Lignin-induced Expression of *Aspergillus oryzae 5992* Genes using Suppression Subtractive Hybridization

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A previous study reported that a novel *Aspergillus oryzae* strain (*CGMCC5992*) can synthesize lignin hydrolytic enzymes for lignin degradation from straw. The present work involves the different gene expression of *A. oryzae 5992* grown in media using lignin and glucose as carbon sources by suppression subtractive hybridization. Surprisingly, peroxidase was found in up-regulation genes, which is the key enzyme for degrading lignin. This shows that *A. oryzae 5992* can secrete peroxidase in the presence of lignin. The functions of up-regulation genes also included gluconeogenesis, repairs, as well as signal and transporter proteins in the cell membrane. In addition, the down-regulation of genes was closely related to the aerobic metabolism of glucose, the fatty acid synthesis of the cell membrane, and the synthesis and utilization of ATP. Therefore, *A. oryzae* could regulate metabolism using lignin as carbon source, including lignin degradation promotion, glucose metabolism inhibition, and glucose regeneration.

Keywords: Suppression subtractive hybridization; Aspergillus oryzae; Lignin; Gene; Different expression; Metabolism

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INTRODUCTION

As a complex, non-crystalline, three-dimensional network of aromatic hetero-polymers (Bugg *et al*. 2011), lignin is the second main constituent of plant biomass after cellulose, accounting for about 20% of plant biomass (Itoh *et al*. 2003; Wang *et al*. 2011). In plant tissue, lignin binds the surrounding cellulose to form a natural rugged barrier, limiting the access of cellulase to cellulose (Shi *et al*. 2008). Therefore, the efficient, rapid, and economically and environmentally friendly destruction of the lignin network is a promising approach for the industrial development and utilization of lignocellulosic substrates, such as bioethanol production and the manufacture of cellulose-based chemicals and materials (Perez *et al*. 2002).

Different methods have been developed to destroy the lignin of lignocellulosic substrates based on physicochemical technologies, such as microwaving, ionizing radiation, steam explosion, acid or alkali dilution, oxidation, and varying combinations of these (Mosier *et al*. 2005). However, these typical physical and chemical techniques require not only high amounts of energy (steam or electricity), but also corrosion-resistant and high-pressure reactors, which lead to increased demand for special equipment and cost of pretreatment, as well as secondary pollution (Keller *et al*. 2003; Brodeur *et al*. 2011).

Microorganisms have been used to destroy the lignin of lignocellulosic substrates, providing the following advantages: decreased environmental pollution; transformation of

waste materials into resources; and realization of the circulatory re-use of resources. Until now, the reported fungi used in lignin degradation can be divided into three categories: white rot fungi, brown rot fungi, and soft rot fungi. White rot fungi have been generally considered to be the relatively stronger fungi for degrading lignin (ten Have and Teunissen 2001; Yelle *et al*. 2008; Hamed 2013). However, white rot fungi generally offer a poor production of lignin hydrolases since these enzymes are secondary metabolites (Tuor *et al*. 1995). Although it is cost-effective to pre-treat lignocellulosic substrates using white rot fungi, a large site area is required, which is not suitable for industrial production (Behera *et al*. 2014). Therefore, it is crucial to discover a strain with the rapid production capacity of lignin hydrolases, which determines whether lignin bio-degradation can be applied to industrial production. Exo-genes of lignin hydrolyases, including manganese peroxidase (MnP) and lignin peroxidase (LiP), have been put into the recipient strains, such as *Escherichia coli*, *Aspergillus niger*, and *Pichia methanolica* using gene cloning technology, and active lignin hydrolyases were successfully obtained (Gu *et al*. 2000). However, these engineering strains cannot tolerate high concentrations of hydrogen peroxide, another substrate of MnP and LiP, nor can they produce hydrogen peroxide. These two drawbacks directly limit the application of these engineering strains in lignin biodegradation.

