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Gene Expression Analysis of *Phanerochaete chrysosporium* During the Transition Time from Primary Growth to Secondary Metabolism

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In order to identify the secondary metabolism-related genes of *Phanerochaete chrysosporium* growing under pure O_2 and nitrogen-limited conditions, 2322 ESTs fragments originated from two suppression-subtractive libraries were analyzed using the cDNA microarray technique. Ten significantly upregulated and 22 significantly downregulated genes were identified in the 72 h cultured mycelia RNA samples (secondary metabolism). According to qPCR, 16 out of the 32 genes were expressed differently in secondary metabolism. Transcripts of secondary metabolism up-regulation genes exhibited homologies to aryl-alcohol dehydrogenase (SSh1554), ABC transporter gene (SSH624), chitinase (SSH963), heat shock protein (SSH1193), catalase (SSH317), cytochrome P450 (SSH331), glucosamine-6-phosphate isomerase (SSH611), and alkyl hydroperoxide reductase (SSH362) genes. Ninety-three genes could be classified by Eukaryotic Orthologous Groups (KOG). Among the genes assigned a function, gene expression patterns were different in both secondary metabolism and primary metabolism. In the group of "Cellular Processes and Signaling," most of the genes were from the primary metabolism library. On the other hand, genes from the secondary metabolism library were found mainly in the "Information Storage" and "Processing and Poorly Characterized" groups. Based on the KOG functional assignments, six genes belong to the ubiquitin system, and all of them were from primary metabolism phase. The presence of the H_2O_2-relevant genes suggested that parts of the genes expressed in 72 h might be involved in the ligninolytic process during secondary metabolism of *P. chrysosporium.*

***Keywords*:** *Phanerochaete chrysosporium*, subtractive cDNA library, microarray, secondary metabolism

As a representative model to study the biodegradation mechanism of lignin, *Phanerochaete chrysosporium* is the first basidiomycetous genome sequenced by the Joint Genome Institute of USA (http://genome.jgi-psf.org/pub/JGIdata/White-Rot) (Broda *et al*., 1996; Martinez *et al*., 2004). Thirty-million base-pair genomes of *P. chrysosporium* have been sequenced and have yielded a prediction of 11,777 genes. However, genes involved in regulating transition from primary metabolism (PM) to secondary metabolism (SM) of *P. chrysosporium* have not yet been clarified (Martinez *et al*., 2004). Recently, some researches showed that extracellular oxidative systems played important roles in *P. chrysosporium* derived lignin-degradation (Kersten and Cullen, 2007). The identification of these genes and revealing their transcriptional regulation will enhance our understanding of lignocellulose biodegradation, a critical process in the global carbon cycle, and will promote bioprocesses development in biomass utilization, organopollutant degradation, and fiber bleaching (Gold and Alic, 1993; Cullen, 1997).

Previous physiological studies demonstrated that the growth of *P. chrysosporium* mycelium under pure O_2 and nitrogen-limited conditions (2.4 mM nitrogen source) was terminated on the second day and that the lignin peroxidase (LIP) activity appears in the extracellular fluid on the fourth day (Boominathan *et al*., 1993; Gold and Alic, 1993; Cullen, 1997). This means that the time between days 2 and 3 is the crucial time point for *P. chrysosporium* to initiate the secondary metabolism (Boominathan *et al*., 1993). The time between nitrogen depletion and the appearance of the lignin-degrading activity suggests an indirect rather than a direct connection between lignin metabolism and nitrogen metabolism. It also suggests that the activity is associated with a physiology shift into "secondary metabolic" metabolism. The genes expressed during this period may be involved in this event. However, no comprehensive studies have been undertaken to characterize differently expressed genes of day two and day three cultures of *P. chrysosporium,* or to clarify the molecular mechanism of the shift from nitrogen metabolism to lignin metabolism.

Although the *P. chrysosporium* genome has been sequenced, functional genomics tools, such as genome-wide microarrays, are not widely available for this species. In this study, we constructed a feasible *P. chrysosporium* cDNA microarray in which ESTs were screened from two suppression subtractive hybridization (SSH) libraries with mycelial RNAs isolated from day two and day three cultures. Within the microarray, which includes 2322 ESTs fragments, we investigated the expression of genes during the transition period (day two to

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day three). We hypothesized that the gene expression pattern differs between primary growth and the secondary metabolism stage.

Materials and Methods

Strain and culture conditions

Phanerochaete chrysosporium BKM-F-1767 was grown in 50 ml low-nitrogen medium in 500-ml Erlenmeyer flasks with rubber stopper at 39°C and flushed with 100% oxygen until the mycelia were harvested at the second and third day. This medium contains only 2.4 mM nutrient nitrogen and is known as the Kirk medium (Zhang *et al*., 1986; Tien and Kirk, 1987).

Lignin peroxidase activity assay methods

To confirm the exact time of the mycelia harvesting, lignin peroxidase activity was assayed spectrophotometrically as the H_2O_2-dependent formation of veratraldehyde from veratryl alcohol. The reaction was started by H_2O_2 addition, and the linear increase in absorbance at 310 nm was monitored over 3∼5 min at room temperature (Tien and Kirk, 1987). Total nitrogen concentrations were determined by the micro-Kjeldahl procedure (Ward, 1963).

Subtractive cDNA library construction

The mycelia were harvested on the second and third day and designated as PM and SM stage samples, respectively. The total RNA of second- and third-day mycelia was extracted by Trizol reagent (Invitrogen, USA). Poly (A) RNA was purified from total RNA using the Oligo-tex-dT mRNA Midi kit (QIAGEN, USA). SSH was processed using the PCR-selected cDNA Subtraction kit (Clontech) according to the manufacture's instructions (Diatchenko *et al*., 1996; Kuang *et al*., 1998). The forward-subtraction starting material consisted of 2 μg of 3 d mRNA as tester and 2 μg of 2 d mRNA as driver. This process resulted in SM-specific products. Conversely, in reverse subtraction, 2 d mRNA was used as tester and 3 d mRNA as driver; it produced PM-specific products. The subtraction efficiency was evaluated by the reduction of glyceraldehydes-3-phosphate dehydrogenase (*gpd*) gene abundance by PCR-selected subtraction (forward primer: 5'-TACCACGCTACCCAGAAGA-3'; reverse primer: 5'-CACGATCGAAGATCGACGAG-3'). All PCR products generated from forward- and reverse-subtraction were directly ligated into a PMD18-T vector (TaKaRa, China) and then transformed into *E. coli* strain Top-10. Transformed *E. coli* cells were plated on LB-Amp media with X-Gal and IPTG. White clones were kept in LB broth containing 15% glycerol and 100 μg/ml ampicillin. Thus, the library constructed from forward-subtraction products contained SM-specific clones. Moreover, the library derived from reverse-subtraction products contained PM-specific clones.

