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Gene Expression in Secondary Metabolism and Metabolic Switching Phase of *Phanerochaete chrysosporium*

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Abstract Ligninolytic enzymes are well-known to play the crucial roles in lignin biodegradation and have potential applications in industrial processes. The filamentous white-rot fungus, *Phanerochaete chrysosporium,* has been widely used as a model organism for studying these ligninolytic enzymes that are able to degrade the lignin during the secondary metabolism. To study the gene expression in secondary metabolism and metabolic switching phase of *P. chrysosporium*, we constructed a metabolic-switching phase suppression subtractive hybridization (SSH) cDNA library and a secondary metabolic phase SSH cDNA library to compare their mRNA expression profiles. We isolated the genes that are specially expressed and subsequently identified four genes that specially expressed during metabolic-switching phase while 22 genes in secondary metabolic phase. Accordingly, these specially expressed genes might play key roles in different metabolic stages, which would offer more new insights into the shift from nitrogen to lignin metabolism.

Keywords Ligninolytic enzymes · *Phanerochaete chrysosporium* · Metabolic-switching phase · Secondary metabolic phase · cDNA library

Introduction

Lignin is a major component of the plant cell wall and the second most abundant natural polymer on the earth [1, 2]. The filamentous white-rot fungus *Phanerochaete chrysosporium* has been widely used as a model organism for the studies on biodegradation of lignin, since it can secrete a variety of extracellular oxidative enzymes known as ligninolytic enzymes during the secondary metabolism [3, 4]. These enzymes are considered to play crucial roles in lignin biodegradation and have potential uses in industrial processes, such as biopulping, biobleaching, and the remediation of recalcitrant organopollutant-contaminated

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soils and effluents [5–7]. The 32.5-Mb haploid genome of *P. chrysosporium* has been sequenced using a pure whole genome shotgun strategy, and a total of 10,048 genes were predicted. Supporting evidence, annotations, and analyses are available online. The results revealed that many genes were potentially involved in the lignin degradation [8, 9]. The *P. chrysosporium* genome sequence represents an important advance in the molecular genetics of basidiomycetes and provides a framework for further investigation on lignocellulose degradation.

Previous physiological studies demonstrated that, under conditions optimized (2.4 mM nitrogen source and pure oxygen) for lignin metabolism, the growth of *P. chrysosporium* showed a reproducible sequence of events after inoculation: 0 to 24 h, germination, linear growth, and depletion of nutrient nitrogen; 24 to 48 h, cessation of linear growth and depression of ammonium permease activity (demonstrating nitrogen starvation); 72 to 96 h, appearance of lignin peroxidase activity [10]. It was suggested that nitrogen was exhausted between day 2 and day 3, and the onset of secondary metabolism was triggered by deficiency of nitrogen [11]. This period was a crucial time for *P. chrysosporium* to enter secondary metabolism [11, 12]. It also indicated that the appearance of lignin peroxidase activity was closely related to the beginning of secondary metabolism in physiology. In recent years, several comprehensive studies have been undertaken to dissect the variation in expression patterns under different culture conditions and time [9, 13–18]. Despite the fact that a substantial amount of work was done, the molecular mechanism regulating the ligninolytic enzyme production remains unclear. Identifying critical genes involved in regulating transition from primary metabolism to secondary metabolism will give us a chance to understand details of the ligninolytic system and help us improve the efficiency on use of the fungus.

Jiang et al. (2009) have studied the differentially expressed genes between day 2 and day 3 in the culture. The results demonstrated that there are significant differences in gene expression pattern between the two time points [19]. However, in the culture from day 2, they have also detected some genes which were usually expressed in secondary metabolism. It was indicated that we could determine the exact time of metabolic-switching phase. We noted that metabolic-switching phase and the period of secondary metabolism was a continuous process, and the gene expression displayed sequentially. Therefore, sampling in multiple time points might be better to observe the specially expressed genes. In the present study, we used suppression subtractive hybridization (SSH) to identify the gene specially expressed during metabolic-switching phase and secondary metabolism. The SSH results were further confirmed by Dot blot analysis.

In this study, we constructed metabolic-switching phase SSH cDNA library and secondary metabolic phase SSH cDNA library. Their mRNA expression profiles were contrasted to isolate genes that are specially expressed. Accordingly, we have identified four genes that specially expressed during metabolic-switching phase and 22 genes specially expressed in secondary metabolic phase. These specially expressed genes may play important roles in different metabolic stage. The result of this study would allow a greater understanding of the molecular mechanisms of the shift from nitrogen metabolism to lignin metabolism.

Materials and Methods

Strains and Culture Conditions

P. chrysosporium BKM-F-1767 (ATCC 24725) was grown under the nitrogen-limited media, and the conidia were obtained as described previously [20]. Three milliliters of the

suspension (1×10^7 spores/ml) were inoculated into 50 ml nitrogen-limited media. The cultures were then grown statically in 500 ml Erlenmeyer flasks with rubber stopper at 39°C, and the mycelia were flushed with 100% oxygen for 2 min at 24-h intervals. Oxygen purge is necessary for optimal lignin degradation culture conditions [4]. The mycelia were harvested at eight different time intervals (40, 48, 56, 64, 72, 96, 120, and 144 h) for RNA extraction, respectively.