Aspergillus oryzae CGMCC5992 has been isolated from the sludge of the Yudai River at the University of Jiangsu and identified according to morphological and molecular biology methods. This strain can produce high amounts of active MnP and LiP (Guo *et al*. 2014), degrade the lignin of lignocellulosic substrates in a relatively short period, and tolerate high concentrations of hydrogen peroxide (Zhang *et al*. 2014). Moreover, it is a promising strain for employment in lignin degradation in industrial applications.

Suppression subtractive hybridization (SSH), which combines suppression PCR with subtractive hybridization methods, was developed by Luda Diatchenko in 1966 (Ren *et al*. 2006). A powerful and efficient hybridization process, SSH can identify two different mRNA populations and isolate genes that are exclusively expressed in one population but not in the other (Diatchenko *et al*. 1996; Basyuni *et al*. 2010). This technique has been successfully applied to the identification of various biotic and abiotic stress-responsive genes in recent investigations (Diatchenko *et al*. 1999; Venkatachalam *et al*. 2009). In this study, we investigated the genes of *A. oryzae CGMCC5992* involved in lignin degradation, explored the molecular mechanism for high-efficiency lignin degradation, and assessed the relationship between gene transcription and the biodegradation of lignin using an SSH-based approach.

EXPERIMENTAL

Materials

Strains and media

A. oryzae CGMCC5992 was isolated in our laboratory and deposited in the China General Microbiological Culture Collection Center (CGMCC). The strain was cultured on potato dextrose agar (PDA) slants at 28 °C for four days, then stored at 4 °C and passaged every seven to nine weeks. The lignin, consisting of 8.7% cellulose, 27.6% hemicelluloses, 48.1% lignin, 7.2% protein or peptone, 4.8% ash, and 3.6% wax, was obtained from an online provider (Yang Hai Chemical Co., Ltd, Ji'nan, Shandong Province, China).

Media

The lignin-degradation medium consisted of 0.5 g/L lignin, 5 g/L peptone, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L ammonium tartrate, 1 g/L NaCl, 0.1 g/L $CoSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2$, 0.1 g/L$CuSO_4 \cdot 5H_2O$, 0.5 g/L $MnSO_4 \cdot H_2O$, 0.01 g/L H_3BO_3, 0.1 g/L $FeSO_4 \cdot 7H_2O$, 0.1 g/L $ZnSO_4 \cdot 7H_2O$, 0.001 g/L glycine, and 0.001 g/L vitamin B_1.

The control medium consisted of 0.5 g/L glucose, 5 g/L peptone, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L ammonium tartrate, 1 g/L NaCl, 0.1 g/L $CoSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2$, 0.1 g/L $CuSO_4 \cdot 5H_2O$, 0.5 g/L $MnSO_4 \cdot H_2O$, 0.01 g/L H_3BO_3, 0.1 g/L $FeSO_4 \cdot 7H_2O$, 0.1 g/L $ZnSO_4 \cdot 7H_2O$, 0.001 g/L glycine, and 0.001 g/L vitamin B_1.

Methods

Preparation of biomass

A. oryzae spores (1×10^6) were inoculated into a 250-mL flask containing 100 mL potato dextrose (PD) medium and incubated in a shaking incubator at 30 °C and 125 rpm for two days. A 10% (v/v) inoculum was aseptically added to the 10-mL lignin-degradation medium and control medium, respectively. The fermentation was carried out in a shaking incubator at 30 °C, 125 rpm for seven days. The biomass was obtained through filtration with four layers of gauze, washed three times with 0.01 M phosphate buffer solution (pH 7.4), and stored at −80 °C prior to RNA extraction. The biomass from the lignin-degradation medium and the control medium was labeled as A and B, respectively. In the forward-subtract process, A and B were used as the "test" and "drive," while they were used as the "drive" and "test" in the reverse-subtract process, respectively.