Construction of cDNA Microarray

DNA samples for microarray spotting were amplified by PCR from each white clone with nested primer 1; 5'-TCGA GCGGCCGCCCGGAGGT-3' and nested primer 2R; 5'-AG CGTGGTCGCGGCCGAGGT-3'. PCR amplifications were performed in the following reaction mixture: 1 μl template, 7 μl 10× Taq reaction buffer, 2 μl 2.5 mM dNTP, 1 μl nested p1 primer, 1 μl nested p2R primer, 0.3 U Taq DNA polymerase, and 58.2 μl ddH_2O. PCR samples were incubated at 96°C for 4 min, and then subjected to 30 cycles of 94°C for 30 sec, 68°C for 30 sec, and 72°C for 2.5 min. A final incubation step of 72°C for 10 min was also carried out. In order to trace the microarray experiment and to determine the detection sensitivity, four *P. chrysosporium gpd* genes (accession number: M81754), two *lip*C genes, (accession number: X55343), and nine yeast intergenic sequences without homology with all known genes were also PCR amplified and spotted as internal and exogenous controls (Lamar *et al*., 1995). PCR products were precipitated with isopropanol, resolved in 15 μl 50% dimethyl sulphoxide (DMSO), and then adjusted to 0.1∼0.5 g/L. A commercial arrayer PixSys 5500 (Cartesian Technologies, USA) was used to array DNA fragments onto poly-L-lysine-coated glass slides. The spotting process was carried out according to a protocol pub-

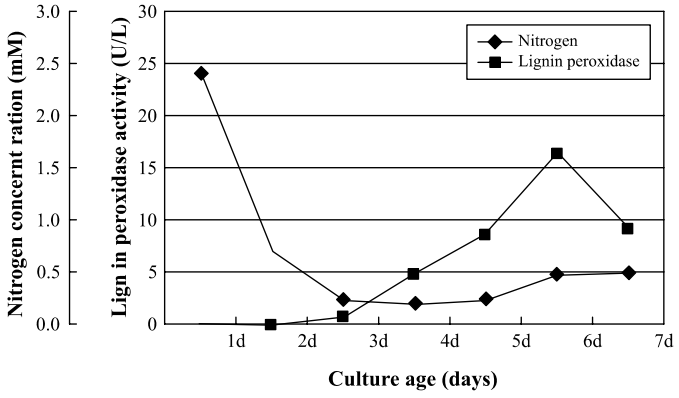

Fig. 1. Relationship between nitrogen starvation and lignin peroxidase activity over 7 days. The concentration of nitrogen dropped rapidly and depleted to the lowest level at some time point between day 2 and day 3, then kept a relative stable level for a few days. The LIP activity was initiated after three day incubation along with the depletion of the nitrogen and maximized between day 5 and day 6 while a small jump of nitrogen concentration was detected

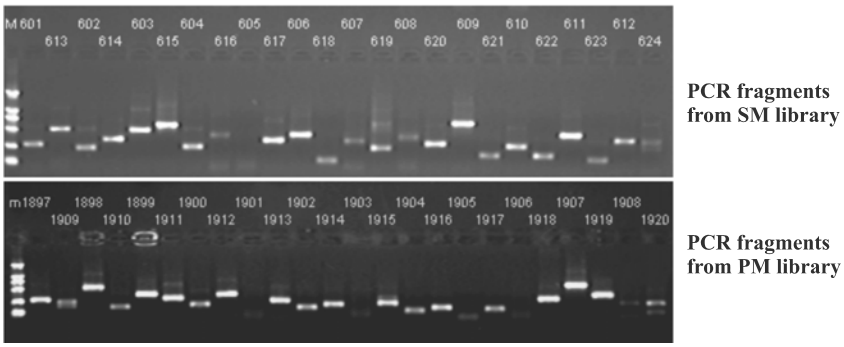

Fig. 2. Inserted fragments amplified by PCR using nested primers and run in a 1% agarose gel. Shown are inserts from clones 601-624 and 1897-1920 which belong to SM and PM libraries, respectively.

lished online at http://cmgm.stanford.edu/pbrown/protocols/.

Preparation of probes and microarray hybridization

Two kinds of fluorescence-labeled probes were prepared to hybridize with the microarray originated from the SSH cDNA library.

(1) Preparation of SSH probe. To evaluate the subtractive efficiency, the forward- and reverse-subtracted samples were labeled with fluorescent dye Cy5/Cy3-dCTP (SSH probe) by PCR amplification according to the protocols of Subtraction kit (Kuang *et al*., 1998).

(2) Preparation of reverse transcription-based probe (RT-probe). To determine the difference in gene expression between the 2 d and 3 d RNA samples, 5 μg total RNA of each was used to produce a Cy5/Cy3-labeled probe using the single primer amplification method described in Smith and Dixon (Dixon *et al*., 1998; Smith *et al*., 2003). The SSH and RT-probe were both labeled by the dye-reverse technique and using duplicate independent samples. Both SSH and RT probes were purified with a PCR purification kit (QIAGEN, USA), resuspended in elution buffer, and OD was checked. Labeled PM and SM probes were quantitatively adjusted based on the efficiency of Cy-dye incorporation and mixed into 12 μl of hybridization solution (50% formamide, 1× hybridization buffer). DNA in the hybridization solution was denatured at 95°C for 3 min prior to loading on a microarray. The array was hybridized at 42°C overnight and washed with two consecutive washing solutions (0.1% SDS, 0.6× SSC at 50°C for 5 min, and 0.03% SSC for 5 min at room temperature). Then, the slides were centrifuged to dry in 50-ml conical tubes at 1,000 rpm for 1 min.

Imaging and data analysis

Hybridized arrays were scanned using a laser confocal scanner (ScanArray Express, PE Company). Images obtained were analyzed with GenePix Pro 3.0 (Axon Instruments). A space- and intensity-dependent normalization based on a LOWESS program (Yang *et al*., 2002) was employed here. Concerning RT-probe hybridization, two independent RNA samples were analyzed, and each hybridization was performed by a dye swap such that each spot produced four data points. We determined the differently expressed genes by t-test ($P<0.05$) with an expression-level alteration criterion of >twofold.

DNA sequencing and sequence analysis

Recombinant colonies were selected and sequenced at United Gene Holdings, Ltd (Shanghai, China). Sequences were trimmed of vector sequences using the GenBank VecScreen facility. Trimmed sequence data were searched against the *P. chrysosporium* genome database and GenBank database (Altschul *et al*., 1997). When matches were found, potential cellular roles for the matching sequence were determined. The interesting sequences and their KOG groups were analyzed on the online service at http://genome.jgi-psf.org/whiterot. EST sequences described in this paper can be found in the NCBI database under accession numbers CK280173-CK 280197, CK327248-CK327308, and CV222660-CV222872.

Real-time RT-PCR

For each cDNA sequence of interest, RT-PCR was perfor-

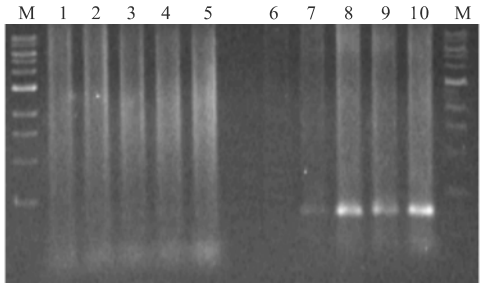

Fig. 3. Reduction of *gpd* gene abundance by PCR-selected subtraction. PCR was performed on subtracted (lanes 1~5) or unsubtracted (lanes 6~10) secondary PCR product with the *gpd* primers. Lanes: 1 and 6, 18 cycles; 2 and 7, 23 cycles; 3 and 8, 28 cycles; 4 and 9, 32 cycles; 5 and 10, 35 cycles. For the unsubtracted cDNA, the *gpd* gene product was observed at about 23 cycles, while the amplified product could not be seen at 35 cycles in the subtracted cDNAs. This indicates that the *gpd* gene was subtracted very efficiently.

med. Briefly, 2 μg of total RNA was used in the first- and second-strand synthesis reactions employing a cDNA synthesis kit (TaKaRa, China). PCR amplifications were performed in the following reaction mixture: 1 μl 1× SYBR Green PCR Master Mix, 3 μl 10× Taq reaction buffer, 0.9 μl 10 mM dNTP, 5 U Taq, 17.8 μl H_2O, and 2 μl 10 mM of each forward and reverse primer. Reactions in 96-well format were performed in the Bio-Rad iCycler (BIO-RAD). The cycling parameter was 2 min at 94°C, followed by 25 cycles of 20 sec at 94°C, 30 sec at 55°C, and 40 sec at 60°C. As an internal control, *gpd* gene fragment was amplified in parallel with the other genes of interest. The primer sequences are shown in supplementary Table 1.