Measurement of Ligninolytic Enzyme Activity

To confirm the exact time of the mycelia harvesting, lignin peroxidase (LIP) activities were analyzed by veratryl alcohol oxidation. The enzyme was incubated with 2.5 mM veratryl alcohol, 25 mM D-tartaric acid (pH 3.0), and 0.5 mM H_2O_2. The extinction coefficient of veratryl aldehyde (oxidized veratryl alcohol) at 310 nm is 9,300 $M^{-1}cm^{-1}$. The reaction was started by H_2O_2 addition, and the linear increase in absorbance at 310 nm was monitored over 3 min at room temperature [21]. Each assay was performed in triplicate. One unit is defined as the amount of the enzyme to oxidize 1 μmol of veratryl alcohol to veratryl aldehyde per minute.

RNA Extraction and Full-Length cDNA Synthesis

Total RNA was isolated from mycelia using the Trizol reagent (Invitrogen, CA) according to the manufacturer's manual. RNA samples were treated with DNase I (Takara, Dalian, China) to remove genomic DNA. The concentrations and qualities of RNA samples were determined by UV-Vis spectrometer and electrophoresis on 1.5% denatured formaldehyde agarose gel. Polyadenylated RNA was purified from total RNA using the oligo-tex-dT mRNA Midi kit (Qiagen, USA). SMART PCR cDNA synthesis kit (Clontech Laboratories, CA) was used to reverse transcribe mRNA extracted from the different stage samples according to manufacturer's instructions. High-quality cDNAs were obtained and confirmed by using PCR analysis of *gpd* and *LIPC* gene expression.

Suppression Subtractive Hybridization

The 48, 56, and 64 h double-strand cDNA were mixed equimolarly and designated as metabolism-switching phase samples (tester1); the 72, 96, 120, and 144 h cDNA were mixed equimolarly and designated as secondary metabolism samples (tester2); the 40 h cDNA (primary metabolism samples) were used as driver. The cDNA of tester1 to subtract the driver cDNA resulted in the metabolism-switching phase (MS) library, and the cDNA of tester2 to subtract the driver cDNA produced the secondary metabolism phase (SM) library. SSH was performed with the PCR-selected cDNA Subtraction kit (Clontech Laboratories, CA), essentially as recommended but with some modification. In brief, the two cDNA pools were digested with *Rsa*I and then precipitated by ethanol and NH_4OAc. Two aliquots of the tester cDNA were ligated to adaptors 1 and 2R, respectively. The first round hybridization was performed 68°C for 8 h, and the second round hybridization involved mixing the two samples from the first hybridization and adding fresh denatured driver cDNA; the second hybridization reactions were incubated at 68°C for 14 h. The resulting cDNA molecules were then subjected to two rounds of PCR to amplify and enrich the desired differentially expressed sequences. Primary PCR condition was 5 min at 72°C, 25 s at 94°C, and 27 cycles of 10 s at 94°C, 30 s at 66°C, and 1.5 min at 72°C. One milliliter of one tenth diluted primary PCR product was added into a new PCR tube for secondary PCR.

The second round of PCR condition was 94 °C for 15 s, 68 °C for 30 s, and 72 °C for 60 s for 18 cycles. The subtracted PCR products generated by SSH were cloned into the pMD20-T vector (Takara) and transformed into *Escherichia coli* JM109 competent cells for blue–white selection. White clones were randomly picked for further identification and characterization. To evaluate the efficiency of the adapter ligation reaction, the relative amount of glyceraldehye-3-phosphate dehydrogenase (*gpd)* cDNA present in adaptor-ligated cDNA populations were compared by PCR using *gpd* gene forward (5′-TACCACGCTACCCAGAAGA-3′) and reverse (5′-CACGATCGAAGATCGACGAG-3′) primers, or *gpd* gene forward primer and PCR primer 1 (5′-CTAATACGACTCACTA TAGGGC-3′, which locates on the adaptor sequences). To evaluate the efficiency of the subtraction, the relative amount of *gpd* cDNA presented in the subtracted and unsubtracted cDNA populations after SSH was examined by PCR amplification using the *gpd* gene forward and reverse primers.

PCR Amplification and Dot Blot Differential Screening of the Selected Clones

The selected white clones from the two subtracted cDNA libraries were grown in a 96-well plate in 200 μl LB medium containing 100 μg/ml ampicillin for 12 h at 37 °C. Then, 1 μl of each bacterial culture was used to amplify the cDNA insert in the recombinant plasmids. A 15-ml aliquot of each PCR product was then denatured by adding an equal amount of 0.4 N NaOH, and 15 μl of this mixture from each clone, was blotted onto Hybond N nylon membrane (Amersham Pharmacia Biotech) using a vacuum blotter (Millipore). Altogether, these blots were subsequently cross-linked by ultraviolet radiation. Three different types of reverse transcription-based probes prepared from primary (40 h) and metabolism-switching phase (48–64 h), as well as secondary metabolic phase (72–144 h) RNA samples were used in the hybridization. The three types probes were labeled with [α-^{32}P] using the 6-bp random primer, respectively. The blots were prehybridized with hybridization buffer (6× SSC, 5×Denhart, 0.5% SDS, and 100 μg/ml sheared denatured salmon sperm DNA) for 3 h at 68 °C with continuous agitation. Hybridizations were performed overnight at 68 °C in the hybridization buffer containing specific [α-^{32}P]-labeled cDNA probes. Following hybridization, blots were washed in a low stringency solution (0.2×SSC/0.1%SDS) at room temperature for 30 min and then a high stringency solution (0.1×SSC/0.1%SDS) at 68 °C for 30 min each. The membranes were then exposed to X-ray film overnight at −80 °C. The screening results showed that there are four types of clones in each SSH library: (1) phase-specific clones only hybridized with their own phase cDNA probes; (2) phase-differential clones hybridized with their own phase cDNA probe and another probe; (3) false-positive clones hybridized with all three probes; and (4) no signal clones which did not display signals with any probes. According to the above results of the dot blot, the phase-specific clones from each library were selected randomly for further analysis.