RNA isolation and cDNA synthesis

Total RNA was extracted using a Fungi RNA kit (R6618, Sigma, USA) following the manufacturer's instructions. The mRNA was extracted and purified using PolyATtract® mRNA isolation system II (25200, Promega, USA) according to the manufacturer's instructions. The cDNA synthesis was carried out using 1 μg mRNA with the PCR-select™ cDNA subtraction kit (Clotech, USA). Differential screening was performed using a PCR-select differential screening kit (BD Biosciences, USA) according to the manufacturer's instructions. Spots exhibiting at least a five-fold higher intensity with forward-subtracted probes compared with the reverse-subtracted probes were scored as positives.

DNA sequencing of different genes

The cDNA of positive spots was sequenced using an ABI 3730 automatic DNA sequencer (USA). A total of 300 positive clones were randomly sequenced at the GenScript Corporation (Nanjing, China). Sequence alignment and homology searches were performed using "TIGR" and "NCBI BLAST" databases available on the J. Craig Venter Institute website (blast.jcvi.org) (Chan *et al*. 2006; Johnson *et al*. 2008). E-value scores of less than 10^{-5} were considered significant, indicating homology between the obtained sequences and database sequences.

Validation of Differential Expression of Genes by Reverse Transcription PCR (RT-PCR)

The differentially expressed genes selected randomly from the ESTs were further validated by RT-PCR using gene-specific primer pairs designed by DNAMAN. The cDNAs of *A. oryzae* biomass growing in two different media were amplified. The quality and specificity of amplified products were confirmed by visualization on a 1% agarose gel.

RESULTS AND DISCUSSION

Purity Analysis of Total RNA and mRNA

UV absorption of RNA from biomasses A and B revealed a uniform peak at 257 nm without any shoulder peaks. Their A260/280 ratios were 1.9 and 2.01, respectively, suggesting little contamination by polysaccharides and proteins (Leite *et al*. 2012). Electrophoresis of the extracted RNA exhibited distinct 28S, 18S, and 5s rRNA bands (Fig. 1a), indicating high purity, integrity, and no obvious degradation of total RNA (Ping *et al*. 2010). The high-quality mRNA strip was obtained from total RNA through electrophoresis on 1.0% agarose gel, and the diffusion band (Fig. 1b) suggested that the size of mRNA ranged from several hundred base pair (bp) to over several thousand bp (kb) and met the requirement of the SSH experiment.

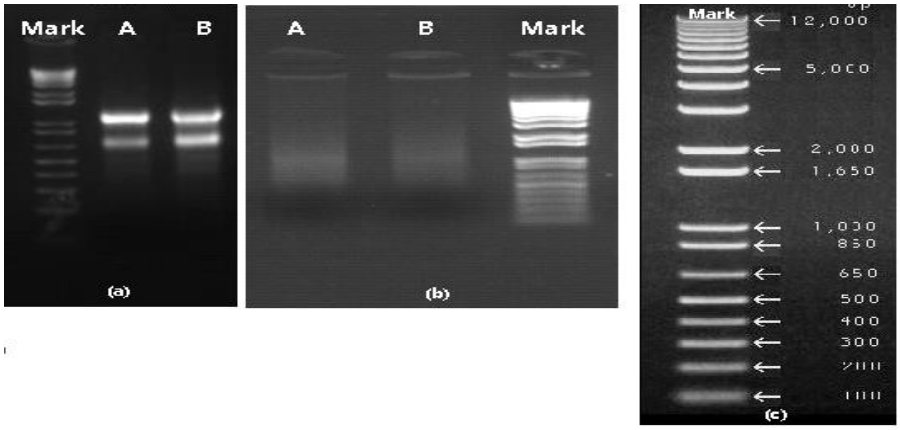

Fig. 1. (a) Agarose gel electrophoresis of total RNA from *Aspergillus oryzae* from the lignin-degradation medium and the control medium. (b) mRNA isolated from total RNA. A: The lignin-degradation medium; B: the control medium.

