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ORIGINAL ARTICLE

# Identification of fungal genes involved in the preinfection events between ectomycorrhizal association (*Pisolithus tinctorius* and *Pinus massoniana*)

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**Abstract** The preinfection stage of establishment of the mycorrhiza before physical contact between symbionts is crucial, as changes that occur throughout mycorrhiza formation are set in motion at this time. To improve the understanding of the molecular mechanisms involved in the fungus *Pisolithus tinctorius* during its interaction with its symbiotic partner *Pinus massoniana*, we set up a cellophane-separated culture system avoiding physical contact between the symbionts. A cDNA subtraction library representing the differentially expressed genes of *P.tinctorius* during the preinfection stage was constructed using suppression subtractive hybridization combined with mirror orientation selection (SSH/MOS). Differential screening of the SSH library allowed us to identify 133 unique cDNA clones. Sequences analysis of the expressed sequence tags (ESTs) showed that they represent 23 differentially expressed genes, of which 10 are involved in metabolism and energy, transcription and protein fate, membrane proteins and signaling, and cell rescue and defense, and 13 encoding hypothetical proteins with unknown function. Some fungal genes have not been previously identified in other ectomycorrhizal associations. Expression of the genes that code for ubiquitin-conjugating enzyme E2, 40S ribosomal protein, maternal g10 transcript, ADP ribosylation factor 6, and cytochrome P450 were evaluated by RT-qPCR, confirming the activation of these genes during the preinfection stage.

**Keywords** Ectomycorrhiza · *Pisolithus tinctorius* · Preinfection stage · Gene expression · Suppression subtractive hybridization

Haibo Li and Huazheng Peng contributed equally to this work.

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## Introduction

Ectomycorrhizae (ECM) are mutualistic symbiotic associations between plant roots and compatible filamentous fungi. ECM formation takes place in four stages: preinfection, colonization, differentiation, and functioning (Martin et al. 1997). In the preinfection stage, plant roots and fungi exchange signals in the soil in order to determine symbiotic compatibility (Martin et al. 2007). The preinfection stage before physical contact between symbionts is crucial, as changes that occur throughout mycorrhiza formation are set in motion at this time (Hilbert et al. 1991). Therefore, in past years, some studies focused on identifying differently expressed or symbiosis-specific fungal genes during the preinfection stage by using suppression subtractive hybridization (SSH) and cDNA-AFLP (Podila et al. 2002; Menotta et al. 2004; Zaretsky et al. 2006; Acioli-Santos et al. 2008; Silva Coelho et al. 2010). Many genes identified from these studies belonged to different functional categories, which suggests that a complex series of molecular mechanisms is activated at the preinfection stage that precedes actual contact between plant and fungus. However, no ectomycorrhiza-specific genes were detected during the symbiosis development, but instead a marked change in gene expression patterns in both partners (Voiblet et al. 2001; Duplessis et al. 2005; Zaretsky et al. 2006). The identification of signaling molecules is important for understanding the complex signaling pathways involved in host–symbiont interactions. Although the recent studies have addressed the specific signals that trigger ECM symbiosis establishment, the nature of the signaling molecules and the molecular basis of the signal perception and transduction in mycorrhiza remain largely unknown (Felten et al. 2012).

*Pisolithus tinctorius* is an almost ubiquitous ectomycorrhizal fungus, which is able to establish symbiosis with a wide range of plants (Marx 1977). Therefore, it is used as a model

mycorrhizal fungus for the analysis of ectomycorrhizal symbiotic interactions (Martin and Tagu 1995; Martin et al. 1999). *Pinus massoniana* is the main timber and pioneer afforestation tree species in China. This unique Chinese tree species completely depends on the fungus *P. tinctorius* to survive and thrive in natural environments. Therefore, *P. tinctorius* is regarded as the most suitable ectomycorrhizal fungi for *P. massoniana* (Hua 1995a). Many physiological and morphological studies on the ectomycorrhizal associations, formed between *P. tinctorius* and *P. massoniana*, have been carried out for many years (Hua 1995b, 2001; Zheng et al. 2003). However, molecular interactions in the *P. tinctorius*–*P. massoniana* system have not been performed up to now.

To improve the understanding of the molecular mechanisms involved in *P. tinctorius* during its interaction with its symbiotic partner *P. massoniana* at the preinfection stage, in the present study, we constructed a subtracted library from cellophane-separated fungus *P. tinctorius* isolates by using a method based on suppression subtractive hybridization (SSH) combined with mirror orientation selection (MOS). To identify and screen the fungal genes differentially expressed at the preinfection stage of the ectomycorrhizal association with *P.massoniana*, the cDNA arrays hybridization as a form of reverse northern blot analysis was used to eliminate false positive clones and to increase selection efficiency. These identified positive clones were sequenced and the expressed sequence tags (ESTs) were analyzed and assigned to functional groups.

## Materials and methods

### Growth conditions of fungal and plant

The ectomycorrhizal gasteromycete *Pisolithus tinctorius* Coker & Couch (synonym *Pisolithus arhizus* (Scop. per Pers.) Rauschert), isolate 7522, was routinely grown on modified Melin Norkans culture medium (MMN) (Marx 1969) and transferred to new culture media every 3 months. For this molecular experiment, *P. tinctorius* isolates were grown for 25 days (about 4.5–5.0 cm in diameter) at 25 °C on Petri dishes containing MMN-medium covered with a piece of cellophane membrane. Before use, the cellophane membrane was treated according to the protocol described by Zaretsky et al. (2006). *P. massoniana* seeds, originated from southeastern China, were soaked in water at 30 °C for 24 h, immersed for 1 min in 70 % ethanol, disinfected for 30 min in $H_2O_2$ 30 %, and rinsed three times with sterilized water. After superficial disinfection, the seeds were transferred to a flask containing 30 ml Murashige-Skoog culture medium supplemented with 0.2 % glucose and 0.8 % agar at 25 °C and 16 h of light at an intensity of 120 μmol $m^{-2}$ $s^{-1}$ photons.

