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Note

Further characterization of hydrophobin genes in genome of *Flammulina velutipes*

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ABSTRACT

This study aimed to identify and further characterize hydrophobin genes in the whole-genome sequence of *Flammulina velutipes*. Ten genes (*Hyd-1*, *Hyd-2*, *Hyd-3*, *Hyd-4*, *Hyd-5*, *Hyd-6*, *Hyd-7*, *Hyd-8*, *Hyd-9*, and *Hyd-10*) sharing eight conserved cysteine residues were identified from 13 previously predicted putative hydrophobin genes in the *F. velutipes* genome. Quantitative real-time PCR (qPCR) analysis showed that these genes were specifically expressed in different developmental stages and tissues. Whereas all of the genes showed relatively higher levels of expression in the primordial stages of the fungus, most of them were expressed at relatively low levels in the mycelial stage.

Flammulina velutipes, also known as winter mushroom or Enokitake, is a common white-rot basidiomycete distributed throughout temperate zones (Hughes et al. 1999). It is one of the six most actively cultivated mushroom species in the world (Psurtseva 2005). In the commercial production of mushrooms, the development of fruiting bodies is induced by controlling environmental factors such as temperature, light and humidity, as well as physical and chemical stimuli. Extensive reports on *F. velutipes* fruit body formation are available (Plunkett 1956; Kitamoto and Gruen 1976; Williams et al. 1985; Terashita et al. 1992) because this fungus readily forms fruit bodies under low-temperature and low-light conditions. Several studies have investigated the molecular basis of fruit body development in this fungus (Kim and Azuma 1999; Kim et al. 1999; Ando et al. 2001; Yamada et al. 2005). We recently determined the whole-genome sequence of *F. velutipes* (Park et al. 2014). Therefore, genome-wide identification of the genes involved in the initiation of fruiting can now be studied to understand this process at the molecular level and develop efficient and reproducible methods of cultivating and inducing fruit body development in valuable basidiomycetes species.

Several fruiting-specific or fruiting induction genes have been isolated in other basidiomycetes, such as *Agaricus bisporus* (De Groot et al. 1996), *Schizophyllum commune* (Mulder and Wessels 1986) and *Agrocybe aegerita* (Salvado and Labarère 1991). Some of these fungal genes belong to a family that encodes hydrophobic proteins called hydrophobins. In addition, two fruiting-specific or fruiting induction genes (*fvh1* and *Fv-hyd1*) encoding hydrophobins were isolated from a *F. velutipes* primordial complementary DNA (cDNA) library (Ando et al. 2001; Yamada et al. 2005).

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Hydrophobins are small, secretory, hydrophobic fungus-specific proteins composed of four loops of disulfide bonds among eight highly conserved cysteine residues that self-assemble into amphipathic layers as hydrophilic–hydrophobic interfaces (Ando et al. 2001; Yamada et al. 2005). Fungal strains usually contain several genes coding for hydrophobins, which play key roles in morphogenesis and fruit body development. Some of these hydrophobin genes are also highly expressed in fruiting bodies (Wösten and Wessels 1997). The aim of the present study was to further identify and characterize hydrophobin genes in *F. velutipes* by using our recently published whole-genome sequence information (Park et al. 2014). Herein, we describe 10 *F. velutipes* hydrophobin genes with deduced amino acid sequences and their regulation at the transcription level during fruiting body development. This comparative study of gene expression at various developmental stages and in different tissues provides valuable insights into the molecular basis of mushroom fruit body development.

Hydrophobin genes in the *F. velutipes* genome

The *F. velutipes* KACC43778 dikaryotic strain was obtained from the Korean Agricultural Culture Collection (KACC; Rural Development Administration, Korea; http://www.genebank.go.kr/) and grown at 26 °C on mushroom complete medium agar (0.2% peptone, 2% glucose, 0.2% yeast extract, 0.05% $MgSO_4$, 0.046% KH_2PO_4, 0.1% K_2HPO_4, and 1.5% agar) for 2 wk. For genomic DNA and total RNA isolation from mycelia, a 300-mL Erlenmeyer flask containing 50 mL mushroom complete medium was inoculated with fresh plugs from the plate (five mycelial plugs/flask) and incubated at 26 °C for 2 wk without agitation.