The Construction of cDNA Subtractive Library

To identify the genes associated with lignin degradation, SSH cDNA libraries (both forward and reverse) were constructed from high-quality mRNA. A total of 6,000 cDNA clones derived from the SSH-cDNA libraries, including 3,200 from the forward library and 2,800 from the reverse one, were successfully amplified.

A total of 87 positive clones with the strongest signals were sequenced, including 55 EST sequences from the forward library and 32 EST sequences from the reversed library. These sequence data had been validated.

Functional Classification of Different Genes

Based on sequence similarities to known genes in *A. oryzae*, 89 different genes in both carbon sources (glucose and lignin) were categorized into several groups according to their functions as follows: (1) hydrolysis; (2) energy metabolism; (3) transporters; (4) synthesis; (5) transcription; (6) protein synthesis; (7) signal transduction; (8) protein destination and storage; (9) division; (10) cell metabolism regulation and control; (11) cell structure; (12) unknown proteins; (13) intracellular traffic; and (14) no significant similarity to the NCBI BLAST search database.

Table 1. Characterization of Differentially Expressed Genes Randomly Selected from the Forward Library

Similar Species	E value	Accession No.
glutathione peroxidase Hyr1 , mRNA	1e-09	XM_001817719
ribosomal RNA	4e-88	KJ809565
A. niger contig An03c0110	8e-137	AM270052
A. oryzae RIB40 DNA，SC206	8e-137	AP007172
Myceliophthora thermophila ATCC 42464 chromosome 7	1e-94	CP003008
Albugo laibachii Alem1, genomic contig CONTIG_1625_Em1_cons_v4_1818_619_641	1e-94	FR834422
Melanopsichium pennsylvanicum 4 genomic scaffold	1e-94	HG529728
A. parasiticus NRRL 502 ITS region	3e-57	NR_121219
A. nomius NRRL 13137 ITS region	3e-57	NR_121218
Penicillium argentinense CBS 130371 ITS region	2e-46	NR_121523
Penicillium euglaucum CBS 323.71 ITS region	2e-46	NR_121517
Penicillium gallaicum CBS 167.81 ITS region	6e-46	NR_103657
Penicillium wellingtonense CBS 130375 ITS region	7e-45	NR_121519
Penicillium nothofagi CBS 130383 ITS region	7e-45	NR_121518
Penicillium ubiquetum CBS 126437 ITS region	7e-45	NR_121514
Penicillium pasqualense CBS 126330 ITS region	7e-45	NR_121513
A. oryzae RIB40 hypothetical protein	2e-20	XM_003189216
Calanus helgolandicus 14-3-3 protein	2e-06	KC521533
Lolium perenne UDP-sugar pyrophospharylase	2e-06	JF747494
Belgica antarctica clone Ba-U01b pacifastin-like mRNA	6e-06	DQ507280
Equus caballus subtracted library fragment 43	2e-05	AY246807
Olea europaea pathogenesis-related thaumatin-like protein	8e-05	JQ711523
Lolium multiflorum purple acid phosphatase	8e-05	EF558901
Warburgia ugandensis clone WarbTPS-g sesquiterpene synthase gene partial cds	3e-04	FJ416155
A. oryzae RIB40 synaptobrevin 1	4e-57	XM_001727395
A. flavus NRRL3357 SNARE protein	4e-57	XM_002375674
A. oryzae cDNA, contig sequence	4e-57	AB225641