### In vitro interactions between fungal and plant

Three 15-day-old *P. massoniana* seedlings were transferred to a growth box (20 cm×15 cm×5 cm) containing 15 ml modified MMN culture medium ($MMN_{1/10}$ culture medium with a 1:10 ratio of nitrogen and phosphorus and no carbohydrate) (Marx 1969). After 24 h, 25-day-old *P. tinctorius* isolates grown on cellophane membranes were placed on top of the roots of the seedlings and incubated at 25 °C and 16 h of light at an intensity of 120 μmol $m^{-2}$ $s^{-1}$ photons. Simultaneously, the 25-day-old *P. tinctorius* isolates were incubated in the growth box containing $MMN_{1/10}$ medium under the above-mentioned conditions, but in the absence of the host plant *P. massoniana* seedlings. *P. tinctorius* isolates were incubated in the presence and obsence of host plant in the growth boxes for 3 days, and afterward, the *P. tinctorius* mycelium was collected from the cellophane membranes and immediately frozen in liquid nitrogen and stored at −80 °C until use. Therefore, two treatments were established: fungus cultured in the presence of the host plant (tester sample), and cultured in the absence of host plant (driver sample).

### RNA extraction and SSH/MOS

Total RNA extraction of *P. tinctorius* mycelium cultured in both treatments (tester and driver samples) was performed using Trizol Reagent (Gibco, Germany) according to the manufacturer's instructions. Messenger RNA (mRNA) was isolated using the Oligotex mRNA Mini Kit (Qiagen, Germany).

Suppressive subtractive hybridization was performed using the PCR-Select cDNA Subtraction Kit (BD Biosciences Clontech, USA) according to the manufacturer's instructions. Double-stranded cDNA was obtained from the mRNA of *P. tinctorius* using the SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech) according to the manufacturer's instructions. cDNAs from testers and drivers were digested with *Rsa* I. Each cDNA from the testers was then separated into two portions and adapters 1 and 2R were each added to one of the two parts. In the first hybridization, testers were hybridized with excess drivers in a ratio of tester: driver 1:30 at 68 °C for 8 h. In the second hybridization, 1 μl driver mixture (1 μl hybridization buffer, 1 μl driver and 2 μl water) were hybridized with the first hybridization solution at 68 °C overnight. PCR amplification was then performed with PCR primer 1 (5′-CTAATACGACTCACTATAGGGC-3′) and followed with nested PCR primers 1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and primer 2R (5′-AGCGTGGTCGCGGCCGAGGT-3′) to amplify differentially expressed cDNA corresponding to the gene population of *P. tinctorius* differently expressed during the pre-symbiotic phase of the ectomycorrhizal association with *P. massoniana*. The MOS

technique was used to eliminate false-positive clones from SSH libraries following the methods of Rebrikov et al. (2000). The products of MOS were cloned in the pMD19-T Vector (Takara, Dalian, Japan) and then transformed into ultracompetent JM109 *Escherichia coli* cells with the ligation reaction according to Inoue et al. (1990). Positive clones were picked and transferred into 384-well plates and incubated at a rotating shaker (2,000 rpm) at 37 °C for 6 h.

### cDNA arrays and reverse northern blot analysis

In order to further confirm the positive clones from SSH/MOS library, reverse northern blots were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Sciences, Germany). The 384 positive clones were amplified by bacteria liquid PCR using the NP2Rs primer (5′-GGTCGCGGCCGAGGT-3′). Then, 2 μl volume of PCR products were spotted onto Hybond-$N^+$ nylon membranes (GE Healthcare, Buckinghamshire, UK) using a 96-pin multiblot replicator (V&P Scientific, San Diego, USA), and then the cDNA was cross-linked to the nylon membranes by a UV cross-linker (GE Healthcare) for 15 min. Two identical membranes with cDNA arrays were prepared for two differential screening cDNA probes, a forward-subtracted cDNA probe and a reverse-subtracted cDNA probe. Probe labeling, hybridization, and detection were performed using the Kit II following the manufacturer's instructions. Briefly, the membranes was pre-hybridized in hybridization buffer at 40 °C for 30 min. The labeled probes were denatured at 100 °C for 5 min before being used for hybridization. After overnight hybridization at 37 °C with the DIG-labeled probe, the membrane was subjected to stringency washes and immunological detection by Chemi Doc XRS imaging system (Bio-Rad, Hercules, CA, USA). A *P. tinctorius* β-tubulin gene was used as an internal standard.

