the β-tubulin is a constitutive gene, the reduced amplification of this gene in the subtracted cDNA means that was reduction of the common genes during the suppressive subtractive hybridization supports the efficiency of the technique. The confirmation of the efficiency of the cDNA library subtraction (Fig. 2) demonstrates that a high-quality cDNA subtractive library was constructed, representing the differentially expressed genes and the suppression of constitutive expression genes.

Size polymorphism of the cloned cDNA fragments (data not shown) indicated low redundancy for the cDNAs, and 274 cDNA fragments were sequenced. After the electropherogram quality analysis and VecScreen program, 168 sequences were selected analyzed by the Cap3 program. Of these, 131 expressed sequence tags (ESTs) were compared to sequences deposited in the NCBI database using the BLASTX algorithm.

A total of 44 translated amino acid sequences of the ESTs presented *e* values $<10^{-4}$ (33.6%), 45 presented $e \geq 10^{-4}$ (34.4%), and 42 presented no similarity with any protein deposited in the database (32%).

Among the 44 EST sequences with an *e* value lower than 10^{-4}, 26 presented similarity with proteins of known function and 18 with hypothetical proteins. Similarity with the proteins of the fungus *L. bicolor* was observed in 36% of the translated amino acid sequences from the 44 ESTs.

Functional characterization of the genes indicated that 7% code for proteins related to carbohydrate metabolism (3-ketoacyl-CoA-thiolase peroxisomal, acetyl-CoA acetyltransferase, amidohydrolase, and MFS monosaccharide transporter); 2% to amino acid metabolism (methylmalonate–semialdehyde dehydrogenase), 11% to energetic metabolism (vacuolar ATP synthase, ATP synthase beta chain, mitochondrial pyruvate dehydrogenase E1 component beta subunit, NADPH dehydrogenase, and AGL064Wp), 9% to transcription and protein synthesis (peptidyl-prolyl cis-trans isomerase, RNA polymerase II largest subunit, 40S ribosomal protein S6, and aspartyl-tRNA synthetase), 7% to cell communication and signal transduction (voltage-dependent ion-selective channel, Apr protease precursor, and Ste20-like serine/threonine kinase), 11% to stress response (2-nitropropane dioxygenase, cytochrome P450-like protein, glutathione-S-transferase, reductase AKOR2, and peroxiredoxin), 5% to transposons (IS10 transposase and transposase), 5% to biogenesis of cell components (hydrophobin 1 and endoglucanase 1 precursor), and 43% code for proteins of unknown function (predicted proteins, symbiosis-related protein, CipC1 protein, among others) (Table 2).

The identification of many genes belonging to nine functional categories leads us to believe that a complex series of molecular mechanisms is altered during the first stage of ectomycorrhiza formation, prior to the contact between plant and fungus, as shown previously (Podila et al. 2002; Krüger et al. 2004; Menotta et al. 2004; Martin et al. 2007).

Since interaction with the roots triggers alteration in the fungal mycelium, the activation of genes related to carbohydrate, energetic metabolism, and transcription and protein synthesis are essential to support these modifications. Another relevant metabolic process that was activated during the pre-symbiotic phase was the process of cell communication and signal transduction, with the activation of genes that code for proteins such as the voltage-dependent protein from the selective ion channel, the precursor of Apr protease, and the Ste20-*like* serine/threonine kinase. The presence of signal genes in the constructed subtractive library is expected, because the cross talk between symbionts is apparently mediated by these genes.

To confirm the differential expression of some genes in the pre-symbiotic phase, five genes were selected and their