A. oryzae Aosnc1 gene for v-SNARE protein	9e-24	AB279870
A. oryzae RIB40 protein SPG20	2e-118	XM_001818144
A. oryzae RIB40 endo-1,3(4)-beta-glucanase	1e-31	XM_001819907
A. flavus NRRL3357 endo-1,3(4)-beta-glucanase	1e-31	XM_002374464
A. oryzae 3.042 mitochondrion, complete genome	3e-94	JX129489
A. flavus NRRL3357 acetate--CoA ligase	1e-155	XM_002374176
A. oryzae RIB40 metalloproteinase	9e-64	XM_001818635
A. oryzae RIB40 hypothetical protein	3e-105	XM_003190352
A. flavus NRRL3357 cytochrome P450 alkane hydroxylase	1e-98	XM_001821443
A. flavus NRRL3357 cytochrome P450 alkane hydroxylase	1e-98	XM_002380767
A. oryzae 3.042 mitochondrion, complete genome	9e-95	JX129489
A. flavus alkaline protease	2e-86	AF324246
A. oryzae RIB40 ubiquitin	1e-115	XM_001820217
A. flavus NRRL3357 polyubiquitin UbiD/Ubi4	1e-115	XM_002374107
A. oryzae RIB40 endo mannanase	3e-89	XM_001827622
Talaromyces stipitatus ATCC 10500 glycosyl hydrolase	7e-06	XM_002477832
A. oryzae RIB40 integral membrane protein	4e-09	XM_003190486
A. oryzae RIB40 plasma membrane proteolipid 3	2e-76	XM_003189153
A. oryzae RIB40 heat shock protein	5e-29	XM_003188968
A. oryzae RIB40 1,3-beta-glucanosyltransferase	4e-57	XM_001823186
Uncinocarpus reesii 1704 glycolipid-anchored surface protein 5	4e-17	XM_002540727
A. oryzae RIB40 C2H2 transcription factor (Rpn4)	9e-33	XM_001727454
A. flavus NRRL3357 cytochrome P450 alkane hydroxylase	1e-98	XM_002380767
A. flavus NRRL3357 alkaline serine protease	2e-86	XM_002374250
Gibberella moniliformis beta-1,3-glucanosyltransferase	2e-04	DQ458798
Lolium multiflorum purple acid phosphatase	0.002	EF558901
Lolium perenne UDP-sugar pyrophospharylase	2e-04	JF747494
A. oryzae RIB40 metalloproteinase	9e-64	XM_001818635
A. oryzae RIB40 glutaminase GtaA	3e-47	XM_001824579

Table 2. Characterization of Differentially Expressed Genes Randomly Selected from the Reversed Library

Similar Species	E Value	Accession No.
A. oryzae RIB40 elongation factor	2e-33	XM_001823791
Escovopsis sp. ugm010407-01 EF-1 alpha gene	5e-25	AY629392
Panulirus stimpsoni clone 54 microsatellite sequence	3e-12	EU557303
A. oryzae RIB40 eukaryotic translation initiation factor eIF-1	2e-24	XM_001824434
Arthroderma otae CBS 113480 translation factor SUI1	1e-21	XM_002842952
A. oryzae RIB40 centromere/microtubule-binding protein	2e-32	XM_001817057
A. flavus NRRL3357 pseudouridylate synthase family protein	2e-32	XM_002382906
A. oryzae RIB40 metallo-beta-lactamase domain protein	2e-22	XM_001820669
A. oryzae RIB40 tubulin-specific chaperone Rbl2	7e-18	XM_001826401
A. flavus NRRL3357 phthalate transporter	7e-27	XM_002377606
A. oryzae RIB40 serine/threonine-protein kinase	4e-24	XM_001823921
A. flavus NRRL3357 calcium/calmodulin-dependent protein	4e-27	XM_002381003
A. flavus NRRL3357 C2H2 transcription factor (Rpn4)	3e-20	XM_002375743
A. oryzae RIB40 aconitate hydratase	3e-29	XM_001819545
A. oryzae RIB40 ATP synthase F1-sector subunit beta	2e-32	XM_001827414
A. flavus NRRL3357 DUF221 domain protein	4e-30	XM_002384593
A. flavus NRRL3357 phthalate transporter	8e-27	XM_002377606
A. oryzae RIB40 stress response protein	3e-19	XM_001819708
Branchiostoma japonicum chitotriosidase-like protein	5e-19	JF932309
A. oryzae RIB40 plasma membrane ATPase 2	1e-57	XM_001824674
A. oryzae RIB40 NADP-specific glutamate dehydrogenase	2e-30	XM_001821367
A. flavus NRRL3357 phosphatidate cytidylyltransferase	8e-49	XM_002376035
A. oryzae RIB40 Inositol oxygenase 1	7e-40	XM_001826169
A. oryzae RIB40 acyl-CoA desaturase	2e-17	XM_001817469
A. flavus NRRL3357 stearic acid desaturase	2e-17	XM_002372564
Branchiostoma belcheri tsingtaunese ferritin mRNA,	3e-15	AY175376