DNA Sequencing and Bioinformatics Analysis

The specific clones from two SSH libraries were sequenced using standard M13 sequencing primers by ABI PRISMTM 377 DNA Sequencer (Sangon, Shanghai, China). Nucleotide sequences were subjected to removal of the vector sequence by Chromos program. Trimmed sequence data were assembled into contigs using CONTIGEXPRESS program with parameters optimized for ESTs rather than genomic sequence. TBLASTX and BLASTN analysis of nucleotide sequences against non-redundant protein sequence databases at the NCBI database and *P. chrysosporium* genome database (http://geno-me.

jgi-psf.org/Phchr1/Phchr1.home.html) were performed. Any similarities with a score over 46 or an e-value of less than 10^{-7} were considered to be significant. A unique contig with significant matches was annotated, based on their most similar functions following the general rules from the Functional Catalogue by KEGG (Kyoto Encyclopedia of Genes and Genomes) [22] gene search system (http://genome.jgi-psf.org/cgi-bin/metapathways?db=Phchr1), KOG (Eukaryotic Orthologous Groups) annotation http://genome.jgi-psf.org/cgi-bin/kogBrowser?db=Phchr1) and with the aid of the Gene Ontology annotation (http://genome.jgi-psf.org/cgi-bin/ToGo?species=Phchr1).

Results

The Transition From Primary Growth to Secondary Metabolic Phase

The relationships among the growth, nutrient nitrogen starvation, and the appearance of ligninolytic activity were examined in stationary batch cultures under nitrogen-limited conditions and oxidative stress. The results demonstrated that the LIP activity was first observed between day 3 and day 4 in the extracellular fluid, and its highest peaks appeared on day 6 [Fig. 1]. This result also indicated that the pivotal time for *P. chrysosporium* to initiate secondary metabolism was between day 2 and day 3. This time point was named as metabolism-switching phase because the nutrient nitrogen was depleted, and the primary growth ceased under this condition. It also implied that the RNA samples isolated from 48 to 64 h mycelia could be used to determine the differentially expressed genes in metabolism-switching phase, and the RNA samples from 72 to 144 h could be used to identify differentially expressed genes in secondary metabolic phase [Fig. 2].

Construction of the Subtracted cDNA Libraries and Evaluation of Subtraction Efficiency

To estimate the efficiency of subtraction, the abundance of *gpd* cDNA was compared before and after subtraction. In nonsubtracted MS libraries, a 350-bp *gpd* specific PCR product is visible by 18 cycles of amplification and becomes saturated at 28 cycles [Fig. 3c left]. In contrast, the *gpd* PCR product was detected only at 28 cycles in the subtracted libraries. The data indicated that the amount of *gpd* transcript was reduced by over 1,000-fold after subtraction. The PCR product of *gpd* appeared to be detectable after amplification for 23 cycles and 18 cycles in the subtracted and the nonsubtracted SM libraries, respectively, indicating that the amount of *gpd* transcript was reduced by 32-fold in the subtracted SM

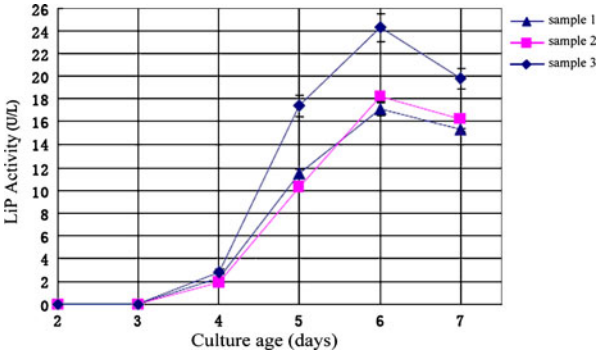

Fig. 1 LIP activity in different samples from different growth stages

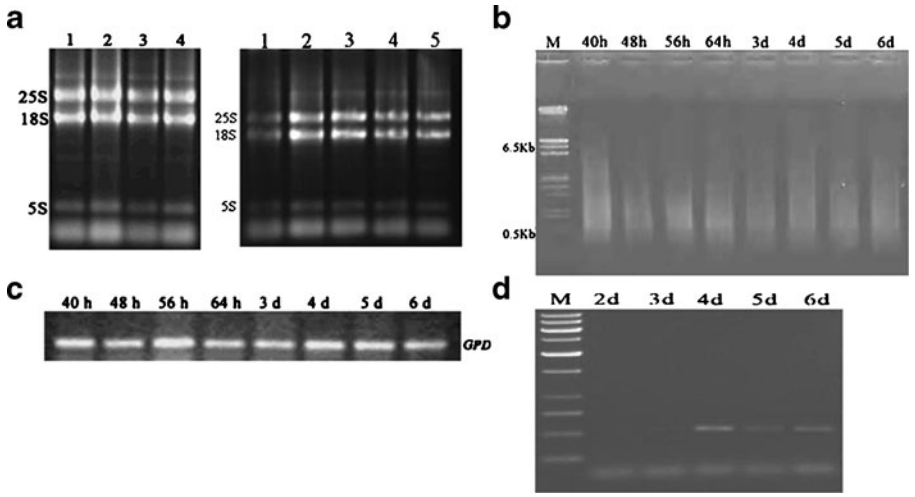

Fig. 2 RNA extraction and full-length cDNA synthesis. **a** Electrophoresis of total RNA of *P. chrysosporium*. The samples of *1–4 lanes* of *left* photo were 40, 48, 56, and 64 h, respectively; and the samples of *1–5 lanes* of right photo were 40 h, 3, 4, 5, and 6 d, respectively. **b** Electrophoresis of ds-cDNA at various growth stages. **c** RT-PCR analysis of GPD transcription at various growth stages. **d** RT-PCR analysis of LIP transcription at various growth stages