### Sequencing of the subtracted cDNA library and sequence annotations

The positive clones obtained through reverse northern blot were sequenced using an automated DNA sequencer (ABI 377; Applied Biosystems, Foster City, CA, USA) with GeneCore BioTechnologies (Shanghai, China). Raw cDNA sequences were initially trimmed for vector, primer and adapter by Chromas 2.30 (Technelysium; Tewantin, Queensland, Australia) or by visual inspection. Sequences shorter than 100 bases and ambiguous sequences were discarded. The trimmed cDNA sequences that represent the SSH/MOS library were then analyzed for homology by Basic Local Alignment Search Tool (BLAST) X algorithm in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST) and in the *P. tinctorius* genome database at the Joint Genome Institute (JGI) (http://genome.jgi-psf.org/). Only the amino acid sequences deduced from the cDNA sequences obtained from subtraction library with E value $<10^{-4}$ were considered to identify known genes or to have partial similarity to known genes. The MIPS database (http://mips.helmholtz-muenchen.de/proj/funcatDB) was used to define the functional categories of the evaluated sequences. Contiguous sequences were obtained using the program ContigExpress software (Vector NTI Advance 9.1.0; Invitrogen).

### Gene transcription analysis through RT-qPCR

The RT-qPCR technique was used to evaluate and compare gene expression in the fungus *P. tinctorius* cultured in the presence and in the absence of the host plant. Total RNA was isolated from fungus using the RNAiso Plus (Takara) according to the manufacturer's instructions. The concentration of RNA was determined photometrically. Synthesis reaction of the first cDNA strand was performed using PrimeScript® RT reagent Kit with gDNA Eraser (Takara) according to the manufacturer's instructions. An amount of 400 ng cDNA was used in each reaction for RT-qPCR. The reagents used to perform these reactions were commercially purchased (SYBR Premix Ex Taq™; Takara). The RT-qPCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems), and the relative transcript abundances of target genes were calculated based on the cycle threshold (CT) $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) using β-tubulin as a constitutive reference transcript for normalization. The oligonucleotide primers for RT-qPCR were determined by using Oligo 6.54 software (MBI, USA) (Table 2). These primers were designed to amplify a 100- to 250-bp region that spans at least one intron in the genomic DNA (based on available gene models). Cycling conditions included an initial incubation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. Specificity of the primers was determined by melting curve analysis. All RT-qPCR experiments were performed in triplicate with RNAs isolated from at least two different cultures.

## Results

Subtraction by SSH combined with MOS allowed the efficient and rapid cloning of differentially expressed transcripts. Using the combination of SSH and MOS, we constructed a cDNA subtraction library representing the differentially expressed genes of the fungus *P.tinctorius* during the preinfection stage. More than 1,200 positive clones were randomly selected from the SSH/MOS cDNA library and subjected to reverse northern dot-blot analysis

(Fig. 1). The reverse northern analysis revealed that nearly 90 % of these clones gave a weakly positive signal or no signal. As a result, only 133 clones that showed a strong positive signal on reverse dot-blot analysis were selected and sequenced. The 133 expressed sequence tags (ESTs) were assembled into 1contig of 1,363 bp in size and 24 singletons of 370–830 bp in size, or 25 non-redundant ESTs. Each non-redundant EST sequence was queried against the NCBI protein database and *P. tinctorius* genome database using the BLASTX algorithm (Tables 1 and 2).

Among the 25 EST sequences, 22 translated amino acid sequences of the ESTs presented *E*-value < $10^{-4}$ (88 %) and three presented *E*-value>$10^{-4}$ (12 %). Nine of 22 EST sequences with *E*-value < $10^{-4}$ showed significant similarity to known genes, 11 to proteins of unknown function (hypothetical proteins, and leucine-rich repeat protein), and 2 to ESTs of unknown function. In addition, among the three translated amino acid sequences of the ESTs with *E*-value> $10^{-4}$, two showed a match with hypothetical proteins and one with a known gene. Functional analysis indicated the ten fungal genes (Clone ID: Pt1–10) expressed exclusively at preinfection stage may be involved in metabolism and energy (Pt1: thioredoxin, and Pt2: S-adenosyl-L-methionine-dependent methyltransferase), transcription and protein fate (Pt3: ubiquitin-conjugating enzyme, Pt4: 40S ribosomal protein, and Pt5: maternal g10 transcript), membrane proteins and signaling (Pt6: cell wall symbiosis regulated acidic polypeptide precursor, Pt7: symbiosis regulated acidic polypeptide, and Pt8: ADP ribosylation factor 6/GTPase SAR1), cell rescue and defense (Pt9: cytochrome P450), and protein with binding function (Pt10: metallothionein), while the other 13 fungal genes code for proteins of unknown function (Pt11-22, and Contig1).

To confirm the differential expression of these identified genes in the pre-symbiotic phase, five of them were selected and their transcriptions were evaluated using the RT-qPCR. An amplified fragment of the expected size (150–250 bp) was obtained from the *P. tinctorius* mycelium cultured in both treatments (tester and driver), which indicated that the target gene is expressed in *P. tinctorius* cultured both in the presence and absence of the host plant. A twofold increase in the transcription of the gene that codes for ubiquitin-conjugating enzyme E2 (Pt3), three of the genes that codes 40S ribosomal protein (Pt4), and five- to tenfold of the genes that codes maternal g10 transcript and (Pt5) and cytochrome P450 (Pt9) were observed in *P. tinctorius* cultured in the presence of host plant (tester) in comparison to the free-living fungus (driver), confirming the activation of these genes in this phase of the association (Fig. 2a). Specially, there was a significant increase (approximately 100-fold) of ADP ribosylation factor 6 (Pt8) in the pre-symbiotic phase (Fig. 2b).