A. oryzae RIB40 C2H2 zinc finger protein	1e-13	XM_001822173
A. oryzae RIB40 tRNA pseudouridine synthase	2e-17	XM_001826376
A. oryzae RIB40 malate synthase	6e-23	XM_001816943
Pestalotiopsis fici W106-1 Ras-related protein ced-10	1e-04	XM_007830866
A. oryzae RIB40 GTP-binding protein rhoA	7e-91	XM_001819256
A. oryzae RIB40 woronin body major protein	2e-50	XM_003190492

The data showed that the up-regulated genes were from the forward library, while the down-regulated genes were from the reverse library. Among the up-regulated genes, the largest functional gene group corresponded to the category of hydrolytic enzymes, including glutathione peroxidase, endo-1,3(4)-β-glucanase, acetate-CoA ligase, cytochrome P450 alkane hydroxylase, alkaline protease, ubiquitin, polyubiquitin UbiD/Ubi4, endo-mannanase, glycosyl hydrolase, 1,3-beta-glucanosyl-transferase, thaumatin-like protein, alkaline serine protease, acid phosphatase, glutaminase, and metalloproteinase. Glutathione peroxidase is a type of oxidoreductase produced by microorganisms or plants that is capable of making phenol and amine compounds to be oxidized. It has been reported that peroxidase can break down lignin (Beckett *et al*. 2015). We obtained sequences from *A. oryzae* in the medium using lignin as carbon source. The gene sequences of glutathione peroxidase were obtained from NCBI database.

The gene sequences of *A. oryzae* from 11 to 86 and 91 to 103 were identical to the sequences of glutathione peroxidase from 569 to 648. The matched sequences are shown in bold red in Figs. 3 and 4.

1 agcgtggtcg cggccgaggt cctgaagcgc gtcaagtgga acttcgaaaa gttcctgatc
61 tcgcccgatg gcaaggtcgt cggtcggtcg ctgggccagt acctgcccgg gcggccgctc
121 gaa

Fig. 3. The gene sequences of *A. oryzae*

1 atgcgatcaa ccctatcgtc tgcgtctatc gtcctacgtg ctttatcatc ccagaaacca
61 ttaatctact tcctcgcgca tataaccagc cctgtcgcaa aatcgccgcg caggtcttct
121 tcaacgctgt caagctccgc taagctgcta ctcaatcatc aacaacctcg tttgagcgca
181 tttcatacgc acaccatggc ttccgcaacc accttcttcg actttgagcc agttgacaag
241 aaaggctctc cttttcccct caccccgctc aagggcaaga ccatcctcgt cgtcaacact
301 gcctccaagt gcggctttac tccccagttc gaaggtctcg agaaactcta ccagaagctg
361 aagtccaagt accccgaaga ctttactatc ctcggattcc cttgcaacca gttcggcggc
421 caggatcccg gttccaacga tcagattcaa gacttctgcc agctgaacta cggtgttaca
481 ttccctgtgt tgggcaagct ggatgtgaac ggaaacgagg cctcaccctt gtggacctgg
541 atgaaggagc agcaacccgg tctgctgggc ctgaagcgcg tcaagtggaa cttcgaaaag
601 ttcctgatct cgcccgatgg caaggtcgtc ggtcgctggg ccagtaccac caagcccgag
661 tcactcgagg acaccatcgt caaggagatt gagaaggcac agaaggccgg aactgcagct
721 tcggttcagg ctaaggaggg agagtctgct gagcaggcta agttgtcgta a