libraries [Fig. 3c right]. These results indicate that the subtractions are greatly effective in decreasing the amount of the gene [Fig. 3].

Two SSH cDNA libraries were constructed for MS- and SM-samples as described in Section 2. The MS library contains 1,123 white clones [Fig. 4a] and the SM library with 1,525 white clones [Fig. 4b]. All the white clones were selected, and the inserts were amplified by PCR. Of the subtracted MS library, 1,045 (93.05%) had inserts, and the subtracted SM library had 1,403 inserts (92.00%). The sizes of inserted fragments in both libraries were between 100 and 650 bp. In each library, 960 PCR products were selected randomly and arrayed on 20 Hybond N nylon membranes for dot blot analysis. Each blotting membrane was hybridized with three different types of probes, respectively. The differences in hybridization signal intensities were shown on the membranes probed with three types of probes, which indicated the success of the SSH procedure in identifying differentially expressed sequences between the two cDNA populations being examined. In the subtracted MS library, there were 150 phase-specific clones (15.62%) which were not hybridized with primary probes and secondary metabolic probes. While in the subtracted SM library, there were 253 phase-specific clones (26.35%) which were not hybridized with primary probes and metabolism-switching probes [Fig. 5]. The nucleotide sequences of 71 and 90 ESTs from subtracted MS library and subtracted SM library were determined by DNA sequencing, respectively.

Functional Classification of ESTs

Based upon the TBLASTX and BLASTN results, 22 ESTs from both subtracted libraries were obtained and could be classified according to Eukaryotic Orthologous Groups (KOG), KEGG, Gene Ontology annotation, and Interpro for identifying ortholog and paralog proteins on the online service at http://genome.jgi-psf.org/whiterot. The functional assignment for each predicted gene was presented in Table 1. There are seven genes

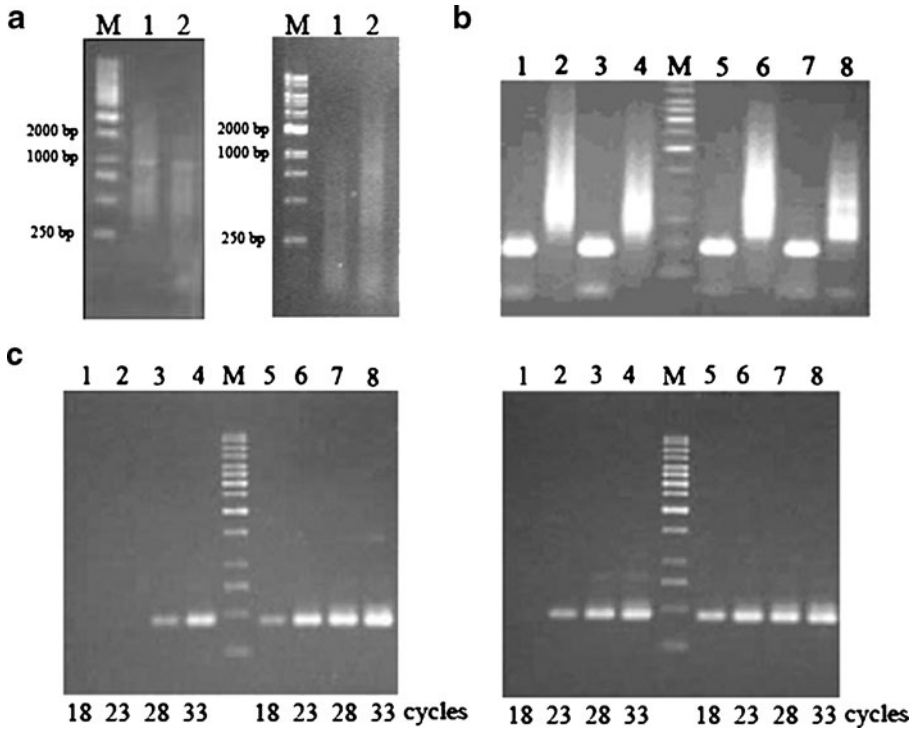

Fig. 3 Suppression subtractive hybridization analysis. **a** Electrophoresis of RasI digested cDNA product. **b** PCR analysis of adaptor-ligation efficiency. The *lanes 1*, *2* and *lanes 3*,*4* were used tester1-1 and tester1-2 as template, respectively, while *lanes 5*, *6* and *lane 7*, *8* were tester2-1 and tester2-2, respectively; The GPD3′- and PCR Primer 1 primer were used for *lane 1*, *3*, *5*, and *7*, while the GPD3′- and GPD5′- primer were used for other lane besides marker lane. **c** PCR analysis of subtraction efficiency. The *left* photo indicated the metabolism-switching phase and *1–4 lanes* indicated the GPD gene amplification after subtraction efficiency while *5–8 lanes* after unsubtraction efficiency. The *right* photo indicated the secondary metabolism phase and each lane was same as the left one

