Identification of Long Non-Coding RNAs and Their Target Genes from Mycelium and Primordium in Model Mushroom \textit{Schizophyllum commune}

Tuheeng Wu\textsuperscript{a,b}, Jian Chen\textsuperscript{a,b}, Chunwei Jiao\textsuperscript{a}, Huiping Hu\textsuperscript{a}, Qingping Wu\textsuperscript{b,c} and Yizhen Xie\textsuperscript{a,c}

\textsuperscript{a}Guangdong Yuewei Edible Fungi Technology Co., Guangzhou, China; \textsuperscript{b}School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, China; \textsuperscript{c}Guangdong Provincial Key Laboratory of Microbial Safety and Health, State Key Laboratory of Applied Microbiology Southern China, Institute of Microbiology, Guangdong Academy of Sciences, Guangzhou, China

\textbf{ABSTRACT}

\textit{Schizophyllum commune} has emerged as the most promising model mushroom to study developmental stages (mycelium, primordium), which are two primary processes of fruit body development. Long non-coding RNA (IncRNA) has been proved to participate in fruit development and sex differentiation in fungi. However, potential IncRNAs have not been identified in \textit{S. commune} from mycelium to primordium developmental stages. In this study, IncRNA-seq was performed in \textit{S. commune} and 61.56 Gb clean data were generated from mycelium and primordium developmental stages. Furthermore, 191 IncRNAs had been obtained and a total of 49 IncRNAs were classified as differently expressed IncRNAs. Additionally, 26 up-regulated differently expressed IncRNAs and 23 down-regulated between mycelium and primordia libraries were detected. Further, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that differentially expressed IncRNAs target genes from the MAPK pathway, phosphatidylinositol signal, ubiquitin-mediated proteolysis, autophagy, and cell cycle. This study provides a new resource for further research on the relationship between IncRNA and two developmental stages (mycelium, primordium) in \textit{S. commune}.

\section{Introduction}

Recently, due to the typical basidiomycete life cycle, available genome sequence information [1], and effective genetic tools (e.g., CRISPR-Cas9 technology [2], knock-out tool based on homologous recombination [3]), \textit{Schizophyllum commune} has been the most important model to study mushroom-forming fungal development. Fruiting body development is triggered by various environmental factors (e.g., light and humidity), and involves a transition from dikaryotic vegetative mycelium to a complex multi-cellular fruiting body in \textit{S. commune} [4]. And mycelium and primordium are two basic developmental processes of fruiting body development. Most studies of fruiting body development focused on the identification and functional study of transcription factors or other proteins. Previous studies showed that transcription factors such as c2h2, gat1, blue light receptor-white collar complex (wc-1 and wc-2), hom1, hom2, and Ras1 had been proved to regulate various aspects of fruiting body formation in \textit{S. commune} [5,6]. Additionally, comparative transcriptome analysis of five basidiomycete fungi including \textit{S. commune} during developmental stages showed that transcription factors had evolutionarily conserved functions across species [7]. It also suggested there is an evolutionarily conserved developmental program in basidiomycete fungi.

Long non-coding RNAs (IncRNAs) are a type of non-protein coding transcripts longer than 200 nucleotides which implicated in chromatin structure [8], transcriptional [9], and post-transcriptional gene regulation [10], and regulation of genomic stability [11]. In addition, IncRNAs have been proved to participate in various biological processes such as morphological transition and sexual differentiation in fungi [12]. For example, in the human fungal pathogen \textit{Cryptococcus neoformans}, an IncRNA \textit{RZE1} regulated the switch from yeast to hypha through the interaction with upstream of the master regulator gene \textit{ZNF2} [13]. Previous research showed that \textit{IRT1} and \textit{IRT2} IncRNAs and the master transcription factor \textit{IME1} direct sexual differentiation in yeast [14]. Wang et al. found that \textit{XRN1}-associated IncRNAs might regulate fungal virulence and sexual development in \textit{Cordyceps militaris} [15]. Interestingly, \textit{HAX1} IncRNA was characterized in...
Trichoderma reesei for its role in cellulase expression [16]. However, none of the lncRNAs have been identified from S. commune and the epigenetic regulation by lncRNAs in S. commune is still unknown.