## Disscusion

Based on the molecular interactions in the *P. tinctorius–P. massoniana* system, we obtained 25 fungal genes differentially expressed at preinfection stage of ectomycorrhizal symbiosis, of which 10 functionally known genes were distributed in different functional categories. This result was similar to those shown by Menotta et al. (2004), Zaretsky et al. (2006), Acioli-Santos et al. (2008), and Silva Coelho et al. (2010), suggesting a complex series of molecular mechanisms is activated at the earliest or preinfection stage that precedes actual contact between plant and fungus.

The thioredoxin (Trx) system consist of thioredoxin and thioredoxin reductase (Holmgren 1995). Trxs are powerful disulfide reductases that play a post-translational regulatory role in proteins involved in an ever-increasing number of cellular processes (Buchanan and Balmer 2005). A gene (Clone ID: Pt1) encoding thioredoxin was identified from the present system. Similarly, Voiblet et al. (2001) identified a fungal gene encoding thioredoxin reductase (Clone ID: 8C7) from the *P. tinctorius–E. globulus* system. The increased expression of the two components of the thioredoxin system in the fungus *P. tinctorius* suggests that the Trx system may be involved in maintaining proteins that play important roles in the early stages of symbiosis in the reduced state, just as shown by Alkhalfioui et al. (2008) for a novel type of Trx in *Medicago truncatula*.

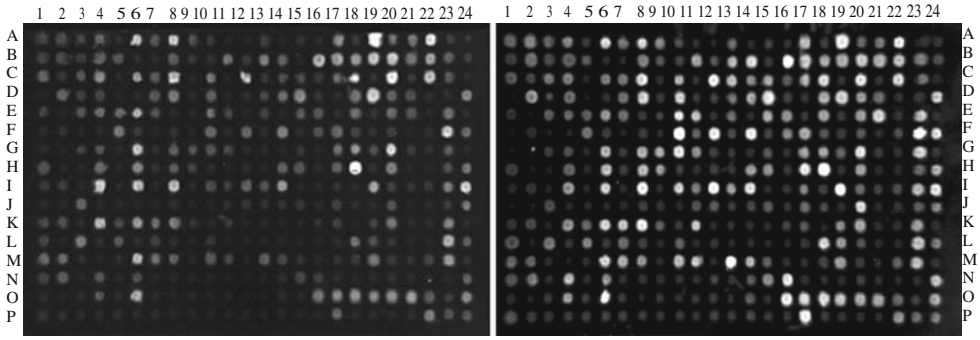


**Fig. 1** Differential screening of positive clones from the subtractive library by using reverse northern blot experiments. Hybridizations were carried out using DIG-labeled cDNA probes obtained from driver and tester samples. **a** Driver samples representing fungus cultured in the absence of host plant; **b** tester sample representing fungus cultured in the presence of the host plant

**Table 1** Identification of the possible proteins coded by the cDNAs of the suppression subtractive library of the fungus *Pisolithus tinctorius* during preinfection stage of its association with *Pinus massoniana*

| Clone/Contig | Length(bp) | GenBank accession number | Database match | *e*-Value | JGI Protein ID |
|---|---|---|---|---|---|
| Metabolism and energy | | | | | |
| Pt1 | 370 | XP_001829510 | Thioredoxin (*Coprinopsis cinerea* okayama7#130) | 4e-32 | 427548 |
| Pt2 | 417 | EIW85136 | S-adenosyl-L-methionine-dependent methyltransferase (*Coniophora puteana* RWD-64-598 SS2) | 1e-46 | 993273 |
| Transcription and protein fate | | | | | |
| Pt3 | 475 | EIM89141 | Ubiquitin-conjugating enzyme E2 (*Stereum hirsutum* FP-91666 SS1) | 9e-80 | 6282 |
| Pt4 | 450 | EIW84125 | 40S ribosomal protein S20 (*Coniophora puteana* RWD-64-598 SS2) | 9e-76 | 196084 |
| Pt5 | 685 | XP_001839135 | Maternal g10 transcript (*Coprinopsis cinerea* okayama7#130) | 2e-87 | 681688 |
| Membrane proteins and signaling | | | | | |
| Pt6 | 762 | AAM14581 | Cell wall symbiosis regulated acidic polypeptide precursor (*Pisolithus microcarpus*) | 4e-128 | 735241 |
| Pt7 | 364 | AAD30995 | Symbiosis regulated acidic polypeptide SRAP32-2 (*Pisolithus tinctorius*) | 8e-61 | 1003161 |
| Pt8 | 535 | EIW62870 | ADP ribosylation factor 6/GTPase SAR1 (*Trametes versicolor* FP-101664 SS1) | 9e-108 | 7781 |
| Cell rescue and defense | | | | | |
| Pt9 | 614 | EIW78658 | Cytochrome P450 (*Coniophora puteana* RWD-64-598 SS2) | 8e-81 | 942282 |
| Protein with binding function | | | | | |
| Pt10 | 343 | AAS19463 | Metallothionein (*Paxillus involutus*) | 0.007 | No hits |
| Proteins of unknown function | | | | | |
| Contig1 | 1,363 | GAA96378 | Hypothetical protein (*Mixia osmundae* IAM 14324) | 6e-64 | 167712 |
| Pt11 | 414 | XP_002392261 | Hypothetical protein (*Moniliophthora perniciosa* FA553) | 6e-45 | 137940 |
| Pt12 | 830 | XP_003614382 | Hypothetical protein (*Medicago truncatula*) | 2e-101 | 628224 |
| Pt13 | 464 | EGN96248 | Hypothetical protein (*Serpula lacrymans* var. lacrymans S7.3) | 1e-53 | 1000906 |
| Pt14 | 411 | EIW78471 | Hypothetical protein (*Coniophora puteana* RWD-64-598 SS2) | 8e-27 | 729421 |
| Pt15 | 495 | XP_001829556 | Hypothetical protein (*Coprinopsis cinerea* okayama7#130) | 7e-32 | 774834 |
| Pt16 | 797 | EIW86565 | Hypothetical protein (*Coniophora puteana* RWD-64-598 SS2) | 0.076 | 9796 |
| Pt17 | 624 | EJF60762 | Hypothetical protein (*Dichomitus squalens* LYAD-421 SS1) | 1e-27 | 903892 |
| Pt18 | 791 | EGO01615 | Hypothetical protein (*Serpula lacrymans* var. lacrymans S7.3) | 4e-98 | 33961 |
| Pt19 | 455 | EIW86863 | Hypothetical protein (*Coniophora puteana* RWD-64-598 SS2) | 9e-08 | 997320 |
| Pt20 | 340 | CCF44426 | Hypothetical protein (*Colletotrichum higginsianum*) | 0.023 | 1004407 |
| Pt21 | 606 | CCD21194 | Leucine-rich repeat protein (*Trypanosoma vivax* Y486) | 1e-10 | No hits |
| Pt22 | 371 | EIW76673 | hypothetical protein (*Coniophora puteana* RWD-64-598 SS2) | 2e-24 | No hits |
| Pt23 | 297 | CB011261 | *Pisolithus microcarpus* EST, *Pisolithus microcarpus* grown for three weeks | 3e-45 | No hits |
| Pt24 | 435 | BF942641 | *Pisolithus tinctorius* EST, 4-day-old *Eucalyptus globulus- Pisolithus tinctorius* | 2e-55 | No hits |