associated with metabolic pathway (31.81%), including one (protein ID 8832) from MS library and six (133953, 136462, 127717, 124398, 3765, and 132198) from the SM library. Six genes (27.27%) involved in protein synthesis and post-translational modification were identified, five among which, 2095, 134927, 7266, 138363, and 4406, are specifically expressed during the MS phase while 37135 is specific to the SM phase. Another three genes (13.63%) involved in RNA processing and modification is consisted of 134,319 (specific to the MS phase), 2,277, and 8,984 (both two only expressed in SM phase). Only two genes (9.09%) could be functionally classified into the signal transduction processes, one (4874) specific to the MS and the other (25929) specific to the SM phase. However, other ESTs could not be assigned functionally based on this analysis.

Discussion

When *P. chrysosporium* was grown under conditions optimized for lignin degradation, the linear growth was terminated on second day, and the lignin peroxidase activity appears in

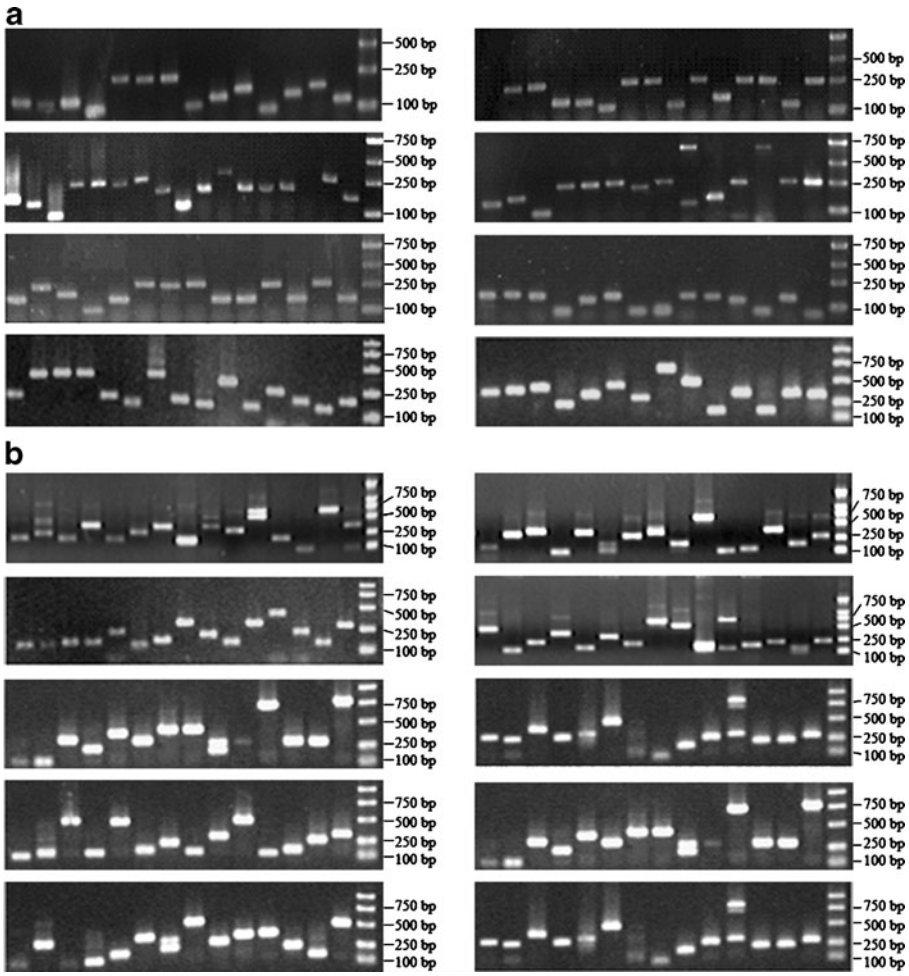

Fig. 4 The electrophoresis of colony PCR products of some clones. **a** Indicated the metabolism-switching phase and **b** the second metabolism phase

the extracellular fluid on the fourth day [12, 23]. It means that the time between day 2 and day 3 are pivotal times for *P. chrysosporium* to initiate secondary metabolism. The purpose of this study was to identify the gene specially expressed during metabolic-switching phase and secondary metabolism by SSH, based on the hypothesis that genes expressed between day 2 and day 3 potentially play important roles in the event of the transition from primary to secondary metabolism of *P. chrysosporium*. Moreover, genes specially expressed after 3 days of incubation were generally considered to be involved in secondary metabolism. In the present research, to monitor more exactly the appearance of specially expressed gene, eight time point RNA samples were sampled at 40, 48, 56, 64, 72, 96, 120, and 144 h. The RNA sample from the time point of 40 h after inoculation was chosen as driver because previous studies showed that some genes usually expressed in secondary metabolism were appeared at 48 h after inoculation. Thus, we proposed that this initial time point showed strong possibility as a starting point to efficiently identify genes involved in the metabolic switching.