Over the years, next-generation sequencing technology has accelerated the discovery of lncRNAs for its low cost and high throughput. Also, this technology has been used widely for lncRNA expression profiling associated with development and sex determination in fungi. For instance, in the plant-pathogenic fungus Fusarium graminearum, expression profiling of 547 lncRNAs from fungal fruiting bodies undergoing sexual development was determined [17]. Totally, 4140 lncRNAs from mycelium and fruiting body stages in C. militaris had been identified [15]. In addition, a total of 402 lncRNA candidates were obtained from three developmental stages in the best-known medicinal fungus Ganoderma lucidum [18]. From a technological perspective, lncRNA sequencing can be applied in the identification of lncRNAs from mycelium and primordia developmental stages in S. commune.

In this study, we identified differentially expressed lncRNAs and their target protein-coding genes from S. commune mycelium and primordia developmental stages using the lncRNA sequencing technique.

2. Materials and methods

2.1. Growth and treatment of S. commune

The S. commune dikaryotic strain W160031 used in this study was provided by the Institute of Microbiology, Guangdong Academy of Sciences, Guangzhou, China. Vegetative mycelium, cultured on PD medium (200 g potato, 20 g glucose, 3 g KH₂PO₄, 1.5 g MgSO₄, 10 g peptone, 20 g agar, 1000 ml water) for 7 days at 25°C in darkness, was harvested (Figure 1). Mycelium was grown on a PD medium for 7 days at 25°C in the dark. After which, the culture was incubated at 25°C for 2 days with white light treatment for primordia formation. Primordia was collected as soon as its formation (Figure 1). Finally, samples from two stages were frozen in liquid nitrogen and immediately stored at −80°C for RNA extraction.

2.2. Preparation of total RNA, cDNA library, and sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by the quality evaluation using 1.5% agarose gels and an Agilent 2100 Bioanalyzer. And then lncRNA Sequencing libraries were generated with NEB Next® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, Ipswich, MA, USA). Next-generation sequencing of these libraries was performed on the Illumina HiSeq platform with a paired-end strategy at Biomarker Technologies (Beijing, China).

2.3. Assembly, annotation, and analysis of differentially expressed protein-coding genes

Clean reads were obtained after removing reads with adapter sequences, low-quality sequences, and duplicate sequences. Subsequently, clean reads were compared with S. commune H4-8 v3.0 reference genome (NCBI project accession number ADMJ00000000) using HISAT2 [19]. Then transcriptome was assembled using StringTie software [20]. All assembled unigenes were functionally annotated based on five databases, including the NCBI non-redundant protein (Nr), Eukaryotic Ortholog Groups (KOG), Protein family (Pfam), Swiss-Prot, and Kyoto Encyclopedia of Genes and Genomes (KEGG). For quantification of gene expression levels, Fragments per kilobase of exon model per million mapped reads (FPKM) were calculated using StringTie software. Differential expression analyses of two developmental stages were performed using DESeq2 [21].
stages were performed using the DESeq R package [21]. Genes with an adjusted p-value <0.01 and absolute value of log2 (Fold change) >1 were defined as differentially expressed genes (DEGs). For functional annotation of differentially expressed genes, Gene Ontology (GO) enrichment analysis was implemented by the topGOseq R package [22]. KOBAS software was used to test the statistical enrichment of differential expression genes in KEGG pathways [23].

2.4. Analysis of differentially expressed IncRNAs and their target protein-coding genes

Computational methods including CPC2/CNCSI/Pfam/CPAT were combined to screen for IncRNAs in the unknown transcripts. LncRNAs must have lengths of more than 200 nt and have more than two exons. The classification of IncRNAs was performed by cuffcompare software [24], according to different types of IncRNAs (lincRNA, intronic lncRNA, anti-sense lncRNA, sense lncRNA). FPKM were calculated for quantification of IncRNAs expression levels. LncRNAs with an adjusted p-value <0.01 and absolute value of log2 (Fold change) >1 were defined as differently expressed lncRNAs. To uncover the potential functions of differently expressed IncRNAs, their neighboring protein-coding genes were predicted and the expression correlation of differently expressed IncRNAs with neighboring target protein-coding genes was investigated as previously reported [25]. GO and KEGG enrichment analyses of these target protein-coding genes were performed as above.