Results

The transition from primary growth to secondary metabolism

The relationship between nutrient nitrogen starvation and the appearance of LIP activity was detected when *P. chrysosporium* was grown under nitrogen-limited conditions and oxidative stress. The results demonstrated that extracellular nitrogen was depleted between the 2nd and 3rd day, and the LIP activity was detected between the 3rd and 4th day. The maximum LIP activity was detected at day 6 (Fig. 1). The results indicated that that day 2 and day 3 are pivotal times for *P. chrysosporium* to enter secondary metabolism. During this period, primary growth ceased and secondary metabo-

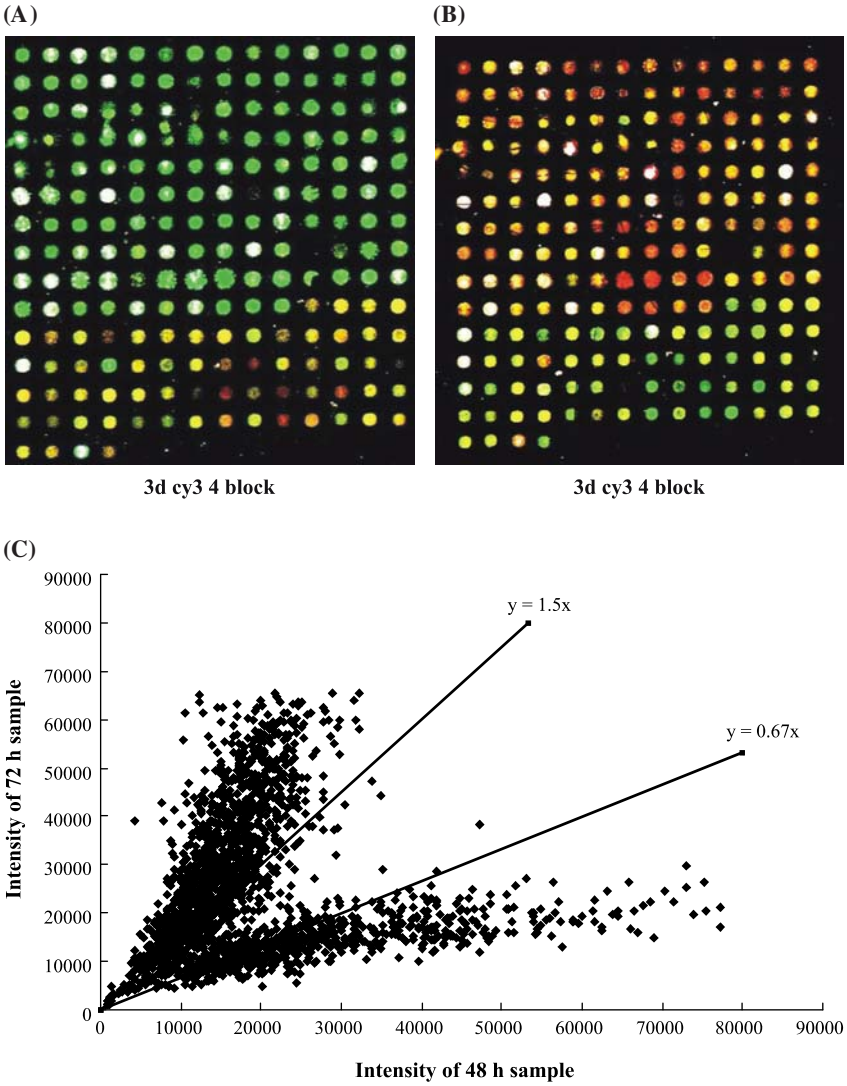

Fig. 4. Evaluation of the subtractive efficiency of cDNA clones on the microarray by hybridizing the microarray using cy5 and cy3 labeled SSH probes. One subarray hybridization image is shown in which the SSH probe from SM sample was labeled with cy3 (green) and PM was labeled with cy5 (red). The top 10 rows in the subarray are clones from SM and the bottom 5 rows from PM (A). Dye-reverse image corresponding to (B); scatter plot analysis of hybridization intensity of SM and PM SSH probe indicated 78% clones on the microarray possess different abundance in the SM and PM SSH probe (C).

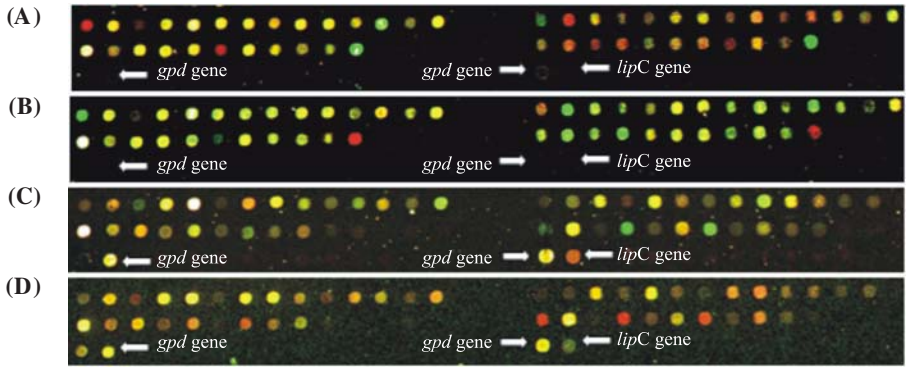

Fig. 5. The fluorescent signal of *gpd* and *lip*C gene fragments. (A) and (B), screened by SSH-probe; (C) and (D), screened by RT-probe (A) and (C), PM cy5, SM cy3; (B) and (D), SM cy5, PM d cy3. The *gpd* gene fragment can't be detected by SSH probe (A and B) but can be detected by the RT-probe (C and D). It indicates that the *gpd* genes were subtracted during the SSH process. RT-probe hybridization showed a SM/PM ratio of the *gpd* gene is 1.32. The corresponding ratio for the *lip*C gene is 2.16. Because the *gpd* gene is a housekeeping gene and *lip*C is a SM specific gene (Lamar, 1995), this indicates that the hybridization result of the RT-probe was satisfactory.