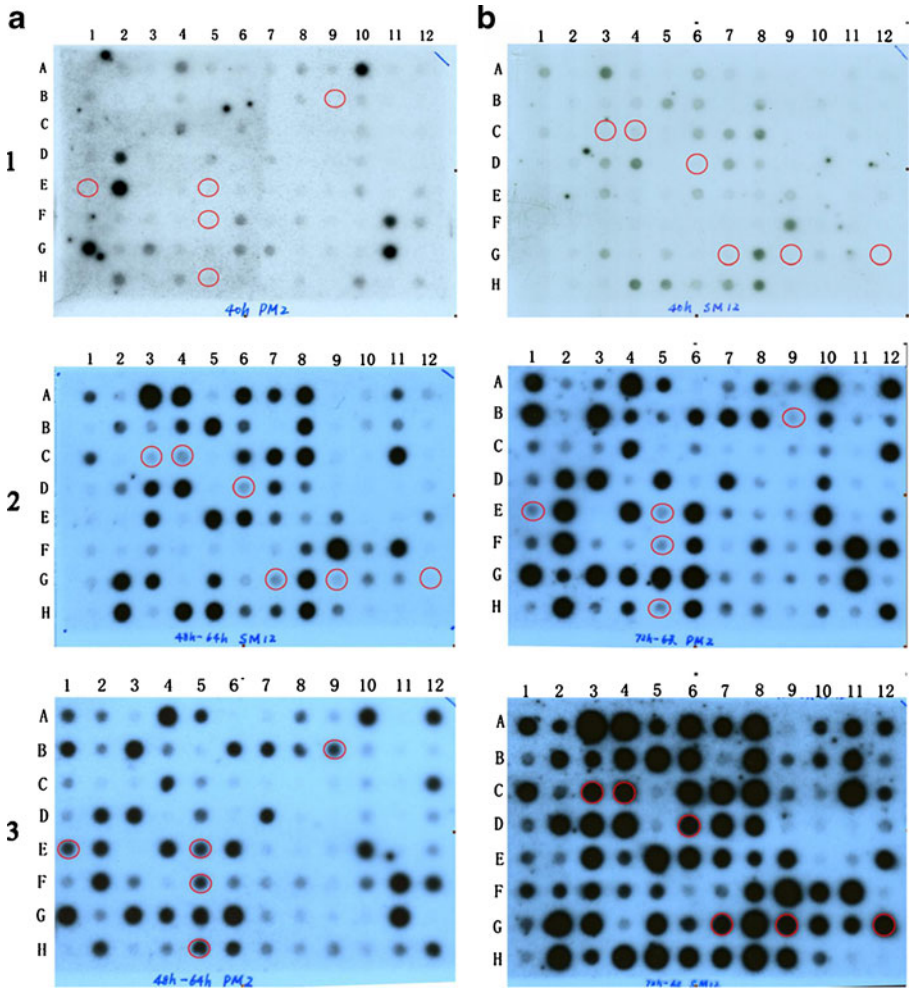

Fig. 5 Dot blot differential screening of clones at different phases. **a** indicated the metabolism-switching phase and **b** the second metabolism phase

SSH has been widely demonstrated to be a very successful technique for screening differentially expressed genes in different samples [24]. SSH overcomes the divergence in mRNA abundance by using a hybridization step during the subtraction that equalizes mRNA levels between the two populations being compared [25]. Applying SSH, the MS subtract cDNA library and SM cDNA library were constructed and used to contrast their mRNA expression profiles to isolate genes that are expressed in different stage. To screen the libraries, we performed three rounds dot blot with three different types of RT-probes for screening the specific clones in the two libraries as a self-hybridization confirmation, which can be used to remove the false-positive clones in a large part. The results confirm the special expression of four and 22 genes in MS and SM libraries, respectively. Bioinformatics searches of available non-redundant databases revealed that all of the genes had the significant hit in the databases examined. Some 84.61% (22) of the identified genes

Table 1 Function analysis of metabolic switching phase and second metabolism phase of different expression genes.

Phase	Protein ID	KOG	KEGG	IPR	GO
Metabolic switching phase	134319	mRNA splicing factor ATP-dependent RNA helicase	ecNum 3.6.1.3	DEAD/DEAH box helicase, N-terminal helicase, C-terminal helicase-associated region DEAD/DEAH box helicase helicase, C-terminal DEAD/DEAH box helicase ATP-dependent helicase, DEAH-box	ATP-dependent helicase activity
		RNA processing and modification	Adenosinetriphosphatase $ATP + H_2O = ADP + phosphate$ Purine metabolism		Helicase activity ATP-dependent DNA helicase activity ATP-dependent RNA helicase activity DNA-dependent ATPase activity RNA-dependent ATPase activity Protein-transporting ATPase activity DNA translocase activity ATPase activity, uncoupled ATPase activity, coupled to Transmembrane movement of ions ATPase activity Coupled to transmembrane movement of substances
	4874	Sexual differentiation process protein ISP4		Oligopeptide transporter OPT superfamily Tetrapeptide transporter, OPT1/isp4 Oligopeptide transporter OPT superfamily Peptidase aspartic	Aspartic-type endopeptidase activity
		Signal transduction mechanisms			Proteolysis and peptidolysis
	37135	Vacuolar sorting protein VPS33/slp1 (Sec1 family) intracellular trafficking, secretion, and vesicular transport		Sec1-like protein	Vesicle docking during exocytosis Vesicle-mediated transport
	8832	Rhodanese-related sulfurtransferase inorganic ion transport and metabolism		Rhodanese-like	

