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Genes encoding FAD-binding proteins in *Volvariella volvacea* exhibit differential expression in homokaryons and heterokaryons

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ABSTRACT

Flavin adenine dinucleotide (FAD)-binding proteins play a vital role in energy transfer and utilization during fungal growth and mycelia aggregation. We sequenced the genome of *Volvariella volvacea*, an economically important edible fungus, and discovered 41 genes encoding FAD-binding proteins. Gene expression profiles revealed that the expression levels of four distinctly differentially expressed genes in heterokaryotic strain H1521 were higher than in homokaryotic strains PYd15 and PYd21 combined. These observations were validated by quantitative real-time PCR. The results suggest that the differential expression of FAD-binding proteins may be important in revealing the distinction between homokaryons and heterokaryons on the basis of FAD-binding protein functionality.

1. Introduction

The Chinese mushroom, *Volvariella volvacea* (Bull.) Singer, is a tropical and subtropical saprophytic fungus in the family *Pluteaceae*, phylum *Basidiomycota*. The lifecycle of *V. volvacea* begins with the germination of spores, which produce homokaryotic mycelia with 2–36 nuclei per vegetative cell (Chang and Chu 1969). This is followed by fusion and nuclei exchange with mating-type-compatible homokaryons to yield heterokaryons. Subsequently, these heterokaryons form primordia and develop fruiting bodies under optimal growing conditions. Homokaryons are generally short-lived (Kauserud et al. 2006), and in *V. volvacea* and other Basidiomycetous fungi, they grow much more slowly than heterokaryons. In addition, homokaryons are significantly different from heterokaryons in terms of colony morphology. In characterizing the differences between homokaryons and heterokaryons, previous studies focused on enzyme activity, the ability to grow on wood as a substrate, and other phenotypic indicators (Fryar et al. 2002; Hiscox et al. 2010; Nazrul and Bian 2011). However, we recently obtained the genome sequence and gene expression profiles of *V. volvacea* using next-generation high-throughput sequencing technology. In this study, we decided to focus on the genes that may be responsible for inducing differences between homokaryons and heterokaryons.

Flavin adenine dinucleotide (FAD) and its related cofactors, nicotinamide adenine dinucleotide (NADH) and adenosine triphosphate (ATP), play an important role in energy production and in many other biological processes. White et al. (2001) reported that an FAD-binding protein gidA, a yellow protein, is involved in development in *Myxococcus xanthus*. The gidA$^-$ mutant inevitably evolves into a stable derivative, gidA*, after several generations. In addition, the gidA* mutant releases a small, heat-stable, protease-resistant molecule that inhibits aggregation and gene expression of wild-type cells during development. In a study on *Schizophyllum commune*, Ohm et al. (2010) found that the genes involved in protein and energy production were up-regulated during the formation of mycelia aggregates, namely, during the formation of a heterokaryon from homokaryons. In other words, FAD-binding proteins play an important role in mycelia aggregation and development in fungi.

With the availability of an increasing number of three-dimensional protein structures in the Protein Data Bank, it is possible to predict the structure and function of a protein based solely on its sequence. If the protein of interest shares at least 30% amino acid identity with another protein, the two proteins generally exhibit similar three-dimensional structures (Doolittle 1986). Such fundamental discriminators may improve our understanding of protein function, in particular for FAD-binding proteins where several tertiary structures, often only distantly related to

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one another, are known to exist. Four different FAD-family folds have been identified, represented by glutathione reductase (GR), ferredoxin reductase (FR), *p*-cresol methyldroxylase (PCMH), and pyruvate oxidase (PO) (Correll et al. 1993; Dym and Eisenberg 2001; Fraaije et al. 1998; Ingelman et al. 1997). Additionally, Dym and Eisenberg (2001) analyzed structure–sequence relationships in 32 FAD-binding proteins and reported the presence of several conserved motifs in each structural family.

Based on the aforementioned studies, it is reasonable to infer that the FAD-binding proteins in *V. volvacea* can be identified using data on three-dimensional protein structures and conserved motifs. Accordingly, we decided to investigate the genes encoding the proteins containing FAD-binding domains in *V. volvacea*, for the purpose of understanding the distinction between homokaryons and heterokaryons in terms of protein function.

2. Materials and methods

2.1. Materials

Homokaryotic strains PYd15 and PYd21 (obtained from monosporous isolations), and heterokaryotic strain H1521 (generated by a cross between homokaryotic strains PYd15 and PYd21) used in this study were obtained from the Mycological Research Center of Fujian Agriculture and Forestry University, China. These strains were maintained on PDA slant medium at 15 °C.