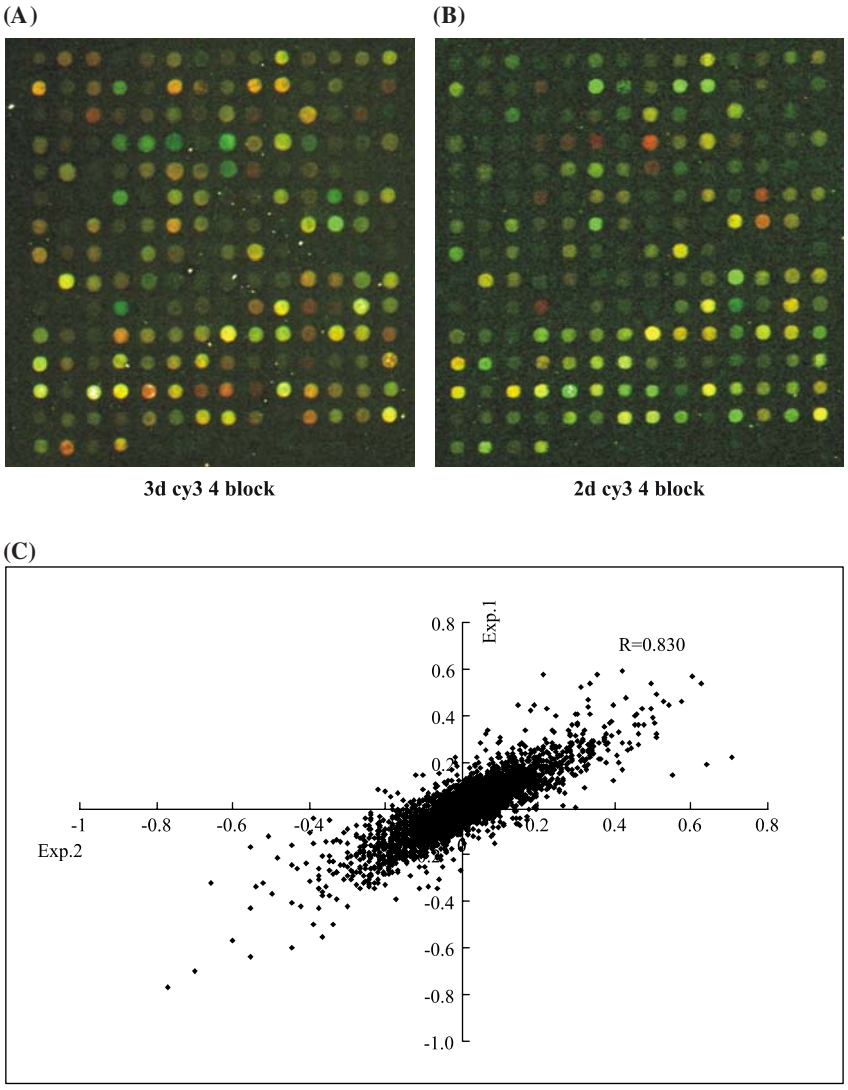

Fig. 6. Identification of differentially expressed gene by RT-probe. (A) The 4th subarray of the images hybridized with 3d cy3 and 2d cy5 probe. (B) The 4th subarray of the images hybridized with 3d cy5 and 2d cy3 probe. (C) Scatter plot analysis showing the reproducibility of two independent experiments when using the RT-probe. Log-transformed fold change of each gene was compared.

lism began. It also implies that RNA samples extracted from 2d and 3d mycelia can be used to detect the difference in gene expression pattern between primary and secondary metabolism.

Construction of subtractive cDNA libraries and evaluation of subtraction efficiency

Two cDNA libraries were constructed for PM- and SM-subtracted samples, respectively. Totally, 2596 clones were obtained. A serial number with prefix SSH was assigned to each of the clones, among which SSH1 to SSH1728 represented clones from the SM library, while SSH1729 to SSH 2596 were PM-specific clones. A total of 1555 cDNA fragments (with an average size of 292 bp) from the SM library were amplified by nested PCR, and 767 fragments with an average size of 282 bp were obtained from the PM library. Parts of the inserts are shown in Fig. 2. All successfully amplified fragments (2322=1555+767) accounted for 89.4% of all clones and were spotted on glass slides by using robotic printing.

The subtraction efficiency of the SSH method was first evaluated by comparing *gpd* gene abundance on the subtracted (lanes 1~5) with the unsubtracted (lanes 6~10) products. The result showed that the commonly expressed *gpd* gene in PM and SM samples had been efficiently excluded through SSH process (Fig. 3).

The subtraction efficiency was also evaluated based on the hybridization results of the SSH probe. Cy5 /cy3-labeled SM and PM probes were hybridized with the microarray. Moreover, one subarray of the hybridization images is shown in Fig. 4. Up to 95.8% of the total spots (2,226 of 2,322 spots) were detected by SSH probes. Scatter plot analysis of self-to-self hybridization using SSH probe indicates that 78% of clones on the microarray (100%-488/2226) differ in abundance in the PM and SM SSH probes (Fig. 4C). Meanwhile, the fluorescent signal of internal *gpd* fragments could not be detected (Fig. 5A and B), which indicates that the *gpd* gene was subtracted efficiently in the PM- and SM-sub-

Table 1. List of differently expression ESTs and their functional annotation ($P \leq 0.05$)

EST ID	Accession no.	Gene model or location on genome	Ratio		Functional (protein) annotation
SH1554	CK280191	fgenesh1_kg.C_scaffold_3000004	69.86	Yes	KOG Desc: Voltage-gated shaker-like K^+ channel, subunit beta/KCNAB
SSH445	CK327253	fgenesh1_pg.C_scaffold_8000049	25.61	Yes	KOG Desc: Sexual differentiation process protein ISP4
SSH624	CV222699	fgenesh1_pg.C_scaffold_5000230	24.69	Yes	KOG Desc: Multidrug resistance-associated protein, ABC superfamily
SSH1572	CK280192	fgenesh1_pg.C_scaffold_13000294	22.02	Yes	Blast X: AB076805 peptidyl-Lys metalloendopeptidase, [Grifola frondosa]
SSH1180	CK280184	fgenesh1_pg.C_scaffold_1000600	14.92	Yes	Blast X: AP002866 hypothetical protein, [Arabidopsis thaliana chromosome4]
SSH963	CK280178	fgenesh1_pg.C_scaffold_11000355	11.04	Yes	KOG Desc: Chitinase
SSH875	CK280177	scaffold_2: 2256344- 2256063	10.09	Yes	Blast N: mCG113607, [Mus musculus], 2.5, EDL33610.1
SSH939	CK280179	scaffold_10: 1357322-1357151	9.04	Yes	No significant similarity found
SSH621	CV222698	gwh2.9.64.1	7.495	Yes	KOG Desc: Sexual differentiation process protein ISP4
SSH227	CK280174	No hits found	7.46	Yes	Blast X: Fusion protein, [Newcastle disease virus], AL57890.1
SSH832	CK327258	fgenesh1_pm.C_scaffold_12000019	5.06		KOG Desc: H^+/oligopeptide symporter
SSH1167	CK327263	e_gwh2.12.114.1	4.35		KOG Desc: Catalase
SSH1193	CK280186	e_gww2.16.109.1	4.22		Blast X: heat shock protein, [Trametes versicolor], BAA33053.1
SSH331	CK327251	fgenesh1_pg.C_scaffold_4000627	3.32		KOG Desc: Cytochrome P450 CYP4/CYP19/CYP26 subfamilies
SSH371	CV222680	scaffold_19: 114021-114252	3.03		Blast X: Catalase (EC 1.11.1.6), [CAT-2 - maize], A55092
SSH98	CK327248	scaffold_3: 768615- 768494	2.74		No significant similarity found
SSH611	CK327256	fgenesh1_pg.C_scaffold_21000077	2.64		KOG Desc: Glucosamine-6-phosphate isomerase
SSH366	CK327252	scaffold_10: 718070-717903	2.25		No significant similarity found
SSH362	CK280175	fgenesh1_pg.C_scaffold_19000036	2.09		KOG Desc: Alkyl hydroperoxide reductase
SSH1355	CK327265	scaffold_5: 635787- 636121	2.04		Blast X: GJ19527 gene product, [Drosophila virilis], XP_002055745.1
SSH328	CK327250	scaffold_10: 717592- 717901	2.01		No significant similarity found
SSH864	CK327259	scaffold_5: 636121 -635866	2.01		Blast X: GJ19527, [Drosophila virilis], XP_002055745.1
SSH124	CK327249	scaffold_6: 1891004-1890719	2		Blast X: GA19298, [Drosophila pseudoobscura pseudoobscura], XP_001360955.2

The function, gene model and KOG Description of each EST were annotated by JGI online service. If no result was got, the remained ESTs were searched against the GenBank then the best hit were provide; Ratio: SM/PM ratio based on the results RT-probe; In column $P \leq 0.05$, "yes" means that the ESTs are significantly up-regulation genes from SM library

tracted products. However, it still could be detected by the RT-Probe because the *gpd* gene was not subtracted in this probe (Fig. 5C and D).