Fig. 4. The gene sequences of glutathione peroxidase

Based on this high homology, one can infer that *A. oryzae* generated peroxidase to degrade lignin in the medium using lignin as a carbon source. In some literature, glutathione peroxidase could catalyze the reaction of converse H_2O_2 into ·OH in the presence of GSH to degrade organic substance (Navrot *et al*. 2006; Passaia and Margis-Pinheiro 2015).

It has been generally accepted that alkaline proteinase and metalloproteinase can catalyze the hydrolysis reaction of protein (Birkedal-Hansen *et al*. 1993; Anwar and Saleemuddin 1998), and ubiquitin can hydrolyze protein (Baarends *et al*. 1999). Glutaminase can hydrolyze glutamine to glutamic acid and participate in transdeamination, by which the amino acids from protein hydrolysis are changed into fatty acids (Koibuchi *et al*. 2000). Based on these ideas, the up-regulation of these four genes indicates the increase of protein hydrolysis using lignin as a carbon source. As two key components in plant cell walls, mannans and various 1,4-β-linked glucan polymers play an important structural role in plants (Whitney *et al*. 1998). The key endo-β-mannanase (EC 3.2.1.78) can randomly hydrolyze mannan by breaking the internal β-1,4-D-mannopyranosyl bond in the mannan backbone (Yuan *et al*. 2007), and endo-1,3(4)-β-glucanase can degrade crystalline cellulose (Nicol *et al*. 1998). The up-regulation of these two enzymes in the presence of lignin reveals that the semi-cellulose and cellulose hydrolysis by the strain was enhanced in response to lignin.

The expression of cytochrome P450 alkane hydroxylase was increased in the presence of lignin. As a heme-thiolate enzyme, it can collectively catalyze a range of relatively specific monooxygenase reactions and transform diverse lipophilic compounds into more polar metabolites (Yadav and Loper 1999). Cytochrome P450 alkane hydroxylase is an inducible enzyme and is not synthesized in the absence of alkane. The up-regulation of this enzyme indicates that alkane existed in the medium. However, there was no alkane in the medium, nor was there the catabolism of protein, cellulose, or semi-cellulose. The only source of alkane was the hydrolysate of the lignin caused by lignin hydrolytic enzymes (Yan *et al*. 2008). Therefore, the presence of cytochrome P450 alkane hydroxylase may indirectly prove the possibility that the strain synthesized lignin hydrolytic enzymes to degrade lignin. These digestive enzymes were synthesized in the cytoplasm and secreted outside the cells in vesicle form.

The second largest gene group corresponded to functions relating to transporters, including synaptobrevin, SNARE protein, v-SNARE protein, C_2H_2 transcription factors, and phthalate transporters. Synaptobrevin, a highly conserved membrane protein originally described as a component of brain synaptic vesicles, may play a general role in membrane trafficking and in the secretion of digestive enzymes (Chin *et al*. 1993). SNARE protein mediates the specificity of vesicle trafficking by defining membranes compatible for docking and fusion (Bock and Scheller 1999). v-SNARE drives Ca^{2+}-triggered membrane fusion at a millisecond-timescale (Kesavan *et al*. 2007). These proteins may involve the transfer of hydrolytic enzymes from intracellular to extracellular status.