In our previous study, genome-wide identification of hydrophobin genes was carried out using several methods, including *ab initio* gene structure prediction (Fgenesh; http://www.softberry.com), a homology-based approach (Fgenesh+; http://www.softberry.com), and transcriptome-based gene identification (cufflinks; http://cufflinks.cbcb.umd.edu/manual.html) with the *F. velutipes* (KACC42780 monokaryotic strain) whole-genome sequence (AQHU00000000) (Park et al. 2014). In the present study, we performed additional gene prediction by using the AUGUSTUS tool (Stanke and Morgenstern 2005) with default parameters trained in *Coprinopsis cinerea*. The genes were compared by using BLAST (version 2.2.17) software with a series of protein databases, including the National Center for Biotechnology Information (NCBI) nucleotide (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and non-redundant (http://blast.ncbi.nlm.nih.gov/Blast.cgi) sets, for functional annotation of the predicted genes.

BLASTP searches within the NCBI-NR database of the amino acid sequences of *F. velutipes* genes, which were previously predicted by using combined approaches (Fgenesh, Fgenesh+, and cufflinks), showed that 12 of the predicted proteins shared sequence similarity with fungal hydrophobins (Park et al. 2014). However, gene prediction performed with the AUGUSTUS tool indicated that the total number of hydrophobin genes in *F. velutipes* was smaller (10 hydrophobin) than that predicted through combined approaches (Table 1). Nine putative hydrophobin genes were found in common among the genes predicted by both the combined approaches and the AUGUSTUS tool. Furthermore, one (*Hyd-10*) of these 10 putative genes was further identified and annotated as a hydrophobin gene in this study (Table 1).

cDNA fragments encompassing all of the putative open reading frames (ORFs) were amplified with reverse transcriptase (RT)-PCR and their sequences were refined. For cDNA synthesis, samples were ground to a fine powder under liquid nitrogen by using a mortar and pestle and stored at −80 °C. Total RNA was prepared from tissue samples (100 mg) with the TRIzol reagent (Invitrogen Life Technologies, USA) according to the manufacturer's instructions. Total RNA (10 μg) was treated for 30 min at 37 °C with 1 U of RQ1 RNase-free DNase (Promega, Madison, WI, USA). Reverse transcription of RNA (1 μg) was performed in a 20-μL reaction volume with oligo-dT18 and ImProm-II reverse transcriptase (Promega, Madison, WI, USA). The reactions were incubated at 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 10 min to inactivate the reverse transcriptase. cDNA was sequenced with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequences were analyzed on an ABI Prism 3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA), and sequence data were analyzed by using the Lasergene software (DNAStar Inc., Madison, WI, USA). The nucleotide and deduced amino acid sequences encoded by the hydrophobin genes were aligned by using the BioEdit program (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The GenBank accession numbers of the sequences reported herein are KT868833 (*Hyd-1*), KT868834 (*Hyd-2*), KT868835 (*Hyd-3*), KT868836 (*Hyd-4*), KT868837 (*Hyd-5*), KT868838 (*Hyd-6*), KT868839 (*Hyd-7*), KT868840 (*Hyd-8*), KT868841 (*Hyd-9*), and KT868842 (*Hyd-10*). Comparisons of the genomic and cDNA sequences of these genes revealed single ORFs of 363 bp, 315 bp, 315 bp, 366 bp, 336 bp, 345 bp, 336 bp, 384 bp, 393 bp, and 354 bp, respectively. In addition, comparisons of genomic DNA and cDNA showed that the genomic DNA of these genes had 3, 4, 4, 4, 4, 4, 4, 5, 5, and 4 introns, respectively, with an average intron size of 55 bp. All of the splicing sites followed the GT–AG rule (Supplementary Fig. S1).

The signal peptide prediction of amino acid sequences encoded by hydrophobin genes in *F. velutipes* was carried out by using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). N-glycosylation sites (Asn-Xaa-Ser/Thr) were identified with the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). Supplementary Fig. S2 shows the deduced amino acid sequences encoded by the 10 ORFs aligned with previously reported fungal hydrophobins (*POH2* from *Pleurotus ostreatus* [GenBank AJ225061], *HypB* from *A. bisporus* [GenBank Y15941], *Le-Hyd2* from *Lentinula edodes* [GenBank AF217808], *SC3* from *S. commune* [GenBank XM_003031216], *CoH1* from *C. cinerea* [GenBank XM_001834396], and *fvh1* from *F. velutipes* [GenBank AB026721]). Hydrophobins are composed of four loops, formed by disulfide bonds, among eight highly conserved cysteine residues (Ando et al. 2001; Yamada et al. 2005). Among the 13 putative hydrophobin genes identified in *F. velutipes*, 10 genes (*Hyd-1–10*) were determined to encode the fungal