Identification of differently expressed genes by RT-probe

The spotted microarrays were also hybridized with the RT probes prepared from the PM- and SM-specific mRNA (Fig. 6A and B). The results of scatter plot analysis indicated that the correlation coefficiency between the two selected hybridizations was 0.83 (Fig. 6C). Detection of the SM/PM ratios of *gpd* and *lipc* gene indicates that the hybridization result of the RT-probe was satisfactory.

We determined the differently expressed gene with an expression-level alteration criterion of >twofold. There were 90 SM upregulation ESTs (SM/PM ratios of spots from 2 to 69.86), among which, based on a t-test ($P \leq 0.05$), 30 spots were significant up-regulation ESTs. Ratios from 0.06 to 0.5 were regarded as PM upregulation ESTs. This includes 141 spots, among which 30 spots were significant upregulation ESTs ($P \leq 0.05$).

DNA Sequence analysis

At first, sequence analysis was conducted for differently expressed ESTs (Table 1 and 2). Seventy-nine SM upregulation ESTs and 80 PM up-regulation ESTs were sequenced. After multiple alignments, 22 genes have been identified from the SM samples; among these, 10 genes ($P \leq 0.05$) were interpreted as differently expressed genes (Table 1, Supplementary Table 2). Fifty-three genes were found among PM upregulation ESTs and 22 genes were interpreted as significant upregulation genes (Table 2, Supplementary Table 3). All the sequences were compared with the GenBank database and the *P. chrysosporium* genome database using the Blastn and Blastx algorithms. Their functional annotations are listed in Table 1 and Table 2.

Among the significant upregulation ESTs from the SM library, five ESTs could be functionally assigned. There were two sexual differentiation process proteins ISP4 (SSH445, SSH621), a multidrug resistance-associated protein/mitooxantrone resistance protein that belongs to the ABC superfamily, a chitinase (SSH963), and a voltage-gated shaker-like K^+ channel, subunit beta/KCNAB that actually encodes an aryl-alcohol dehydrogenase in *P. chrysosporium* (SSH1554) (Table 1, Supplementary Table 2). Most of the ESTs from the PM library could not be functionally assigned. ESTs that can be functionally assigned are as follows: halotolerance protein HAL3 (SSH1740); inositol polyphosphate multikinase (SSH1837), which is a component of the ARGR transcription regulatory complex; glucosamine-phosphate N-acetyltransferase (SSH1891); a voltage-gated shaker-like K^+ channel subunit beta/KCNAB (SSH2216); Ca^{2+}/H^+ antiporter VCX1 and related proteins (SSH2269); cyclin-dependent kinase inhibitor (SSH2345) (Table 2 and Supplementary Table

Table 2. List of significantly up-regulated ESTs from PM library and their functional annotation ($P \leq 0.05$)

Clone ID	Accession no.	Ratio	Gene model	Functional (protein) annotation
SSH1740*	CK280194	0.225	fgenesh1_pg.C_scaffold_4000666	KOG Desc: Halotolerance protein HAL3
SSH1755*	CK280195	0.154	scaffold_7:143190-143512	Blast X: Refse protein kinase BRPK
SSH1773*	CK280194	0.24	Scaffold_110:77965-78195	No significant similarity found
SSH1812*	CK325777	0.235	genscan.22.66.1	Blast X: hypothetical protein TTHERM_00415830
SSH1836*	CK280198	0.235	fgenesh1_pg.C_scaffold_11000492	Blast X: ape:APE0915 315aa long hypothetical protein
SSH1837	CK280199	0.18	gww2.1.775.1	KOG Desc: Inositol polyphosphate multikinase
SSH1891	CK327276	0.39	fgenesh1_pg.C_scaffold_12000235	KOG Desc: Glucosamine-phosphate N-acetyltransferase
SSH1894	CK280200	0.17	Scaffold_22:51835-52400	Blast N: cDNA sequence BC051142, isoform CRA_b, [Mus musculus]
SSH1977	CK280202	0.09	fgenesh1_pg.C_scaffold_10000146	No significant similarity found
SSH2030	CK280203	0.135	No hits found	Blast N: orf231, [Spizellomyces punctatus]
SSH2081	CK280205	0.145	scaffold_2:1103243-1103403	No significant similarity found
SSH2111	CK280206	0.23	scaffold_16:372322-372677	Blast N: putative ubiquinol-cytochrome C reductase iron-sulfur subunit, [Corynebacterium efficiens YS-314]
SSH2189	CV222821	0.13	fgenesh1_pg.C_scaffold_18000022	KOG Desc: Actin regulatory protein (Wiskott-Aldrich syndrome protein)
SSH2202	CK280207	0.10	Scaffold_115:30392-30552	Blast X: RIKEN cDNA 2900016D05 gene
SSH2216	CK280208	0.16	e_gwh2.7.161.1	KOG Desc: Voltage-gated shaker-like K+ channel, subunit beta/KCNAB
SSH2269	CK280209	0.25	fgenesh1_pg.C_scaffold_10000139	KOG Desc: Ca2+/H+ antiporter VCX1 and related proteins
SSH2345	CK280210	0.245	fgenesh1_pg.C_scaffold_2000384	KOG Desc: Cyclin-dependent kinase inhibitor
SSH2395	CK280212		e_gww2.28.21.1	Blast X : NCU00855.1predicted protein, [Neurospora crassa]
SSH2354	CK280211	0.23	fgenesh1_pg.C_scaffold_22000072	Blast X: NCU00855.1 predicted protein, [Neurospora crassa]
SSH2401	CK280213	0.145	scaffold_2:476974-477194	Blast N: nonagouti, [Mus musculus]
SSH2482	CK280215		Scaffold_22:85971-86140	No significant similarity found
SSH2494*	CK280217	0.175	scaffold_7:1146952-1147052	No significant similarity found

The function, gene model and KOG Description of each ESTs were annotated by JGI online service. If no result was got, ESTs were searched against the GenBank then the best hit was provided; Ratio: SM/PM ratio based on the results RT-probe.

3). Here, we would not discuss the remained ESTs' functions, although we list all of the blast results against the nr and *P. chrysosporium* data set.

To gain more information, up to 754 clones randomly selected from the SM (478) and PM (276) libraries were sequenced besides the upregulation ESTs. Multiple alignment results showed 78 and 129 ESTs among the sequenced ESTs from the SM and PM libraries, respectively (Supplementary Table 2 and 3). Among the sequenced ESTs, 15 ESTs of the PM library occurred repeatedly at frequencies ranging from 2 to 11. Thirty-six clones of the SM library occurred repeatedly at frequencies ranging from 2 to 56. The most abundant clones, which occurred 56 times, were from the SM library (Table 3).