S-adenosyl-L-methionine-dependent methyltransferases (SAM-Mtases) may be involved in stabilization of DNA, RNA, and proteins, celluar signaling pathways, and protein synthesis (Clarke and Banfield 2001). Plant SAM-Mtases are the key enzymes in phenylpropanoid, flavonoid, and many other metabolic pathways of biotechnological importance (Joshi and Chiang 1998). A differentially expressed gene (Clone ID: Pt2) encoding SAM-Mtase was identified from the present system. Voiblet et al. (2001) identified a most highly regulated plant gene encoding for 7-O-methyltransferase (Clone ID: 8E9) from 4-day-old ectomycorrhizas in the *P. tinctorius–E. globulus* system, which exhibited strong activation (6.8-fold) in mycorrhizal eucalypt roots. These results confirm that the phenylpropanoid pathways in both fungi and plants are activated in the early stages of ectomycorrhiza formations, just as shown by Weiss et al. (1997) for accumulation of phenylpropanoids in larch mycorrhizas.

In eukaryotic cells, the ubiquitin system is a major pathway for regulated protein degradation. This pathway regulates key biological processes such as cell division, metabolism,

**Table 2** Oligonucleotides used for gene expression evaluation and subtractive library validation of the pre-symbiotic interaction between *Pisolithus tinctorius* and *Pinus massoniana* through RT-qPCR

| Clone | Primers | Oligonucleotides 5′-3′ |
|---|---|---|
| Pt3 | Pt3-1F | ACCATCACCGCATCTACAAGCA |
| | Pt3-1R | ACATGTTGTCACCTATCGGACCA |
| Pt4 | Pt4-1F | AAATCCGCATTACCTTGACGAG |
| | Pt4-1R | TAGCGATCCCATGTCTTTGAACC |
| Pt5 | Pt5-1F | CAACATAGTTTCTCGTAGCCTGT |
| | Pt5-1R | AAAATGAGTCCCACGAAGGCAAA |
| Pt8 | Pt8-1F | TTTCAACGTCGAGACTGTCACCT |
| | Pt8-1R | GTCCTGCTTGTTCGCAAAGACCA |
| Pt9 | Pt9-1F | GGAAAACTTTCGCGTCTTCTCCC |
| | Pt9-1R | AAGAACCGACGTATGACCAGCAA |
| | Pttub-1F | CATACCCTCCTGCGTGTACCAG |
| | Pttub-1R | ATTCTGCATATTTCGTCGAGTGGA |

immune response, and apoptosis (Peters et al. 1998). The twofold increased expression of transcripts coding for fungal ubiquitin-conjugating enzyme E2 (Pt3) was observed in the present system. Similarly, this component of ubiquitin pathway was also identified from plants in the *P. tinctorius–E. globulus* system (Clone ID: 6E3 and 6C7), and its increased expression suggested degradation of some down-regulated proteins (e.g., metallothionein, ATP synthase, cytochrome c oxidase, Zn-binding protein, etc.) in ectomycorrhizal tissues probably involved the ubiquitin pathway (Voiblet et al. 2001). Previous studies have shown that plant transcripts corresponding to ribosomal proteins were up-regulated during ectomycorrhiza formation (Johansson et al. 2004). The increased expression (threefold) of 40S ribosomal protein S20 (Pt4) observed in the present *P. tinctorius–P. massoniana* system and in the *Paxillus involutus–Betula pendula* system (Morel et al. 2005) strongly suggests that the up-regulated ribosomal proteins supports the fungal cells to rearrange their structure and metabolism to form a functional ectomycorrhiza. A gene-encoding maternal g10 (Pt5) from the present *P. tinctorius* showed high homology to those from *Coprinopsis cinerea*, a model fungus to evaluate genes underlying sexual development in basidiomycetes. Maternal g10 showed an increased expression (five- to tenfold) in the symbiotic mycelium. However, the maternal g10 homologues have not been found in previous studies on ectomycorrhizal symbiosis (Podila et al. 2002; Menotta et al. 2004; Zaretsky et al. 2006; Acioli-Santos et al. 2008). Human maternal g10 is responsible for cell fate and polarity of the embryo (Dworkin and Dworkin-Rastl 1990). So the potential function of the fungal gene encoding for maternal g10 at early stages of symbiosis formation deserves further analysis.