Second metabolism phase	133953	Gamma-glutamylcysteine synthetase coenzyme transport and metabolism	ecNum 6.3.2.2 glutamate–cysteine ligase ATP + L-glutamate + L-cysteine = ADP + phosphate + gamma-L-glutamyl-L-cysteine.	Glutamate–cysteine ligase catalytic subunit	Glutamate-cysteine ligase activity, glutathione biosynthesis, glutamate-cysteine ligase complex
	136462	Fructose 1,6-bisphosphate aldolase carbohydrate transport and metabolism	ecNum 4.1.2.13 Fructose-bisphosphate aldolase D-fructose 1,6-bisphosphate = glycerone phosphate + D-glyceraldehyde 3-phosphate	Ketose-bisphosphate aldolase, class II, fructose-bisphosphate aldolase, class II, yeast/*E. coli* subtype	Fructose-bisphosphate aldolase activity, glycolysis, zinc ion binding
	10872	FOG/RRM domain General function prediction only		RNA-binding region RNP-1 (RNA recognition motif)	Nucleic acid binding
	2028	FOG/WD40 repeat General function prediction only		G-protein beta WD-40 repeat	
	4406	Microtubule-associated protein dynactin DCTN1/glued cell cycle control, cell division, chromosome partitioning, cytoskeleton			
	127717	Amino acid transporters Amino acid transport and metabolism		Amino acid permease-associated region , Amino acid/polyamine transporter I, amino acid permease	Amino acid-polyamine transporter activity, amino acid transport, membrane, integral to membrane, transport
	2277	mRNA cleavage and polyadenylation factor II complex, subunit PTA1 RNA processing and modification	ecNum 2.7.7.6 DNA-directed RNA polymerase. N nucleoside triphosphate = N diphosphate + {RNA}(N).		DNA-directed RNA polymerase activity, DNA-directed RNA polymerase I activity, DNA-directed RNA polymerase II activity, DNA-directed RNA polymerase III activity
	9362			Hemopexin	
	8984	Nuclear localization sequence binding protein transcription		Cytochrome b/b6, N-terminal	Electron transport, membrane, oxidoreductase activity
	25929	Sexual differentiation process protein ISP4 Signal transduction mechanisms		Oligopeptide transporter OPT superfamily, tetrapeptide transporter OPT1/isp4, peptidase aspartic, active site	Aspartic-type endopeptidase activity, proteolysis and peptidolysis

Table 1 (continued)

Phase	Protein ID	KOG	KEGG	IPR	GO
	124398	Catalase Inorganic ion transport and metabolism	ecNum 1.11.1.6 Catalase. $2H_2O_2 = O_2 + 2H_2O$.	Catalase	Catalase activity, electron transport, response to oxidative stress
	3765	Beta-1,6-*N*-acetylglucosaminyltransferase, contains WSC domain Posttranslational modification, protein turnover, chaperones, Carbohydrate transport and metabolism			
	138363	Subtilisin-related protease/vacuolar protease B Posttranslational modification, protein turnover, chaperones	ecNum 3.4.21.48 cerevisin Hydrolysis of proteins with broad specificity, and of BZ-Arg-OET > Ac-Tyr-OET	Peptidase S8 and S53, subtilisin, kexin, sedolisin proteinase inhibitor I9, subtilisin propeptide	Subtilase activity, proteolysis and peptidolysis, protein self-binding, negative regulation of enzyme activity, cerevisin activity
	2095	Predicted NAD synthase, contains CN hydrolase domain Coenzyme transport and metabolism	NAD + synthase (glutamine-hydrolysing) ATP + deamido-NAD_+ +L-glutamine + H_2O = AMP + diphosphate + NAD_+ +L-glutamate	Nitrilase/cyanide hydratase and apolipoprotein *N*-acyltransferase, NAD + synthase	Nitrogen compound metabolism, hydrolase activity, acting on carbon–nitrogen (but not peptide) bonds, NAD + synthase (glutamine-hydrolyzing) activity, ATP binding, NAD biosynthesis
	130095	40S ribosomal protein S25 translation, ribosomal structure and biogenesis		S25 ribosomal protein	

134660	Translation elongation factor EF-1 alpha/Tu translation, ribosomal structure and biogenesis		Protein synthesis factor, GTP-binding, elongation factor Tu, C-terminal, Elongation factor Tu, domain 2,Translation elongation factor EF-1, alpha subunit, protein synthesis factor, GTP-binding	GTP binding, protein biosynthesis, translation elongation factor activity, cytoplasm, translational elongation, GTPase activity, protein-synthesizing GTPase activity
132198	Glyceraldehyde 3-phosphate dehydrogenase Carbohydrate transport and metabolism	ecNum 1.2.1.12 glyceraldehyde 3-phosphate dehydrogenase (phosphorylating) D-glyceraldehyde 3-phosphate + phosphate + NAD(+)= 3-phospho-D-glyceroyl phosphate + NADH	Glyceraldehyde 3-phosphate dehydrogenase	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity Glycolysis, NAD binding, glucose metabolism Glyceraldehyde-3-phosphate dehydrogenase activity
7266	Thioredoxin Posttranslational modification, protein turnover, chaperones		Thioredoxin, thioredoxin-related	Electron transporter activity electron transport thioredoxin-disulfide reductase activity
134927	40S ribosomal protein S24 Translation, ribosomal structure and biogenesis		Ribosomal protein S24e	Structural constituent of ribosome Intracellular Ribosome Protein biosynthesis