Most of the ESTs could be matched with the *P. chrysosporium* database (*P. chrysosporium* v2.0) by using blast algorithms (E value: 1e-5). Only 10 ESTs in the PM library and 1 in the SM library could not be located in the *P. chrysosporium* genome sequence, although many sequenced ESTs could not be functionally assigned. Ninety-three ESTs could be classified according to Eukaryotic Orthologous Groups (KOG), Cluster of Orthologous Groups (COG) for identifying ortholog and paralog proteins on the online service at http://genome.jgi-psf.org/whiterot. The proportion of predicted genes with an assigned Orthologous Groups was presented in Supplementary Table 4. Thirty-four genes could be functionally classified into the "Cellular Processes and Signaling" group (36.56%); 20 in the "Information Storage and Processing" group (21.51%); 27 in the "Metabolism" group (28.72%); and 12 in the "Poorly Characterized" group (12.90%). Among the 93 genes that were assigned Orthologous Groups, 53 genes (56.99%) were from the PM library and 40 (43.01%) came from the SM library. The genes that were expressed in the SM library are quite different from those expressed in the PM library. For example, genes from the PM library were distributed mainly in "Cellular Processes and Signaling". On the other hand, genes from the SM library were distributed mainly in the "Information Storage" and "Processing and Poorly Characterized" groups.

Evaluation of PM and SM differently expressed genes by qPCR

The expression level of 8 PM and 8 SM differently expressed genes were confirmed by qPCR (Fig. 7). The results showed that the expression levels of all selected genes in the SM stage were higher than those in the PM stage and the SSH1554 has the highest expression level among the SM samples. This suggests that these genes might be the specific expressed genes when *P. chrysosporium* started secondary metabolism. Genes from the PM samples were also confirmed by qPCR. These results showed that SSH 1755, SSH1891, SSH1977, SSH2030, SSH2189, and SSH2202 are PM upregulation genes (Fig. 7B).

Discussion

The growth of *P. chrysosporium* mycelium under pure O_2 and nitrogen-starvation conditions ended on the second day. When *P. chrysosporium* was grown under conditions optimized for lignin metabolism, a reproducible sequence

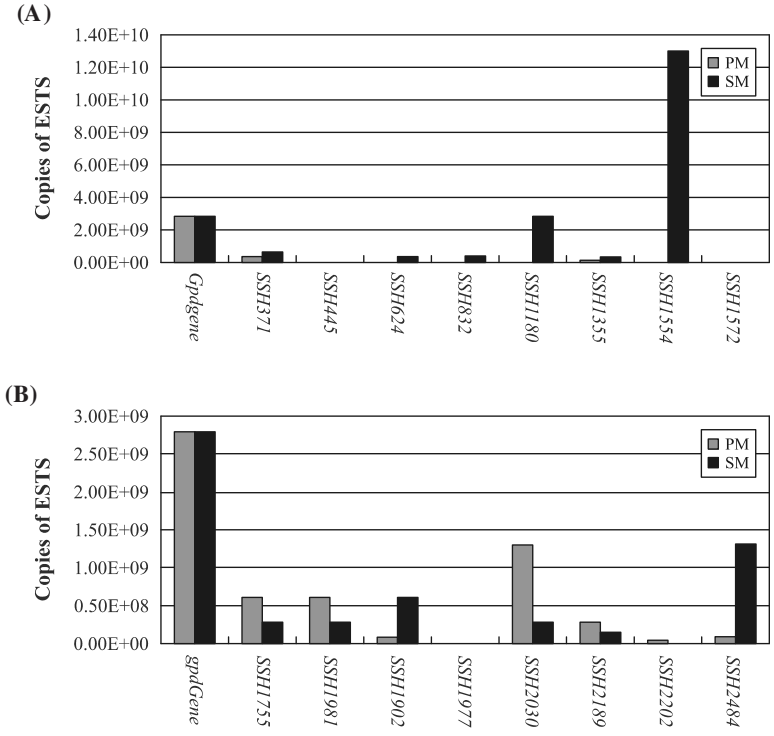

Fig. 7. qPCR analysis confirmed the expression level of selected genes when *P. chrysosporium* from primary growth to initiation secondary metabolism. (A) SM up-regulation ESTs. (B) PM up-regulation ESTs.

of events followed 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); and 72 to 96 h, appearance of lignin peroxidase activity (Keyser *et al*., 1978; Boominathan *et al*., 1993). The previous investigation and our results showed that nitrogen is exhausted between day 2 and day 3 when *P. chrysosporium* is incubated in nitrogen-limited medium (2.4 mM nitrogen source) and the onset of secondary metabolism is triggered by deficiency of nitrogen (Keyser *et al*., 1978; Singh and Chen, 2008). The genes expressed between day 2 and day 3 are believed to be involved in the molecular mechanism of the switch from primary to secondary metabolism of *P. chrysosporium*. Genes expressed after three days of incubation, generally, are considered as the differently expressed genes of secondary metabolism. For example, SSH362 encodes a homologous protein of peroxiredoxin in *P. chrysosporium* that is expressed in nitrogen-limited cultures (Jiang *et al*., 2005). Furthermore, we also found other lignin degradation-related genes that were expressed commonly in ligninolytic culture. Aryl-alcohol dehydrogenase (SSH1554) and copper radical oxidase variant A (SSH1539, SSH1405) were also found in the SM-specific library (Table 1 and Supplementary Table 2). These results are similar to the results of previous studies; most LIP activity appeared during the period from 72 to 96 h (Boominathan *et al*., 1993).

It was interesting that many reactive oxygen species-(ROS) related genes were found in this study. The peroxiredoxins (Prxs, SSH362) belonged to a newly defined family of antioxidant proteins whose catalytic activity and amino acid sequences are markedly different from those of conventional antioxidant enzymes for protection against ROS such as H_2O_2 (Sarah *et al*., 2001; Jiang *et al*., 2005; Kersten and Cullen, 2007). Both SSH371 and SSH1167 encode catalase, although they have different sequences (Wysong *et al*., 1998; Kwon and Anderson, 2001). Microarray data showed that the transcripts of SSH371 and SSH 1167 increased more than threefold and fourfold in the SM stage compared to the PM stage, although both of them are not significantly differently expressed genes (Table 1 and Supplementary Table 2). In fungi, catalases can convert H_2O_2 into oxygen and water. Our results agree with other work that suggested that the catalase gene is upregulated during ligninolytic metabolism (Daniel *et al*., 1995). SSH1193 has a homologous heat shock protein in the white-rot fungus *Coriolus versicolor* (Nover, 1991; Iimura and Tat, 1997) (Table 1 and Supplementary Table 2). Previous studies showed that treatment with H_2O_2 and other oxidative stress can induce the heat shock response in both prokaryotes and eukaryotes. The protein sequence of SSH624 was similar to ABC transporters (Table 1, 3) (Assmann *et al*., 2003). ABC transportation is a common biological defensive mechanism and is a "driving" power for conservation of homeostasis of cells. It likely plays a role in detection or uptake of extracellar metabolites by ligninolytic *P. chrysosporium.* In this study, the transcript of ABC increased 25-fold in the SM stage which is related to ligninolysis. In order to trigger LIP expression, *P. chrysosporium* was starved (via nitrogen or carbon depletion) and exposed to a pure O_2 atmosphere. This can be done by subjecting the fungus to a ROS-rich environment (Bar-Lev and Kirk, 1981). ROS results in damage toward cellular constituents including DNA, lipids, plasma membranes, and proteins. Thus, these are highly toxic and stressful to living cells (Moradas-Ferreira *et al*., 1996; Tose and Fracisca, 2000). Therefore, we speculate that oxidative stress and the occur-