Two members (Clone ID: Pt6–7) of symbiosis-regulated acidic polypeptides (SRAPs) mannoprotein multigene families were abundantly identified from the present system. SRAPs were found at the surface of interacting fungal cells, and were candidate markers for symbiosis-related changes in the cell wall during the early stages (Laurent et al. 1999). Genes encoding the members of SRAP32 mannoproteins, are up-regulated in symbiotic tissues, which was well documented in earlier studies (Laurent et al. 1999; Voiblet et al. 2001). SRAPs are considered to have a considerable role in the construction of new symbiotic interface and in the communication between the two partners (Felten et al. 2012).

The sequence of clone Pt8 shared 99 % homology to the gene-encoding ADP ribosylation factor 6/GTPase SAR1 from the white-rot basidiomycete *Trametes versicolor*. Genes encoding cell signaling and cell transduction components such as ADP ribosylation factor (ARF), heterotrimeric GTPases, calmodulin, and ras were highly expressed in 4-day-old *Pisolithus–Eucalyptus* ectomycorrhiza (Voiblet et al. 2001). Pt8 showed an approximately 100-fold increased expression in this present system. These results confirm that the increased expression of small GTP-binding proteins (e.g., ARF and GTPase SAR) in both fungi and plants are involved in the signaling pathways at the stages of preinfection and mycorrhiza establishment. Ras-like small GTP-binding genes function as signaling molecules regulating vast number of biological processes including early and late secretory pathway, abiotic

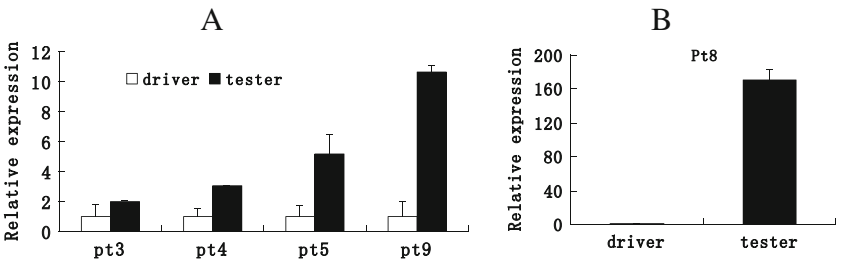


**Fig. 2** Gene expression of *Pisolithus tinctorius* cultured in the presence of plant (tester) in relation to that of fungus cultured in the absence of plant (driver). The *x* axis presents the target genes and the *y* axis the level of gene expression correspondent to the expression of the target genes in relation to the calibrator (tester), normalized by the β-tubulin endogenous control. The evaluated genes possibly control **a** ubiquitin-conjugating enzyme (Pt3), 40S ribosomal protein (Pt4), maternal g10 transcript (Pt5), cytochrome P450 (Pt9); **b** ADP ribosylation factor 6 (Pt8). Data are representative of results obtained in three independent experiments. *Error bars* average ± SE

and biotic stress signal transduction pathways, and mitotic spindle assembly in eukaryotic cells (Yuksel and Memon 2009). Two types of small GTP-binding proteins, ARF and secretion-associated and Ras-related (SAR), are major regulators of vesicle biogenesis in intracellular traffic (Memon 2004). ARF6 is involved in peripheral vesicle trafficking, such as endocytosis and exocytosis, and the organization of the actin cytoskeleton (Memon 2004; Gillingham and Munro 2007). GTPase SAR1 functions as a molecular switch to control the assembly of protein coats (COPII) that direct vesicle budding from endoplasmic reticulum (ER) (Memon 2004). However, the distinct cellular roles of ARF6/SAR1 on ECM formation and development between the two partners are poorly understood. Therefore, the fungal gene ARF6/SAR1 obtained from the present system remains for further study. In addition, studies on how the early signal pathways trigger the expression of symbiosis-regulated genes (e.g., ARF6/SAR1, SRAP32, etc.) that assist in partner recognition and ECM symbiosis establishment will provide further highlights into the signaling networks and early gene regulation processes involved in ectomycorrhiza development.

The cytochrome P450 gene family are important in the oxidative, peroxidative, and reductive metabolism of numerous endogenous compounds (e.g., steroids, fatty acids, phytoalexins, and plant hormones), as well as that of the xenobiotics in the environment (Nelson 1999). The gene encoding the protein cytochrome P450 (Clone ID: Pt9) was identified from the present *P. tinctorius–P. massoniana* system, from *Tuber borchii–Tilia americana* (Menotta et al. 2004) and *Hydnangium* sp.*–Eucalyptus grandis* systems (Silva Coelho et al. 2010) during the pre-symbiotic phase. So the increased expression (five- to tenfold) of cytochrome P450 may be involved in cellular detoxification processes, which suggests the recognition mechanism of the plant and defense mechanism of the fungus are activated in the very early stages of ectomycorrhiza formation.