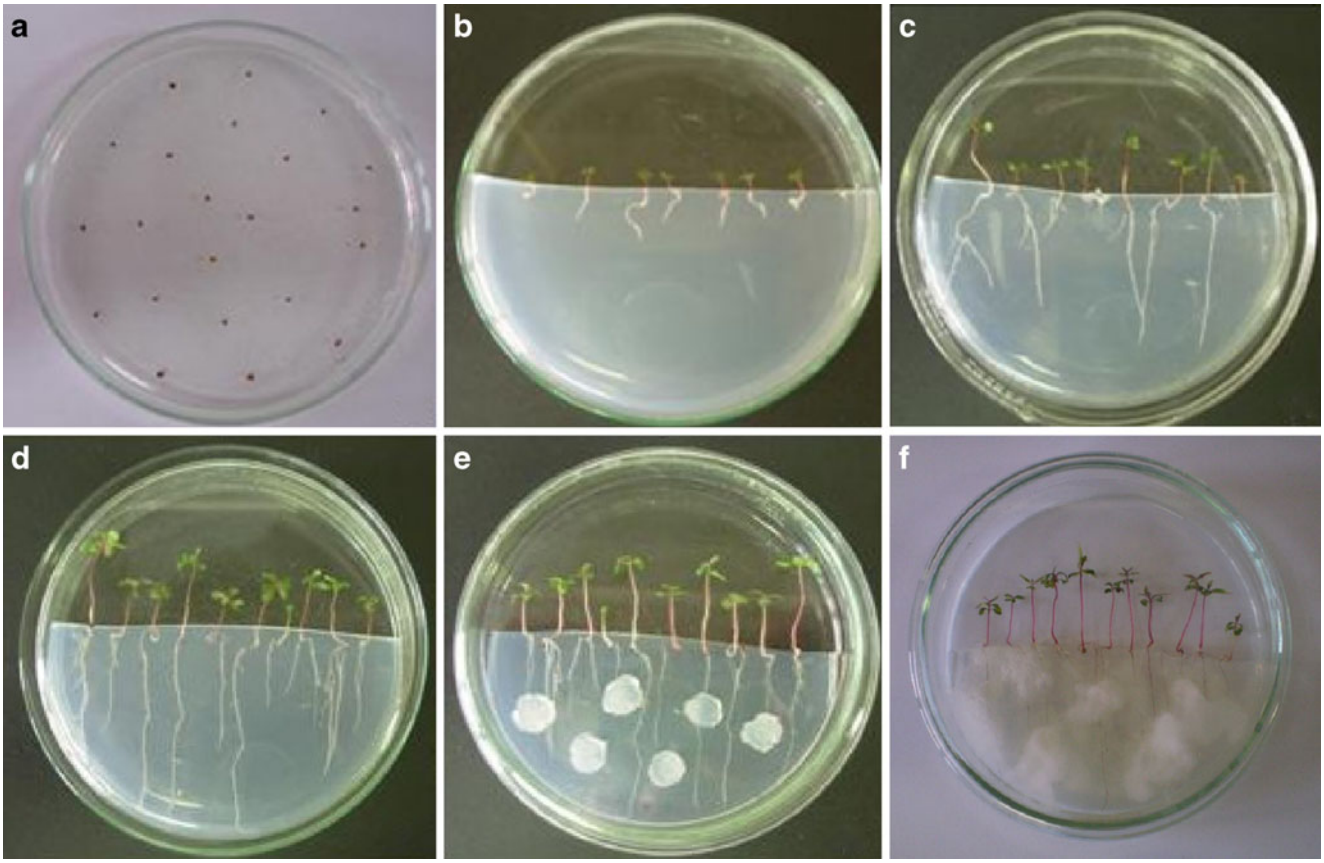

Fig. 1 *In vitro* mycorrhization. *E. grandis* seeds placed over the synthesis medium after disinfection (**a**), *E. grandis* seedlings after 1 day (**b**), 7 days (**c**), and 15 days (**d**) of being transferred to the synthesis medium. *E. grandis* seedlings after 15 days after transfer on and inoculated with *Hydnangium* sp (**e**) and 15 days after inoculation (**f**)

transcription evaluated using the RT-qPCR. The standard curve demonstrated amplification efficiency between 90% and 120% for all oligonucleotides evaluated (data not shown). An amplification efficiency of >90% is typically desired of optimal results. Greater than 100% efficiency suggests that more than one product was amplified in the reaction, primer–dimer products. In addition to the primary sequence of the primer sets, the major contributors to primer–dimer formation are: a high molar level of primers in the assay specific master mix; the order of addition of reactants; and the time period during which a complete amplification reaction admixture. Many tests to optimize these reactions have been done. A six-fold increase in the transcription of the gene that codes for ATP synthase, one of the genes that codes hydrofobin, and 0.5 of the genes that codes pyruvate dehydrogenase, voltage-dependent ion-selective channel, and acetyl-CoA acetyltransferase were observed in *Hydnangium* sp. cultured in the presence of host plant roots (tester) in comparison to the free-living fungus (driver), confirming the activation of these genes in this phase of the association (Fig. 3).