2.5. Verification of IncRNA sequencing using RT-qPCR

Totally five differently expressed IncRNAs were chosen to validate this IncRNA sequencing data (Table S1). Total RNA was isolated from mycelium and primordia using the HiPure Fungal RNA Mini kit (Magen, Guangzhou, China). First-strand cDNA was synthesized by HiScript® III All-in-one RT SuperMix Perfect for qRT-PCR (+gDNA wiper) (Vazyme Biotech, Nanjing, China). The qPCR was performed using ChamiQ Universal SYBR qPCR Master Mix (Vazyme Biotech). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the reference gene. The conditions for qPCR were as follows: initiation was conducted at 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. This experiment was conducted on the Applied Biosystems ABI 7500 (Applied Biosystems, Foster City, CA, USA) containing three technical replicates and three biological replicates. The relative expression of selected lncRNAs was calculated with the $2^{-\Delta\Delta CT}$ method [18,26]. Primers used in this work were synthesized by Guangzhou Tsingke (Table S1).

3. Results

3.1. Transcriptomic landscape of the development from mycelium to primordium

To obtain more information on lncRNAs of mycelium and primordium stages in S. commune, we performed lncRNA sequencing and identified protein-coding genes firstly. After quality control and filtering, a total of 61.56 Gb clean data were generated from 6 samples (2 developmental stages, 3 replicates), and there were an average of 9.54 Gb clean data per sample (Table S2). The Q30 values were all above 94.01%, suggesting that the quality and accuracy of lncRNA sequencing data were sufficient for further identification of protein-coding genes.

A total of 1569 protein-coding genes were predicted by lncRNA sequencing, including 145 novel protein-coding genes. Then these protein-coding genes were mapped to databases for revealing their possible biological functions. The numbers of these genes annotated in the COG, GO, KEGG, KOG, NR, Pfam, Swiss-prot, and eggNOG databases were 668, 1085, 549, 805, 1172, 936, and 1356, respectively. 1600 DEGs were identified from mycelium and primordium in this study. Among them, 793 up-regulated DEGs and 807 down-regulated DEGs between mycelium and primordium libraries were detected (Figure S1). Additionally, GO and KEGG pathway analyses were applied to study the functions of DEGs. GO enrichment analysis results showed that 362, 107, and 278 DEGs were categorized into GO terms “intrinsic component of membrane,” “regulation of biological process,” “heterocyclic compound binding,” respectively (Figure S2). KEGG analysis revealed that KEGG pathways with the most DEGs were amino sugar and nucleotide sugar metabolism, biosynthesis of amino acid, ribosome, and fatty acid metabolism (Figure S3). Finally, protein-coding genes identified from mycelium and primordium stages are sufficient for further lncRNA analysis.

3.2. Computational identification of lncRNAs and association of lncRNAs with gene expression

Recent research showed that lncRNAs are a class of non-protein coding RNAs in fungi that participate in various biological processes. In this study, a total of 191 lncRNAs have been successfully identified based on the results of lncRNA sequencing. The vast majority of lncRNAs originated from antisense regions (61.8%, 118 lncRNAs), whereas 15.7% (30 lncRNA) and 22.5% (43 lncRNA) were lincRNA.
Nevertheless, none of the 191 IncRNAs were intronic (Figure 2). Furthermore, the comparison of IncRNAs to protein-coding genes was performed. The major length of IncRNAs was 400 nt, with the length ranging from 400 to 1000 nt. mRNA were longer in length than IncRNAs in  S. commune (Figure S4). All IncRNAs had lengths of open reading frame <150 bp (Figure S5). In addition, all of IncRNAs contained fewer exons than mRNAs and most of IncRNAs had only two or three exon (Figure S6). The expression level of IncRNAs was lower than that of the mRNAs (Figure S7). Alternative splicing events is one of the most reported functional processes by IncRNAs [9]. We found that major types of alternative splicing events were alternative 5’ first exon and alternative 3’ last exon in this study. 

Furthermore, by comparing the IncRNAs abundance at two stages, a total of 49 differently expressed IncRNAs were detected under adjusted p-value <0.01 and absolute value of log2 (Fold change) >1. Among these detected IncRNAs, 26 up-regulated differently expressed IncRNAs and 23 down-regulated between mycelium and primordia libraries were found (Figure 3). Importantly, five IncRNAs (MSTRG1419.1, MSTRG3512.1, MSTRG3675.1, MSTRG6616.2, MSTRG7349.1) were found to be specifically expressed in mycelium, while five IncRNAs (MSTRG1439.1, MSTRG438.2, MSTRG5066.2, MSTRG5300.3, MSTRG660.1) were only expressed in primordium, suggesting that these IncRNAs might play an important role in the developmental process.