Metallothioneins (MTs) are known to respond to a variety of stresses and likely involved in metal transport, cellular detoxification, and heavy metal tolerance (Lanfranco et al. 2002). cDNAs coding for putative MT-like polypeptides have been identified in the ectomycorrhizal fungi *P. tinctorius* (Voiblet et al. 2001; Peter et al. 2003; Duplessis et al. 2005), *P. involutus* (Courbot et al. 2004), and arbuscular mycorrhizal (AM) fungi *Gigaspora rosea* (Stommel et al. 2001), *G. margarita* (Lanfranco et al. 2002), and *Glomus intraradices* (González-Guerrero et al. 2007). Both plant and fungal MTs showed significant decreased expression in ectomycorrhizal tissues compared to the free-living *P. tinctorius* and *E. globulus* (Voiblet et al. 2001; Peter et al. 2003). The *GmarMT1* gene of AM fungus *G. margarita* was expressed in both presymbiotic spores and symbiotic mycelia, even in the absence of metal exposure, but was significantly less abundant in the latter stages (Lanfranco et al. 2002). The putative MT-like gene (Clone ID: Pt10) showed increased expression during the preinfection stage. These studies suggest the expression of fungal metallothionein gene is activated in the early stages of ectomycorrhiza or presymbiotic phase. This might be due to the fact that the mycorrhizal fungi suffers from both C and N starvation stress before physical contact, and such a metal-unrelated stress situation occurring within the presymbiotic mycelia might activate the expression of MTs. Similarly, Tamai et al. (1994) showed MT (CUP1) genes in brewer's yeast was activated by glucose starvation. However, during the symbiotic phase, more favorable conditions for mycorrhizal tissues to form and grow might lead to the significant decreased expression of MTs in both plants and fungi.

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## References

Acioli-Santos B, Sebastiana M, Pessoa F, Sousa L, Figueiredo A, Fortes AM, Baldé A, Maia LC, Pais MS (2008) Fungal transcript pattern during the preinfection stage (12 h) of ectomycorrhiza formed between Pisolithus tinctorius and Castanea sativa roots, identified using cDNA microarrays. Curr Microbiol 57:620–625

Alkhalfioui F, Renard M, Frendo P, Keichinger C, Meyer Y, Gelhaye E, Hirasawa M, Knaff DB, Ritzenthaler C, Montrichard F (2008) A novel type of thioredoxin dedicated to symbiosis in legumes. Plant Physiol 148:424–435

Buchanan B, Balmer Y (2005) Redox regulation: a broadening horizon. Annu Rev Plant Biol 56:187–220

Clarke S, Banfield K (2001) S-adenosyl-L-methionine-dependent methyltransferases. In: Carmel R, Jacobsen DW (eds) Homocysteine in health and disease. Cambridge University Press, Londen, pp 63–78

Courbot M, Diez L, Ruotolo R, Chalot M, Leroy P (2004) Cadmium-responsive thiols in the ectomycorrhizal fungus *Paxillus involutus*. Appl Environ Microbiol 70:7413–7417

Duplessis S, Courty PE, Tagu D, Martin F (2005) Transcript patterns associated with ectomycorrhiza development in *Eucalyptus globulus* and *Pisolithus microcarpus*. New Phytol 165:599–611

Dworkin MB, Dworkin-Rastl E (1990) Functions of maternal mRNA in early development. Mol Reprod Dev 26(3):261–297

Felten J, Martin F, Legué V (2012) Signalling in ectomycorrhizal symbiosis. In: Perotto S, Baluska F (eds) Signaling and communication in plant symbiosis. Springer, Berlin, pp 123–142

Gillingham AK, Munro S (2007) The small G proteins of the Arf family and their regulators. Annu Rev Cell Dev Biol 23:579–611

González-Guerrero M, Cano C, Azcón-Aguilar C, Ferrol N (2007) *GintMT1* encodes a functional metallothionein in *Glomus intraradices* that responds to oxidative stress. Mycorrhiza 17:327–335

Hilbert JL, Costa G, Martin F (1991) Ectomycorrhizin synthesis and polypeptide changes during the early stage of eucalypt mycorrhiza development. Plant Physiol 97:977–984

Holmgren A (1995) Thioredoxin. Annu Rev Biochem 54:237–271

Hua XM (1995a) Introduction to mycorrhiza. In: Hua XM (ed) Studies on mycorrhiza of forest trees. Chinese Sci Tech Press, Beijing, pp 1–20

Hua XM (1995b) Nutritional source study for super-strain of ectomycorrhizal fungus, *Pisolithus tinctorius*. For Res 8(6):597–604

Hua XM (2001) Mycorrhizal biotechnology of forest trees. World For Res 14(1):22–29

Inoue H, Nojima H, Okayama H (1990) High efficiency transformation of *Escherichia coli* with plasmids. Gene 96:23–28

Johansson T, Le Quéré A, Ahren D, Söderström B, Erlandsson R, Lundeberg J, Uhlen M, Tunlid A (2004) Transcriptionnal responses of *Paxillus involutus* and *Betula pendula* during formation of ectomycorrhizal root tissue. Mol Plant-Microbe Interact 17:202–215

Joshi CP, Chiang VL (1998) Conserved sequence motifs in plant S-adenosyl- l-methionine-dependent methyltransferases. Plant Mol Biol 37:663–674