Table 3. Clones that are stage specific and occurred more than five times

Clone ID	Accession no.	Best homology against GenBank database	SM[a]	PM[b]
SSH1	CK280173	Predicted protein, [Laccaria bicolor S238N-H82], XP_001878268.1	56	−
SSH992	CV222722	Hypothetical protein FG01737.1, [Gibberella zeae PH-1], XP_381913.1	56	−
SSH158	CV222672	No significant similarity found	30	−
SSH624	CV222699	multidrug resistance-associated ABC transporter, [Laccaria bicolor S238N-H82], XP_001877236.1	39	−
SSH1098	CV222726	Unnamed protein product, [Tetraodon nigroviridis], >embCAF99150.1	23	−
SSH108	CV222666	Hypothetical protein CC1G_01887, [Coprinopsis cinerea okayama7#130], XP_001830251.1	23	−
SSH362	CK280175	Peroxiredoxins [Phanerochaete chrysosporium], AAV53576.1	22	−
SSH371	CV222680	catalase (EC 1.11.1.6), [CAT-2 - maize], A55092	22	−
SSH243	CV222675	Predicted protein, [Laccaria bicolor S238N-H82], XP_001880129.1	20	−
SSH526	CV222692	Actin-related protein ARPC3, [Laccaria bicolor S238N-H82], XP_001875441.1	12	−
SSH587	CK280187	Predicted protein, [Laccaria bicolor S238N-H82], XP_001884087.1	10	−
SSH459	CV222687	Actin-related protein ARPC3, [Laccaria bicolor S238N-H82], XP_001875441.1	8	−
SSH1358	CV222738	Predicted protein, [Coprinopsis cinerea okayama7#130], XP_001837680.1	7	−
SSH363	CV222678	SocE, [Myxococcus xanthus], AF263243_1	10	
SSH2272	CV222835	SocE, [Myxococcus xanthus], gbAAF91388.1	−	11
SSH1858	CV222778	Hypothetical protein MGL_3949, [Malassezia globosa CBS 7966], XP_001728955.1	−	10
SH1729	CK327267	Predicted protein, [Monosiga brevicollis MX1], XP_001748237.1	−	8
SSH1755	CK280195	PREDICTED: Hypothetical protein, [Homo sapiens], XP_937443.2	−	7
SSH1848	CV222775	unknown protein, [Oryza sativa Japonica Group], dbjBAD07868.1	−	6

[a] Redundancy number of the ESTs in SM library
[b] Redundancy number of the ESTs in PM library

rence of ROS are common in *P. chrysosporium* during secondary metabolism. *P. chrysosporium* may employ one or several systems to handle these toxic substances that are produced as byproducts of the extracellular lignin-degrading system when *P. chrysosporium* is exposed to pure oxygen. It is conceivable that H_2O_2 or other ROS produced during lignin degradation are involved in the regulation of *P. chrysosporium's* initiation of secondary metabolism (Assmann *et al*., 2003). Based on this study, several genes may be involved in this process. We can deduce that the co-appearance of ROS related genes at the onset of secondary metabolism suggests coregulation between the ROS protection system and the trigger of secondary metabolism in *P. chrysosporium* (Miura *et al*., 2004).

Since the main goal of our experiment is to indentify the significantly differently expressed genes at the onset of secondary metabolism, more SM library ESTs have been sequenced (478), although only 276 clones from the PM library have been sequenced. An interesting phenomenon was observed. Only 78 genes were included in the 474 sequenced SM clones, but in 283 PM-origin clones, there are 126 genes. This suggests that many ESTs from the SM library are redundant (Table 1). For instance, SSH1 and SSH 992 repeated 56 times in the sequenced clones, and they have homology to a putative protein in *Laccaria bicolor* and *Gibberella zeae* (Table 1). The remaining high redundancy clones exhibited homologies to ABC Transporter (SSH624), peroxiredoxins (SSH362), catalase (SSH371), actin-related protein ARPC3 (SSH526, SSh459), and so on. In the PM library, there are fewer redundant genes. The most redundant ESTs are SSH 2272 (SocE), SSH1858 (hypothetical protein MGL-3949), and SSH1729 (predicted protein) (Table 3).

Fifteen genes have been assigned to the Eukaryotic Orthologous Groups (KOG) "Posttranslational Modification, Protein Turnover, and Chaperones" (Supplementary Table 4). It is interesting that 11 out of the 15 genes were from the PM library. Among them, six genes are functionally involved in the ubiquitin system (Supplementary Table 4: SSH1966, SSH2049, SSH2330, SSH2291, SSH2412, and SSH2477). Ubiquitin is a 76-residue protein and mediates protein degradation (Hershko *et al*., 2000). Protein degradation through the ubiquitin pathway is involved in a multitude of process, including cell growth and division, signal transduction, DNA repair, and the transport of substances across membranes. The occurrence of ubiquitin system-related genes in the PM library is consistent with early physiological studies: the growth of *P. chrysosporium* mycelium under nitrogen-limited conditions (2.4 mM nitrogen source) ceases by day 2. However, the role of the ubiquitin system in the PM library still needs to be studied. It can be deduced that the ubiquitin system may be involved in the degradation of protein when nitrogen is exhausted and this may be an extra nitrogen source for *P. chrysosporium*. We also found that most (76.47%) of the genes in the "Cellular Processes and Signaling" category are from the PM library. For example, in the "Intracellular Trafficking, Secretion, and Vesicular Transport" category, all of the genes are from the PM library, and in "Signal Transduction Mechanisms," only two genes are from the SM library. Conversely, in "Information Storage and Processing," most (61.90%) of the genes are from the SM library. A total of 93 genes can be assigned to the KOG groups, among them 53 genes (56.38%) from the PM library and 41 (43.62%) from the SM library. We found that both of the PM-origin genes in "Cellular Processes and Signaling" and SM-origin genes in "Information Storage and Processing" exceeded their total ratio. This indicates that the gene expression pattern in the SM and PM stages are different.

The SSH method has allowed the isolation of differentially expressed cDNA clones by comparison with two RNA populations. However, individual clones should be confirmed by Northern Blot analysis. If the background is high, picking random clones from the subtractive library for Northern Blot analysis is time consuming, tedious, and inefficient (Diatchenko *et al*., 1996; Kuang *et al*., 1998). Combining SSH and cDNA microarray technology can provide an efficient resolution to this problem. Although researchers often considered that the labeled probe from the first PCR products of SSH process was more efficient than the probe prepared from reversed mRNA (Yang *et al*., 1999), but we thought it is better to screen the microarray by using both the SSH and RT-probe. After the differentially expressed genes are selected, qPCR can be used as the most accurate method to analyze mRNA expression of low numbers of genes or to confirm key relationships identified by microarray analysis. In this study, the results of qPCR validate the results of the microarray. Some suggestions can be proposed for future work if SSH and microarray technologies are combined: 1) It is better to screen the microarray by using the RT-probe and the SSH-probe can be used as a self-hybridization confirmation. In addition, most of the time the RT-probe is a mere nicety; 2) It is better to use qPCR as an accurate method to evaluate the results of the microarray.