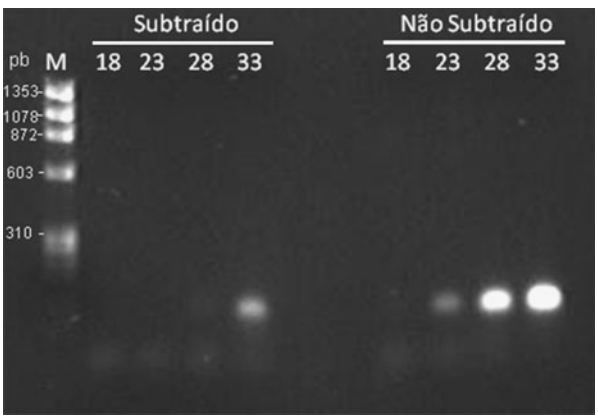

Fig. 2 Efficiency test of the cDNA subtractive library. Electrophoresis in 2% agarose gel of the subtracted and unsubtracted cDNA amplification products with the BTUBHYD1 and BTUBHYD2 oligonucleotides at cycles *18*, *23*, *28*, and *33*. *M* φX174/*Hae*III marker

Discussion

The formation of ectomycorrhiza is characterized by the regulated succession of morphological modifications during root colonization by the fungal hyphae (Smith and Read

Table 2 Identification of the possible proteins coded by the cDNAs of the suppression subtractive library of the fungus *Hydnangium* sp. during the pre-symbiotic phase of its association with *E. grandis*

Clone/Contig	Length (bp)	GenBank acession number	Database match	*e* Value
Carbohydrate metabolism				
H01SH3	733	XP_001876645	3-ketoacyl-CoA-thiolase, peroxisomal/*Laccaria bicolor* S238N-H82	2.00E−65
D09SH3	378	AAK26620	Acetyl-CoA acetyltransferase/*Laccaria bicolor*	1.00E−11
Contig9	276	YP_445294	Amidohydrolase/*Salinibacter ruber* DSM 13855	1.00E−10
F04SH3	216	XP_001874568	MFS monosaccharide transporter/*Laccaria bicolor* S238N-H82	1.00E−17
Amino acid metabolism				
F07SH3	459	XP_001873960	Methylmalonate-semialdehyde dehydrogenase/*Laccaria bicolor* S238N-H82	1.00E−63
Energy metabolism				
D10SH3	594	XP_572836	Vacuolar ATP synthase/*Cryptococcus neoformans*	2.00E−68
A05SH3	416	XP_001833020	ATP synthase beta chain/*Coprinopsis cinerea okayama*7#130	1.00E−62
A02aSH2	591	XP_001881742	Mitochondrial pyruvate dehydrogenase E1 component beta subunit/*Laccaria bicolor* S238N-H82	1.00E−59
D11SH1	305	NP_592817	NADPH dehydrogenase (predicted)/*Schizosaccharomyces pombe*	4.00E−14
A09aSH2	300	NP_986602	AGL064Wp/*Ashbya gossypii* ATCC 10895	5.00E−06
Transcription and protein synthesis				
G05SH2	588	XP_754866	Peptidyl-prolyl cis-trans isomerase/*Aspergillus fumigatus*	2.00E−53
E03aSH3	345	AAW72748	RNA polymerase II largest subunit/*Climacodon septentrionalis*	1.00E−32
B11SH2	548	XP_963431	40S ribosomal protein S6/*Neurospora crassa*	3.00E−14
H09SH2	814	XP_001938116	Aspartyl-tRNA synthetase/*Pyrenophora tritici-repentis*	4.00E−06
Cell communication and signal transduction				
Contig14	1,110	XP_569804	Voltage-dependent ion-selective channel/*Cryptococcus neoformans*	1.00E−14
H10bSH2	283	CAH03670	Apr protease precursor/*Bacillus licheniformis*	1.00E−14
C08SH1	441	XP_001876676	Ste20-like serine/threonine kinase/*Laccaria bicolor*	3.00E−11
Stress response				
H11SH3	477	XP_572454	2-nitropropane dioxygenase/*Cryptococcus neoformans*	1.00E−34
A11SH1	469	ACB69805	Cytochrome P450-like protein/*Heterobasidion annosum*	2.00E−22
C12SH2	333	ACF15452	Glutathione-S-transferase/*Phanerochaete chrysosporium*	7.00E−20
A07SH1	353	AAS46751	Reductase AKOR2/*Pleurotus djamor*	3.00E−19
F03SH2	335	XP_001879250	Peroxiredoxin/*Laccaria bicolor* S238N-H82	1.00E−18
Transposons				
A11SH4	674	NP_058298	IS10 transposase/*Salmonella typhi*	5.00E−26
E07SH1	778	AAT00497	Transposase/*Escherichia coli*	1.00E−05
Biogenesis of cell components				
Contig17	609	ABA46363	Hydrophobin 1/*Heterobasidion annosum*	2.00E−23
H11SH2	366	XP_001936021	Endoglucanase 1 precursor/*Pyrenophora triticirepentis* Pt-1C-BFP	6.00E−13
Proteins of unknown function				
Contig8	600	XP_001876256	Predicted protein/*Laccaria bicolor* S238N-H82	1.00E−44
D11SH3	623	XP_001828572	Hypothetical protein CC1G_11224/*Coprinopsis cinerea okayama7*#130	2.00E−43
D01SH2	375	XP_001878658	Predicted protein/*Laccaria bicolor* S238N-H82	4.00E−33
F09aSH2	510	XP_001881785	Predicted protein/*Laccaria bicolor* S238N-H82	8.00E−24
E02SH3	548	AAB53650	Symbiosis-related protein/*Laccaria bicolor*	1.00E−18
Contig18	517	XP_001830463	Predicted protein/*Coprinopsis cinerea okayama7#130*	4.00E−17
Contig4	828	XP_001879340	CipC1 protein/*Laccaria bicolor*	5.00E−15
H06aSH2	374	XP_001272266	Conserved hypothetical protein/*Aspergillus clavatus* NRRL 1	5.00E−15
G08SH2	391	XP_001728955	Hypothetical protein MGL_3949/*Malassezia globosa* CBS 7966	2.00E−11
F08SH1	292	XP_001877199	Predicted protein/*Laccaria bicolor* S238N-H82	6.00E−10
H08SH3	263	XP_001882666	Predicted protein/*Laccaria bicolor* S238N-H82	3.00E−10