2.2. Colony characteristics

All vegetative mycelia production and growth rate tests were performed on complete agar medium at 35 °C. The distance from the initial lines to the mycelial frontiers were measured (mm) on the fourth day after inoculation. The cultivation of *V. volvacea* on cotton waste was performed as described by Rajapakse (2011).

2.3. Isolation of genomic DNA, and genome sequencing and assembly

Mycelia of homokaryotic strain PYd21 was used for genome-wide *de novo* sequencing. Genomic DNA was isolated as described (Möller et al. 1992) and sequenced on the Illumina Cluster Station and Illumina GAII platform at BGI-Shenzhen (Shenzhen, China). The data were assembled using the SOAPdenovo (Li et al. 2010) assembler (http://soap.genomics.org.cn/). All genes in the genome of *V. volvacea* were predicted with the software Eukaryotic GeneMark-ES (version2.3) (Atlanta, Georgia, USA) (Ter-Hovhannisyan et al. 2008).

2.4. Characterization of FAD-binding proteins in V. volvacea

FAD-binding proteins were identified using the Clusters of Orthologous Groups (COG) Database (Tatusov et al. 2003). Proteins of *V. volvacea* were assigned to orthologous groups using OrthoMCL version 2.0 (Li et al. 2003). Members of such groups were designated as orthologs (in the case of proteins from another species) or inparalogs (in the case of proteins from *V. volvacea*). All-*versus*-all BLASTP analysis was performed using NCBI standalone BLAST version 2.2.20, with an *E* value of 10^{-5} as a cutoff.

2.5. DGE library construction and sequencing

Total RNA was extracted from mycelia of strains PYd15, PYd21 and H1521 using pBIOZOL Plant Total RNA Extraction Reagent, according to the manufacturer's protocol (BioFlux, China). The isolated RNA was treated with RNeasy plant mini kit to remove genomic DNA contamination, based on the manufacturer's protocol (QIAGEN). The integrity and the concentration of the isolated RNA were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

For RNA library construction and sequencing, a 6 μg sample of RNA from each of the three strains was submitted to BGI-Shenzhen (Shenzhen, China) for library construction and sequencing. Sequence tag preparation was carried out using the Illumina Gene Expression Sample Prep Kit according to the manufacturer's protocol (Illumina, San Diego, CA, USA). The mRNAs were separately isolated with oligo(dT) ligated beads, and were then reverse transcribed into double-stranded cDNA. The cDNAs were digested by the restriction enzymes NlaIII and MmeI, ligated with sequencing adapters, and amplified by PCR. The three mRNA tag libraries were sequenced on the Illumina Cluster Station and Illumina HiSeq™ 2000. Image recognition and base calling were performed using Illumina Pipeline (Illumina, San Diego, CA, USA).

2.6. Quantitative analysis of gene expression

The expressed sequence tags of the genes encoding our proteins of interest were obtained by mapping the tag library with ZOOM Studio 1.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) (Lin et al. 2008), using the genome sequences as the respective reference sequences. All expressed sequence tags were mapped to the reference sequences and all distinct, uniquely mapped tags were selected using the Basic Local Alignment Search Tool (BLAST) (blast-2.2.25+), while tags mapped to multiple genes were filtered out. The number of tags for the reference sequences were calculated and normalized to the number of transcripts per million clean tags (TPM) (Morrissy et al. 2009; 't Hoen et al. 2008). TPM is a standardized indicator for the number of transcript copies in one million clean tags (Subramanian et al. 2005).

2.7. RNA isolation for reverse transcription PCR

Total RNA of homokaryotic strains PYd15, PYd21 and heterokaryotic strain H1521 was isolated using the Plant RNA Kit (OMEGA, Norcross, GA, USA). DNA/RNA quality and concentration were assessed by agarose gel electrophoresis and spectrophotometric analysis (NanoDrop ND-1000, Wilmington, DE, USA), respectively.

2.8. Reverse transcription PCR

First strand cDNA was synthesized with a RevertAid First Strand cDNA Synthesis Kit, using the manufacturer's protocol. Each 20 μL reaction mixture contained 2 μL RNA template, 1 μL Random Hexamer Primer, 4 μL 5×Reaction Buffer, 1 μL RiboLock RNase Inhibitor, 2 μL 10 mM dNTP Mix, 1 μL RevertAid M-MuL V Reverse Transcriptase, and 9 μL nuclease-free water. The mixture was mixed gently and then centrifuged prior to cDNA synthesis. For random hexamer primer synthesis, incubations were conducted for 5 min at 25 °C. For cDNA synthesis, the mixture was then incubated for 60 min at 42 °C. The reaction was terminated by holding the mixture at 70 °C for 5 min. The resultant cDNA samples were stored at −70 °C.

2.9. Quantitative real-time PCR

Reaction mixtures (20 μL final volume) for quantitative real-time PCR contained 0.4 μL of each primer (forward/reverse