were assigned as functional proteins. However, four genes had no predicted function, perhaps indicating that these genes are unique to *P. chrysosporium*. Among the genes screened from the subtracted MS cDNA library, the most notable one is the rhodanese-related sulfurtransferase, which occurred 67 times. The sulfurtransferase catalyzes the transfer of a sulfane sulfur atom from an anionic donor to a thiophilic acceptor via an enzymatic persulfide intermediate and often is localized in the matrix of mitochondria [26, 27]. In some cases, rhodanese acts as a thioredoxin oxidase and can utilize reduced thioredoxin as a sulfur-acceptor substrate or catalyzes the direct oxidation of reduced thioredoxin by reactive oxygen species (ROS) [28]. As we know, thioredoxin is a ubiquitous protein that uphold the redox state of the cell, and it can protect the cell against ROS such as H_2O_2 and oxygen free radicals [29–31]. It is also interesting that many nitrogen starvation and oxidative stress relate genes are also found in the subtracted SM cDNA library. Among them are gamma-glutamylcysteine synthetase, thioredoxin, and catalase. As an antioxidant scavenging/defense protein, γ-glutamylcysteine synthetase belongs to the glutathione (GSH) redox system which is one of the most important cytoplasmic redox systems in many organisms [32]. GSH is an essential antioxidant in *Saccharomyces cerevisiae* and is required for protection against H_2O_2 [33]. *S. cerevisiae* mutants in the coding of GSH reductase and GSH synthetase are sensitive to H_2O_2 [34]. When the yeast was at nitrogen starvation, more than 90% of the total GSH shifted towards the central vacuole from the Golgi apparatus, and the GSH synthesis occurred by the consecutive action of γ-glutamylcysteine synthetase and glutathione synthetase [35]. The accumulated GSH in the central vacuole was further degraded and seems to play the role as a reservoir to provide the yeast cell with amino acids for subsequent growth requirements [35]. During the processes, γ-glutamylcysteine synthetase was regulated stringently by the transcript factors Yap1P and Skn7P [36]. If the GSH system cannot get rid of ROS effectively, the thioredoxin system would be the second line of defense, and the thioredoxin was also regulated by Yap1P and Skn7P [36]. In fungi, catalase plays an important role in response to the oxidative stress, and it can protect the cell against of ROS by converting the H_2O_2 to CO_2 and H_2O [37]. In our results, the catalase gene was specially expressed in second metabolism, and its sequence was detected for 44 times in the subtracted SM cDNA library. This result agrees with pervious cytochemical staining assay of *P. chrysosporium* hyphae showing that catalase activity is up-regulated during ligninolytic metabolism [38]. Hammel et al., reported the catalase was upregulated in the ligninolytic cultures by the LC/MS/MS [15]. With qRT-PCR analysis, they found that transcripts encoding this catalase were increased more than 35-fold in ligninolytic cultures in comparison with non-ligninolytic cultures. The appearance of these ROS related genes is not coincident. To trigger LIP expression, liquid cultures of *P. chrysosporium* must be subjected to nitrogen starved and exposed to a pure O_2 atmosphere. Higher O_2 concentration presumably leads to production of much more ROS in relation to that during normal metabolism, so the fungus must suffer from a ROS-rich environment, together with the toxic oxygen radical intermediates produced in the lignin-degrading system [38]. Although ROS is essential for LIP expression, excessive ROS leads to disorganization of the cellular ultrastructure and chlamydospore development [23]. Therefore, we conclude that the *P. chrysosporium* may apply one or several means to handle the excessive toxic substances produced as byproduct in the ligninolytic phase. Previous studies indicated that ROS present during lignin degradation was potentially involved in the regulation of initiation of secondary metabolism [39]. Accordingly, we speculate that the co-appearance of ROS-related genes at the onset of secondary metabolism suggests co-regulation between the ROS protection system and the trigger of secondary metabolism in *P. chrysosporium*.

Two oligopeptide transporter (OPT) genes were determined in our studies. The first one was detected in subtracted MS library, and the other was in the MS library. Recent physiological researches showed large families of OPT genes in *P. chrysosporium* and *Coprinopsis cinereus*. These transmembrane proteins were potentially involved in the transport of small molecular weight nitrogenous compounds [40]. Differential regulation of OPT genes has been observed in response to nitrogen limitation and different growth stages in *C. cinereus* and *Laccaria bicolor* [40, 41]. There are 17 putative OPT genes in *P. chrysosporium* genome, and no physical clustering of OPT genes were observed [8, 42]. In the previous studies, Cullen et al., have found that eight OPT genes were upregulated under defined ligninolytic and cellulolytic conditions [17]. In our results, these two OPT genes were located at different scaffolds, respectively. They share similarities in sequence composition and intron–exon boundaries, which suggested that they may belong to the same sub-family. By using the TMHMM v2.1 (www.cbs.dtu.dk/services/TMHMM/), both of the two OPTs have typical transmembrane structures, with subtle difference. The first OPT protein has 15 transmembrane regions, the N-terminal and C-terminal are located on both sides of membrane while the second one contains 16 transmembrane regions with two terminals located on outside membrane. It suggested that these two OPT genes, which were specially expressed in the two cDNA libraries, potentially played different roles in different metabolic stages under nitrogen-limited condition.

In summary, the combination of SSH and Dot blot analysis provides an effective method to compare the expression profile of *P. chrysosporium* among different metabolic phase. Four specially expressed genes in MS library, and 22 genes in SM library were identified. Most of the genes were potentially involved in the ROS metabolism, indicating that they may play important roles in the regulation of initiation of secondary metabolism. Further investigations will be aimed at demonstrating the biological function of the specially expressed genes during the transition time and second metabolism.

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