Table 2 (continued)

Clone/Contig	Length (bp)	GenBank acession number	Database match	*e* Value
H12aSH2	417	XP_001876100	Predicted protein/*Laccaria bicolor* S238N-H82	9.00E−09
E03SH2	571	XP_001841141	Hypothetical protein CC1G_08285/*Coprinopsis cinerea okayama*7#130	2.00E−08
B12aSH3	245	XP_570733	Hypothetical protein/*Cryptococcus neoformans var. neoformans* JEC21	2.00E−08
Contig12	312	XP_760163	Hypothetical protein UM04016.1/*Ustilago maydis* 521	3.00E−07
B10SH4	350	BAD07869	Hypothetical protein/*Oryza sativa* Japonica Group	3.00E−07
G12aSH3	316	XP_001884448	Predicted protein/*Laccaria bicolor* S238N-H82	7.00E−06
Contig10	399	XP_001877734	Predicted protein/*Laccaria bicolor* S238N-H82	7.00E−05
E02SH4	288	XP_001828791	Predicted protein/*Coprinopsis cinerea* okayama7#130	1.00E−05

1997; Tagu et al. 2002; Martin et al. 2007). In the pre-symbiotic phase that precedes the contact between plant and fungus, compounds produced by the symbionts elicit cell responses that lead to the determination of symbiosis compatibility and have a crucial role in ectomycorrhiza formation.

Genetic expression tools such as DNA microarrays and suppression subtractive hybridization have been used to evaluate the development and functioning of ectomycorrhizae at the molecular level, allowing identification and functional analyses of genes. Because of the large number of genes and signals involved, these tools are adequate to study molecular events that occur in symbiotic interactions (Podila et al. 2002; Menotta et al. 2004; Duplessis et al. 2005; Acioli-Santos et al. 2008; Heller et al. 2008).

In the pre-symbiotic phase, besides the genes related to transcription and protein synthesis, cell communication and stress response genes that code for proteins related to the metabolism of carbohydrates and energy were activated (Table 2).

Genes that code for proteins related to carbohydrate and energy metabolism were identified in the subtractive library of *Hydnangium* sp. (Table 2), such as acetyl-coA acetyltransferase and genes related to the electron transport chain, e.g., ATP synthase. The reorientation of the fungal metabolism in the pre-symbiotic phase is expected to favor and allow the formation and functioning of the ectomycorrhiza, with a significant raise in carbohydrate metabolism and electron transport chain activation, which must be connected with the activation of the carbohydrate metabolism throughout the citric acid cycle.