3.3. Analysis of differentially expressed IncRNAs and their target protein-coding genes

Previous studies showed that IncRNAs can regulate gene expression through the interaction with their neighboring genes in cis [27]. We defined 10 Kb neighboring protein-coding genes as target protein-coding genes in cis. Then differently expressed IncRNAs target protein-coding genes in cis were predicted. GO and KEGG pathway analyses were applied to study the functions of these target protein-coding genes in cis. For GO analysis, in the “Biological process” category, differently expressed IncRNAs target protein-coding genes in cis were enriched in “proteolysis,” “retrograde transport,” and “endosome to golgi” GO terms. In the “Cellular component” category, most of the target protein-coding genes in cis belonged to “cytosol” and “mitochondrial inner membrane.” In the “Molecular Function” category, most of the target protein-coding genes in cis belonged to “serine-type endopeptidase activity,” “O-methyltransferase activity,” “RNA polymerase I activity,” and “ubiquitin binding” (Figure 4(A)). Additionally, KEGG pathways enrichment analysis showed that “spliceosome,” “MAPK signaling pathway-yeast,” “pyrimidine metabolism,” and “Ribosome biogenesis in eukaryotes” as the most enriched terms (Figure 4(B)).
LncRNAs also can regulate gene expression through the correlation in expression between lncRNAs and their target protein-coding genes [27]. In this study, we identified differently expressed lncRNAs target protein-coding genes in trans. GO and KEGG pathway analyses were also applied to study the functions of these target protein-coding genes in trans. In the “Biological process” category, these target protein-coding genes in trans were mainly enriched in the following GO terms: “Small GTPase mediated signal transduction,” “Protein dephosphorylation,” “tricarboxylic acid cycle,” “intracellular signal transduction,” “MAPK cascade.” Moreover, they were also enriched in the “Cellular...
component” category such as GO terms “Intracellular,” “mitochondrion,” “nucleolus,” and “cell division site.” In the "Molecular Function" category, most of the target protein-coding genes in trans belonged to “ATP binding” and “GTP binding” GO terms (Figure 5(A)). KEGG pathway enrichment analysis revealed that pathways with the most neighboring protein-coding genes in trans were “Carbon metabolism,” “Biosynthesis of amino acids,” “Endocytosis,” and “Spliceosome” (Figure 5(B)).

Further KEGG analysis results showed that important differently expressed lncRNAs mainly targeted genes from the MAPK pathway, phosphatidylinositol signal, ubiquitin-mediated proteolysis,
autophagy, and cell cycle. All target genes are listed in Supplementary information Table 1.

3.4. Verification of lncRNA-seq results with RT-qPCR

Five differently expressed lncRNAs were selected to perform qRT-PCR to validate the results obtained from lncRNA sequencing. For each lncRNAs, the qRT-PCR results exhibited similar expression patterns between groups compared with the FPKM values of lncRNA-seq data (see Table S3). It suggested that lncRNA sequencing results in this study were reliable.

4. Discussion

_Schizophyllum commune_ has been used as the most important model organism to uncover the mechanism of fruiting in mushroom-forming fungi. The developmental stages of mycelium and primordia are two basic processes of fruiting in _S. commune_. Current studies focus on transcription factors and pathways involved in mycelium and primordia developmental stages [5,6]. Previous studies showed that lncRNAs are recognized as an important class of epigenetic regulatory non-coding RNAs in fungi, such as yeast and _Neurospora crassa_ [12,28]. However, the characteristics of lncRNAs identified from mycelium and primordia stages are unclear. In this study, we performed genome-wide identification of lncRNAs from mycelium and primordium stages in _S. commune_ by lncRNA sequencing, providing a first look at the landscape of lncRNAs. Totally 191 lncRNAs had been obtained in this study. Importantly, a total of 49 differently expressed lncRNAs were detected. Moreover, 26 up-regulated differently expressed lncRNAs and 23 down-regulated between mycelium and primordia stages were detected. Protein-coding genes potentially targeted by differently expressed lncRNAs were predicted. After which, these protein-coding genes were annotated in the KEGG and GO database.