Lanfranco L, Bolchi A, Ros EC, Ottonello S, Bonfante P (2002) Differential expression of a metallothionein gene during the presymbiotic versus the symbiotic phase of an arbuscular mycorrhizal fungus. Plant Physiol 130:58–67

Laurent P, Voiblet C, Tagu D, De Carvalho D, Nehls U, De Bellis R, Balestrini R, Bauw G, Bonfante P, Martin F (1999) A novel class of ectomycorrhiza-regulated cell wall polypeptides in *Pisolithus tinctorius*. Mol Plant-Microbe Interact 12:862–871

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C (T)) method. Methods 25:402–408

Martin F, Tagu D (1995) Genetics and molecular biology. Genetic transformation of ectomycorrhizal fungi. In: Varma A, Hock B (eds) Mycorrhiza: structure, molecular biology and biotechnology. Springer, Berlin, pp 51–74

Martin F, Lapeyrie F, Tagu D (1997) Altered gene expression during ectomycorrhiza development in the mycota. In: Lemke P, Caroll G (eds) Plant relationships. Springer, Berlin, pp 223–242

Martin F, Laurent P, De Carvalho D, Voiblet C, Balestrini R, Bonfante P, Tagu D (1999) Cell wall proteins of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*: identification, function, and expression in symbiosis. Fungal Genet Biol 27:161–174

Martin F, Kohler A, Duplessis S (2007) Living in harmony in the wood underground: ectomycorrhizal genomics. Curr Opin Plant Biol 10:204–210

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Marx DH (1977) Tree host range and world distribution of the ectomycorrhizal fungus *Pisolithus tinctorius*. Can J Microbiol 23:217–223

Memon AR (2004) The role of ADP-ribosylation factor and SAR1 in vesicular trafficking in plants. Biochim Biophys Acta 1664:9–30

Menotta M, Amicucci A, Sisti D, Gioacchini AM, Stocchi V (2004) Differential gene expression during pre-symbiotic interaction between *Tuber borchii* Vittad. and *Tilia Americana* L. Curr Genet 46:158–165

Morel M, Jacob C, Kohler A, Johansson T, Martin F, Chalot M, Brun A (2005) Identification of genes differentially expressed in extraradical mycelium and ectomycorrhizal roots during *Paxillus involutus-Betula pendula* ectomycorrhizal symbiosis. Appl Environ Microbiol 71:382–391

Nelson DR (1999) Cytochrome P450 and the individuality of species. Arch Biochem Biophys 369:1–10

Peter M, Courty PE, Kohler A, Delaruelle C, Martin D, Tagu D, Frey-Klett P, Duplessis S, Chalot M, Podila G, Martin F (2003) Analysis of expressed sequence tags from the ectomycorrhizal basidiomycetes *Laccaria bicolor* and *Pisolithus microcarpus*. New Phytol 159:117–129

Peters JM, Harris IR, Finley D (1998) Ubiquitin and the biology of the cell. Plenum, New York

Podila GK, Zheng J, Balasubramanian S, Sundaram S, Hiremath S, Brand J, Hymes M (2002) Molecular interactions in ectomycorrhizas: identification of fungal genes involved in early symbiotic interactions between *Laccaria bicolor* and red pine. Plant Soil 244:117–128

Rebrikov DV, Britanova OV, Gurskaya NG, Lukyanov KA, Tarabykin VS, Lukyanov SA (2000) Mirror orientation selection (MOS): a method for eliminating false positive clones from libraries generated by suppression subtractive hybridization. Nucleic Acids Res 28:E90

Silva Coelho I, Queiroz MV, Costa MD, Kasuya MCM, Araújo EF (2010) Identification of differentially expressed genes of the fungus *Hydnangium* sp. during the pre-symbiotic phase of the ectomycorrhizal association with *Eucalyptus grandis*. Mycorrhiza 20:531–540

Stommel M, Mann P, Franken P (2001) EST-library construction using spore RNA of the arbuscular mycorrhizal fungus *Gigaspora rosea*. Mycorrhiza 10:281–285

Tamai KT, Liu X, Silar P, Sosinowski T, Thiele DJ (1994) Heat shock transcription factor activates yeast metallothionein gene expression in response to heat and glucose starvation via distinct signalling pathways. Mol Cell Biol 14:8155–8165

Voiblet C, Duplessis S, Encelot N, Martin F (2001) Identification of symbiosis-regulated genes in *Eucalyptus globulus-Pisolithus tinctorius* ectomycorrhiza by differential hybridization of arrayed cDNAs. Plant J 25(2):181–191

Weiss M, Mikolajewski S, Peipp H, Schmitt U, Schmidt J, Wray V, Strack D (1997) Tissue-specific and development-dependent accumulation of phenylpropanoids in larch mycorrhizas. Plant Physiol 114:15–27

Yuksel B, Memon A (2009) Legume small GTPases and their role in the establishment of symbiotic associations with *Rhizobium* spp. Plant Signal Behav 4(4):257–260

Zaretsky M, Sitrit Y, Mills D, Roth-Bejerano N, Kagan-Zur V (2006) Expression of fungal genes at preinfection and mycorrhiza establishment between *Terfezia boudieri* isolates and *Cistus incanus* hairy root clones. New Phytol 171:837–846

Zheng LY, Li WD, Cheng XF, Hua XM (2003) Study on Ecto-endomycorrohizae Associated by *Pisolithus tinctorius* with Pine. For Res 16(3):262–268