A cDNA corresponding to the acetyl-coA acetyltransferase gene of *L. bicolor* was expressed during the pre-symbiotic phase of the interaction with the roots of *P. resinosa* (Podila et al. 2002), and these results combined with microarray analyses revealed the coordinated expression of the malate

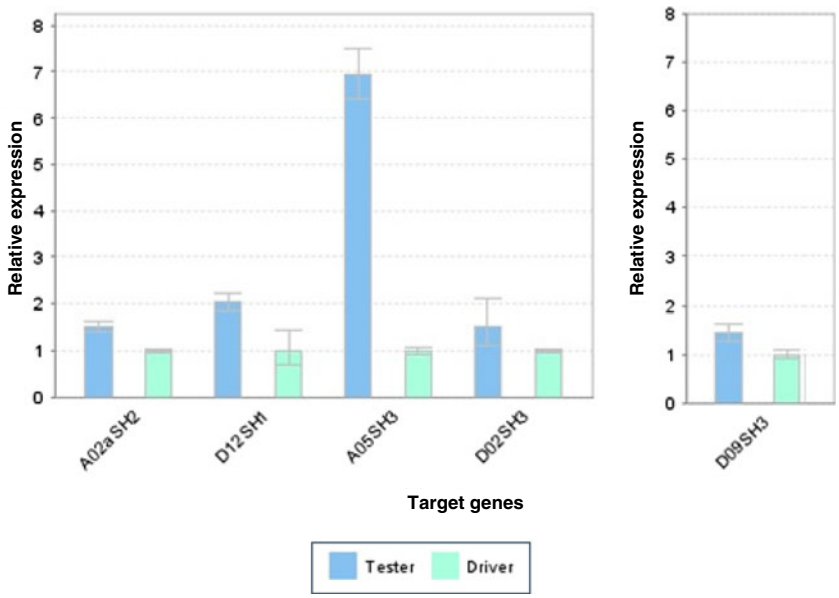

Fig. 3 Gene expression of *Hydnangium* sp. cultured in the presence of plant (*tester*) in relation to that of fungus cultured in the absence of plant (*driver*). The *x* axis presents the target genes and the y axis the level of gene expression correspondent to the expression of the target genes in relation to the calibrator (tester), normalized by the β-tubulin endogenous control. The evaluated genes possibly control pyruvate dehydrogenase (*A02aSH2*), hydrophobin (*D12SH1*), ATP synthase (*A05SH3*), voltage-dependent protein from the selective ion channel (*D02SH3*), and acetyl-CoA acetyltransferase (*D09SH3*)

synthase enzyme and other genes related to lipid metabolism with acetyl-CoA acetyltransferase, suggesting that this enzyme is important in the pre-infection process of the ectomycorrhizal symbiosis and in the transfer and use of carbon by the fungus (Hiremath et al. 2006).

In the subtractive library constructed, genes that code for proteins such as peptydil-prolyl cis-trans isomerase, aspartyl-tRNA sythase, large subunit RNA polymerase II, and 40S ribosomal protein were also observed (Table 2). As the fungal cells need to reorganize their structure and metabolism to form the functional ectomycorrhiza and different metabolic processes are activated in the pre-symbiotic phase, an increased expression of genes that code for proteins related to transcription and protein synthesis is expected to support the increase in fungal metabolism.

Contrary to our results, in the *Pisolithus tinctorius*–*Castanea sativa* system, at 12 h of the interaction, two kinds of downregulated gene in the *P. tinctorius* mycelium represented the carbon metabolism and energy category during the pre-symbiotic phase (Acioli-Santos et al. 2008). The difference in the results can be due to the mycorrhization system and the duration of the interaction between the fungus and plant. In our study, we collected the mycelia after 15 days while in the Acioli-Santos et al. (2008), the mycelia were collected after 12 h.

Another relevant metabolic process that was activated during the pre-symbiotic phase was the process of cell communication and signal transduction, with the activation of genes that code for proteins such as the voltage-dependent protein from the selective ion channel, the precursor of Apr protease, and the Ste20-*like* serine/threonine kinase (Table 2). The presence of signal genes in the constructed subtractive library is expected because the cross talk between symbionts is apparently mediated by these genes.

In the group of proteins related to stress in *Hydnangium* sp., we may observe those related to the synthesis of reactive oxygen species, such as 2-nitropropane deoxygenase and a reductase similar to the AKOR2 reductase of *Pleurotus djamor*, and to cell detoxification, such as glutathione S-transferase, cytochrome P450, and peroxiredoxins. As previously mentioned, a series of changes occur in the symbionts during the pre-symbiotic phase, and the activation of recognition mechanisms of the plant and defense mechanism of the fungus is suggested. A good example is the activity of the glutathione S-transferase enzyme, which has been described as a possible virulence factor in *Botrytis cinerea*, since the presence of this enzyme resulted in fungal protection against fungitoxic compounds produced by the plant (Prins et al. 2000).