The third gene group consisted of cell structure proteins, including integral membrane proteins, plasma membrane proteolipids, and glycolipid-anchored surface proteins. The up-regulation of these genes indicates that lignin as a carbon source altered the content of different components in the cell membrane. The β-1,3-glucanosyl transferases internally split the β-1,3-glucan molecule and transferred the newly-generated reducing end to the non-reducing end of another β-1,3-glucan molecule, resulting in the elongation of the β-1,3-glucan side chains (Caracuel *et al*. 2005). The existence of UDP-sugar pyrophospharylase implies that the biomass of *A. oryzae* synthesized the glycogen

as energy storage from other materials (Schnurr *et al*. 2006). Therefore, the up-regulation of these two enzymes shows that *A. oryzae* increased gluconeogenesis when it degraded lignin. The up-regulation of the heat shock protein shows that the *A. oryzae* mycelia enhanced the function to protect the cell itself and to repair the damage caused by various stimuli (Whitley *et al*. 1999). Here, the heat shock protein mainly protected cells from damage from H_2O_2, which was synthesized by the mycelia itself, and it also acted as another substrate of lignin by enzymatic hydrolysis. The other genes corresponded to cell division and some transcription. In conclusion, the up-regulation of these genes indicates that the hydrolytic effect, gluconeogenesis, and the repairing function were significantly enhanced and their protein contents in the cell membrane were also increased.

Protein kinases contain a highly conserved catalytic region and a less conserved regulatory domain, and they are usually modular enzymes that play a role in nearly every aspect of cell biology (Lakshminarayan *et al*. 2008; Shen *et al*. 2014). C_2H_2 zinc finger protein binds to specific DNA sequences *via* its zinc fingers, and it modulates gene expression during diverse biological processes such as cell growth, differentiation, tumorigenesis, embryogenesis, and apoptosis (Jiang *et al*. 2007). It is believed that GTP-binding proteins are the main regulators of vesicular transport in eukaryotic cells, by which glucose is transferred from the outer membrane to the site where glucose is decomposed (Rothman 1994). These proteins are all concerned with glucose metabolism; therefore their down-regulation suggests that glucose metabolism was limited. Another vital functional gene group corresponded to the category of metabolism, including acyl-CoA desaturase, stearic acid desaturase, phosphatidate cytidylyltransferase, malate synthase, aconitate hydratase, ATP synthase F1-sector subunit beta, and plasma membrane ATPase 2. Acyl-CoA desaturase and stearic acid desaturase are the key enzymes of fatty acid catabolism (Hodson and Fielding 2013). Phosphatidate cytidylyltransferase catalyzes the synthesis reaction of triglyceride, which is mainly in the cell membrane (Longmuir and Johnston 1980). The down-regulation of these genes further revealed the changes in cell membrane components. Aconitate hydratase and malate synthase are the rate-limiting enzymes of the tricarboxylic acid cycle and glyoxylate cycles, respectively (Umemura *et al*. 1997). Their down-regulation indicated that the aerobic metabolism, ATP synthesis, and utilization were inhibited, which was demonstrated by the down-regulation of ATP synthase F1-sector subunit beta and plasma membrane ATPase 2. Taken together, the down-regulation of genes demonstrates that glucose catabolism, lipid synthesis metabolism, and energy metabolism were all suppressed, and the components of the cell membrane were also changed.

CONCLUSIONS

1. The present study is the first report of the different gene expressions of *A. oryzae* grown in the medium containing lignin and glucose, respectively.
2. The discovery of peroxidase from up-regulation genes in the medium using lignin as a carbon source explained that *A. oryzae* could degrade lignin in the straw. This finding provided evidence of the micromechanism interacting between the microorganism and lignin.
3. From the number of over-expressed unigenes, it was also highly convincing that *A. oryzae* could hydrolyze protein, cellulose, and hemicelluloses in the straw.

4. Surprisingly, the strain possessed the capability for gluconeogenesis and changed the components of the cell membrane in the presence of lignin, including the increase of protein components and the decrease of lipids.
5. Lignin, as a carbon source, suppressed the aerobic metabolism of glucose, and it also reduced the synthesis and utilization of energy. The ability of mycelia's resistance to various stimuli and self-repair was significant.

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