In conclusion, the combination of SSH and cDNA microarray technology can provide an efficient way to compare the different gene expression patterns of *P. chrysosporium* in different metabolic stages. Ten SM and twenty-two PM significantly differently expressed genes were indentified. The results also indicate that the gene expression patterns are different during the transition time. Many reactive oxygen species (ROS)-related genes were found in the SM library and the coappearance of ROS-related genes during the transition time suggests that they may be involved in the regulation of *P. chrysosporium's* initiation of secondary metabolism. KOG functional annotation results showed that genes from the SM and PM libraries have different distributions. Most ESTs belonging to the KOG subgroup "Cellular Processes and Signaling" are from the PM library and most of the genes in the "Information Storage and Processing" group are from the SM library. Future work in our lab will aim at studying the biological function of the differently expressed genes during the transition time.

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References

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389-3402.

Assmann, E.M., L.M. Ottoboni, A. Ferraz, J. Rodriguez, and M.P. De Mello. 2003. Iron-responsive genes of *Phanerochaete chrysosporium* isolated by differential display reverse transcription polymerase chain reaction. *Environ. Microbiol.* 5, 777-786.

Bar-Lev, S.S. and T.K. Kirk. 1981. Effects of molecular oxygen on lignin degradation by *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* 99, 373-378.

Boominathan, K., T.M. D'souza, P.S. Naidu, C. Dosoretz, and C.A. Reddy. 1993. Temporal expression of the major lignin peroxidase genes of *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 59, 3496-3950.

Broda, P.P., R. Brooks, and P.F. Sims. 1996. lignocellulose degradation by *Phanerochaete chrysosporium*: gene families and gene expression for a complex process. *Mol. Microbiol.* 19, 923-932.

Cullen, D. 1997. Recent advances on the molecular genetics of ligninolytic fungi. *J. Biotechnol*. 53, 273-289.

Daniel, G., P. Binarova, J. Volc, and E. Kubatova. 1995. Cytochemical and immunocytochemical studies on sites of H_2O_2 production and catalase activity in hyphae of *Phanerochaete chrysosporium* grown in liquid culture and on wood. *In* E. Srebotnik and K. Messner (ed.), Proceedings of the Sixth International Conference at Vienna, Austria.

Diatchenko, L., Y.F. Lau, A.P. Campbell, A. Chenchik, F. Moqadam, B. Huang, and S. Lukyanov. 1996. Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* 93, 6025-6030.

Dixon, A.K., P.J. Richardson, K. Lee, N.P. Carter, and T.C. Freeman. 1998. Expression profiling of single cells using 3 prime end amplification (TPEA) PCR. *Nucleic Acids Res*. 26, 4426-4431.

Gold, M. and M. Alic. 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Microbiol. Rev.* 57, 605-622.

Hershko, A., A. Ciechanover, and A. Varshavsky. 2000. The ubiquitin system. *Nature* 6, 1073-1081.

Iimura, Y. and S.K. Tat. 1997. Isolation of mRNAs induced by a hazardous chemical in white-rot fungus, *Coriolus versicolor*, by differential display. *FEBS Lett*. 412, 370-374.

Jiang, Q., Y.H. Yan, G.K. Hu, and Y.Z. Zhang. 2005. Molecular cloning and characterization of a peroxiredoxin from *Phanerochaete chrysosporium. Cell Mol. Biol. Lett*. 210, 659-668.

Kersten, P. and D. Cullen. 2007. Extracellular oxidative systems of the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Fungal Genet. Biol*. 44, 77-87.

Keyser, P., T.K. Kirk, and J.G. Zeikus. 1978. Ligninolytic enzyme system of *Phanaerochaete chrysosporium* synthesized in the absence of lignin in response to nitrogen starvation. *J. Bacteriol.* 135, 790-797.

Kuang, W.W., D.A. Thompson, R.V. Hoch, and R.J. Weigel. 1998. Differential screening and suppression subtractive hybridization identify genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line. *Nucleic Acids Res*. 26, 1116-1123.

Kwon, S.I. and A.J. Anderson. 2001. Catalase activities of *Phanerochaete chrysosporium* are not coordinately produced with ligninolytic metabolism: catalases from a white-rot fungus. *Curr. Microbiol.* 42, 8-11.

Lamar, R.T., B. Schoenike, A.V. Wymelenberg, P. Stewart, D.M. Dietrich, and D. Cullen. 1995. Quantitation of fungal mRNAs in complex substrates by reverse transcription PCR and its application to *Phanerochaete chrysosporium*-colonized soil. *Appl. Environ. Microbiol*. 61, 2122-2126.

Martinez, D., L.F. Larrondo, N. Putnam, M.D. Gelpke, K. Huang, J. Chapman, and K.G. Helfenbein. 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. *Nat. Biotechnol*. 22, 695-700.

Miura, D., H. Tanaka, and H. Wariishi. 2004. Metabolic differential display analysis of the white-rot basidiomycete *Phanerochaete chrysosporium* grown under air and 100% oxygen. *FEMS Microbiol. Lett.* 234, 111-116.

Moradas-Ferreira, P., V. Costa, P. Piper, and W. Mager. 1996. The molecular defences against reactive oxygen species in yeast. *Mol. Microbiol*. 19, 651-658.

Nover, L. 1991. Inducers of HSP synthesis: Heat shock and chemical stressors. *In* L. Nover (ed.), Heat Shock Response, p. 5-40. CRC Press, Boca Raton, FL, USA.

Sarah, J.R., J.F. Victoria, M. Panagiota, and A.M. Brian. 2001. Thioredoxin peroxidase is required for the transcriptional response to oxidative stress in budding yeast. *Mol. Biol. Cell*. 11, 2631-2642.

Singh, D. and S. Chen. 2008. The white-rot fungus *Phanerochaete chrysosporium:* conditions for the production of lignin-degrading enzymes. *Appl. Microbiol. Biotechnol*. 2008 Sep 20. [Epub ahead of print].

Smith, L., P. Underhill, C. Pritchard, Z. Tymowska-Lalanne, S. Abdul-Hussein. H. Hilton, and L. Winchester. 2003. Single primer amplification (SPA) of cDNA for microarray expression analysis. *Nucleic Acids Res.* 31, E9.

Tose, M.M. and M.S. Fracisca. 2000. Role of reactive oxygen species in apoptosis: implications for cancer therapy. *Int. J. Biochem. Cell Biol.* 32, 157-170.

Tien, M. and T.K. Kirk. 1987. Lignin peroxidase of *Phanerochaete chrysosporium*. *Methods Enzymol.* 161, 238-249.

Ward, P.G. 1963. A micro-Kjeldahl procedure for field use. *J. Med. Lab. Technol.* 20, 191-195.

Wysong, D.R., L. Christin, A.M. Sugar, P.W. Robbins, and R.D. Diamond. 1998. Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. *Infect. Immun.* 66, 1953-1961.

Yang, Y.H., S. Dudoitm, P. Luum, D.M. Linm, V. Pengm, J. Ngaim, and T.P. Speedm. 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res*. 30, 38-41.

Yang, G.P., D.T. Ross, W.W. Kuang, P.O. Brown, and R.J. Weigel. 1999. Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes. *Nucleic Acids Res*. 27, 1517-1523.

Zhang, Y.Z., G.J. Zylstra, R.H. Olsen, and C.A. Reddy. 1986. Identification of cDNA clones for ligninase from *Phanerochaete chrysosporium* using synthetic oligonucleotide probes. *Biochem. Biophys. Res. Comm.* 137, 649-656.