A gene that codes for hydrophobin was also identified, which is a protein related to the biogenesis of cell components. Hydrophobins are hydrophobic proteins that present approximately 100 amino acids commonly found in filamentous fungi. They are among the genes expressed more abundantly in differentiating ectomycorrhiza. Genes that code for hydrophobins and mannoproteins are activated in the symbiotic tissue and are candidate markers for symbiosis-related changes (Tagu et al. 1996; Laurent et al. 1999; Voiblet et al. 2001; Peter et al. 2003). Genes that code for hydrophobins were activated in the beginning of the ectomycorrhizal association between *P. microcarpus* and *E. globulus* when the root tips were colonized but had the constitutive expression level established within 12–21 days (Duplessis et al. 2005).

A large number of amino acid sequences available in the databases that present similarity with the amino acid sequences translated from our subtractive library do not have a known function. Among these, the protein CipC (a C-type protein induced by kanamycin) was identified in *L. bicolor*, and despite having an unknown function, an increase in its synthesis was observed when *Aspergillus nidulans* was cultured in medium containing concanamycin, promoting drastic changes in hyphal morphology (Melin et al. 2002). The quantity of transcribed genes that code for this protein was 2.41 times greater in the ectomycorrhiza after 12 days of interaction between *Paxillus involutus* and *Betula pendula* than in the free-living mycelium. It is believed that the activation of the *cip*C gene is linked to changes in morphology during fungal growth (Morel et al. 2005).

Another protein of unknown function identified in the suppression subtractive library of *Hydnangium* is a symbiosis-related protein previously described in *L. bicolor*. A gene that codes for a symbiosis-related protein in *L. laccata* (*aut*7) may be involved in vacuolar transport and autophagocytosis. The transcription of this gene was doubled in the ectomycorrhiza in relation to the extra-root mycelium of *P. involutus* and *B. pendula* (Morel et al. 2005). The *aut*7 transcript was not detected in the free-living mycelium but was detected in the symbiotic interaction with the host plant. The protein coded by this gene has a crucial role during fungal root colonization (Kim et al. 1999).

The occurrence of a large quantity of sequences that present no similarity with any protein in the database has been observed in many ectomycorrhizal fungi (Voiblet et al. 2001; Podila et al. 2002; Krüger et al. 2004; Menotta et al. 2004; Zaretsky et al. 2006; Frettinger et al. 2007; Acioli-Santos et al. 2008; Heller et al. 2008). It has been suggested that these genes are exclusive to the studied fungus species, or that they represent rare transcripts not yet identified or characterized (Podila et al. 2002). Furthermore, these sequences may represent genes that are only transcribed during the mycorrhization process, i.e., ectomycorrhiza-specific genes. Many effector-type small secreted proteins (SSPs) with unknown function, several of which are only expressed in symbiotic tissues were detected in the

Laccaria genome. The most highly expressed SSP accumulates in the proliferating hyphae colonizing the host root, probably having a decisive role in the establishment of the symbiosis (Martin et al. 2008).

As expected, among the 44 EST sequences translated with an *e* value below 10^{-4}, a large number (16) present similarity with proteins of the fungus *L. bicolor*, the first Basidiomycota fungus to have its genome sequenced (Martin et al. 2008). Since *Laccaria* and *Hydnangium* sp. are the only members of the family Hydnangiaceae, the available information regarding the genome sequences of *Laccaria* may be applied to the study of *Hydnangium*, allowing gene comparison studies between the two fungi.

As the construction of cDNA suppression subtractive library allows the identification of different genes expressed in different conditions, all transcripts of common expression in both phases of fungal growth may not be removed, besides the nondetection of repressed genes. In order to confirm the differential transcription of the identified genes, techniques such as membrane arrays and RT-qPCR have been used. Five selected genes had their transcription confirmed in this study, and they presented an increased expression in the mycelium of *Hydnangium* sp. cultured in the presence of plant (tester) in relation to the mycelium cultured in the absence of plant (driver), thus confirming the activation of these genes in the pre-symbiotic phase (Fig. 3).

Gene identification through the construction of a subtractive library of *Hydnangium* sp. will allow the study of different genes related to mycorrhization, thus aiding in understanding the molecular mechanisms involved in the ectomycorrhizal association. The identified symbiosis-regulated genes might be especially interesting targets for functional analysis of symbiosis, as these genes predetermine the next steps in the development of ectomycorrhiza.

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