Previous research showed that mitogen-activated protein kinase (MAPK) cascades are highly conserved in fungi and play crucial roles in the regulation of developmental processes [29,30]. In this study, all 10 lncRNAs, which be specifically expressed in one developmental stage, were involved in MAPK signaling pathway-yeast (ko04011). In addition, lncRNAs (e.g., MSTRG.660.1) targeted more than two genes from the MAPK pathway, implicating that regulatory crosstalk occurs between lncRNAs and target protein-coding genes. In MAPK signaling pathway yeast, ten lncRNAs targeted the key regulator of osmoregulation Hog1 coding gene. Four lncRNAs targeted small GTP-binding protein Rho1 coding gene _Rho1_, which is essential for vegetative growth and regulation of β-1,3-glucan synthesis [31]. Furthermore, five lncRNA targeted 1,3-beta-glucan synthase Fks2 coding gene and three lncRNAs targeted guanine
nucleotide-binding protein alpha-1 subunit Gpa1 coding gene. Six lncRNAs were found to target the cell wall integrity MAP kinase Mkk1/2 coding gene, suggesting that lncRNAs might regulate cell wall remodeling through the cell wall integrity pathway. More lncRNA target genes from MAPK signaling pathway yeast (ko04011) could be archived in Figure S8. In addition, MAPKK (Ste11, Bck1, Ssk2,22), MAPKK (Ste7, Mkk1,2, Pbs2, Ste7), MAPK (Fus3, Hog1, Kss1) constituted MAPK cascades through phosphorylation. In conclusion, differently expressed lncRNAs might target genes from the MAPK pathway, resulting in cell wall remodeling and morphological change.

Phosphoinositides are minor constituents of membranes while participating in diverse cellular processes including regulation of development [32]. In this study, nine lncRNA were found to target PI4K (phosphatidylinositol 4-kinase) coding gene from the “Phosphatidylinositol signal system” KEGG pathway (ko04070). One lncRNA targeted PTEN (phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase) coding gene and four lncRNAs targeted PLC (phosphatidylinositol phospholipase C) coding gene. These results indicated that lncRNAs might regulate genes involved in the phosphatidylinositol signal pathway via phosphoinositides metabolism.

Proteins modified with ubiquitin will be destructed by the ubiquitin-proteasome system, resulting in post-translational modification for the developmental process in fungi [33]. In the “Ubiquitin mediated proteolysis” KEGG pathway (ko04120), six and three lncRNAs targeted UBLE1B (ubiquitin-like 1-activating enzyme E1 B) and BUBE1C (ubiquitin-like 1-activating enzyme E1 C) from E1 (Ubiquitin-activating enzyme) section, respectively. On the other hand, autophagy is considered a highly conserved cellular recycling process via the degradation of proteins and organelles, which plays important role in the fungal development process [34]. In this work, 12 lncRNAs targeted the ATG12 coding gene in the “Autophagy-animal” KEGG pathway (ko04140). Also, five and four lncRNAs targeted ATG13 and LC3 coding genes, respectively. Above all, the degradation of proteins through ubiquitin-mediated proteolysis and autophagy might play important roles in lncRNA-related cellular homeostasis.

Linking the cell cycle to cell fate decisions is an important topic in animal cell biology [35]. And the connection between the regulation of the cell cycle and morphogenesis was also investigated currently [36,37]. In the “Cell cycle-yeast” KEGG pathway (ko04111), unique expressed up-regulated MSTRG.5300.3 targeted one component of the origin recognition complex (ORC) Orc4 coding gene. Unique expressed up-regulated MSTRG.1439.1 targeted PP2A (serine/threonine-protein phosphatase 2A catalytic subunit), Cdc20 (cell division cycle 20) and Clb1/2 (G2/mitotic-specific cyclin 1) coding gene. These results indicated that lncRNAs might directly regulate the expression level of genes in the cell cycle in S. commune.

Finally, the conservation of S. commune lncRNAs compared with other fungi especially G. lucidum will be investigated. Due to the availability of an efficient transformation system, we will further validate the functions of differently expressed lncRNAs by genetic tools such as CRISPR-Cas9 and knock-in. In summary, results of lncRNA sequencing suggested that differently expressed lncRNAs and target protein-coding genes might play important roles in S. commune mycelium and primordia developmental stages, providing a broad repertoire and useful resource for future functional studies.

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Author contributions

Tuheng Wu performed most of the bioinformatics analysis. Jian Chen carried out the sample collection and RNA extraction. Chuenwei Jiao, Qingping Wu, and Huiping Hu designed the studies. Tuheng Wu analyzed the data and wrote the manuscript. Yizhen Xie conceived and designed the project. All authors have read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The raw data of lncRNA-seq generated in this study had deposited in NCBI associated with bioproject PRJNA862197. In addition, data are also available from the corresponding author upon reasonable request.

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