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RESEARCH ARTICLE

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Gene Expression Analyses of Mutant *Flammulina velutipes* (Enokitake Mushroom) with Clogging Phenomenon

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ABSTRACT

Regulation of proper gene expression is important for cellular and organismal survival, maintenance, and growth. Abnormal gene expression, even for a single critical gene, can thwart cellular integrity and normal physiology to cause diseases, aging, and death. Therefore, gene expression profiling serves as a powerful tool to understand the pathology of diseases and to cure them. In this study, the difference in gene expression in *Flammulina velutipes* was compared between the wild type (WT) mushroom and the mutant one with clogging phenomenon. Differentially expressed transcripts were screened to identify the candidate genes responsible for the mutant phenotype using the DNA microarray analysis. A total of 88 genes including 60 upregulated and 28 downregulated genes were validated using the real-time quantitative PCR analysis. In addition, proteomic differences between the WT and mutant mushroom were analyzed using two–dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). Interestingly, the genes identified by these genomic and proteomic analyses were involved in stress response, translation, and energy/sugar metabolism, including HSP70, elongation factor 2, and pyruvate kinase. Together, our data suggest that the aberrant expression of these genes attributes to the mutant clogging phenotype. We propose that these genes can be targeted to foster normal growth in *F. velutipes*.

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Flammulina velutipes; clogging phenomenon; gene expression profiling

1. Introduction

Flammulina velutipes, also known as velvel shank, has several names, including *enokitake* in Japan, *golden needle mushroom* in China, *futu* in India, and *paengi beoseog* in Korea [1]. Because it is low in calories yet rich in savory flavors and nutrients, including essential amino acids, minerals, and polysaccharides, *F. velutipes* is one of the most popular edible mushrooms [2–4]. This popularity has led to an increasing demand and the artificial cultivation of *F. velutipes* in recent years [3]. Although wild *F. velutipes* grows on stumps of *Celtis sinensis*, commonly called Enoki, cultivated mushrooms are grown in a dark, carbon dioxide-rich environment. This peculiar environment induces the development of their long thin stems, which often cause overcrowding of mushrooms (referred to "mutants with clogging phenomenon" from this on). The overcrowdedness results in poor yields of *F. velutipes* [5,6]. One of important steps to overcome this problem in the artificial cultivation of *F. velutipes* is to identify the genes or mutations responsible for the clogging phenomenon.

Since the initiation of the Human Genome Project in 1990s, the genomes of diverse fungal species have been sequenced. Recently, the Joint Genome Institute commenced the 1000-fungal genome project, which focuses mainly on plant feedstock health, biorefinery, and fungal diversity [7,8]. Furthermore, with the ongoing development of omics analyses, high-throughput sequencing and proteomics analyses have been widely used [9,10]. Expressed sequence tags (ESTs), short sub-sequences of cDNA from a cDNA library, contain considerable genetic information, including specific developmental ages, tissues, and environmental conditions [11]. Using ESTs, precise probes can be designed for DNA microarrays, which can be used to analyze gene expression profiles [12]. Since Chang introduced the concept and methodology of microarrays

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in 1983 [13], as a multiplex lab-on-a-chip, DNA microarrays have been widely used to test large amounts of biological materials using high-throughput miniaturized, multiplexed, and parallel processing [14]. In addition, Wilkins invented proteomics [15], a powerful tool for examining differences in gene expression in the protein level [11,16]. For example, specific growth stages at which some proteins are expressed could indicate morphological changes, unique functions, or developmental requirements [17]. This approach is complementary to the DNA microarray experiment in that both of these techniques examine which genes are activated or repressed under certain conditions. Furthermore, the information provided by these experiments is invaluable for constructing three-dimensional protein maps and investigating the regulation of protein function [18].

For example, metabolic changes of *F. velutipes* mycelia in response to cold and light stresses were examined through proteomics approaches [19,20]. In these studies, proteome changes in *F. velutipes* using iTRAQ-coupled two-dimensional liquid chromatography-tandem mass spectrometry identified differentially expressed proteins upon cold or light stresses. The identified proteins were analyzed through Kyoto encyclopedia of genes and genoomes (KEGG) to identify the affected pathways under these conditions.

During the research for the enhanced production of artificially grown *F. velutipes*, the traits specific to the clogging phenomenon in the mushrooms have been identified [6,21,22]. Compared with wild type (WT) *F. velutipes*, mutant mushrooms exhibit changes in their fruiting body, featuring irregular lengths or shapes of the cap, forming colonies with a fluffy surface, and abnormal growth with less density and weight. Due to these unfavorable traits, the mutant mushroom apparently fails to grow appropriately. To prevent and cure this mutation, identifying the genetic problems underlying the clogging phenomenon is necessary [21]. Therefore, we performed the gene expression profiling and identified differentially expressed genes (DEGs), comparing the WT and mutant mushrooms in this study. We believe that our genomic and proteomic analyses identified the genes involved in growth and fruiting body formation.

2. Materials and methods

2.1. Sample collection

We obtained the mycelia of WT and mutant *F. velutipes* from Green-Peace Mushroom Farm (Cheongdo, Republic of Korea). The samples were cultivated in potato dextrose broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 25 °C for 15 d. After cultivation, the clogging phenomenon was observed in the mycelia of mutant *F. velutipes*.

2.2. Total RNA isolation

To isolate total RNA from mycelium and fruiting body of *F. velutipes*, RNeasy Maxi kit (Qiagen, Valencia, CA, USA) was used. All isolated mycelium and fruiting body samples were freeze–dried to facilitate RNA isolation results due to large amounts of sugars [4,22]. One gram of mycelium and fruiting body tissues of *F. velutipes* were grinded with liquid nitrogen. The samples were dissolved in the buffer with 2-mercaptoenthanol and grinded by homogenizer (Daihan, Gangwon, Republic of Korea). Then, samples were centrifuged at 3000 *g* for 10 min at 15 degree and the supernatant was separated. Remaining pellets were mixed with 70% ethanol and the mixture was centrifugated at 3000 *g* for 5 min at 15 degree. After washing twice, the total RNA was eluted.

2.3. Array hybridization and data analysis

Total RNAs were mixed with Bacterial alkaline phosphatase (TakaRa, Tokyo, Japan) and Tabbaco acid pyrophosphatase (TakaRa). Then, synthetic oligomer that specifically reacts to uncleaved mRNA having phosphate group was added. From the total RNA mixture, mRNA was isolated using Oligotex mRNA purification kit (Qiagen, Valencia, CA) [23,24]. cDNA was synthesized from the isolated mRNA by SuperScript double–stranded cDNA synthesis kit (Invitrogen, Waltham, MA) and amplified by XL PCR kit (PerkinElmer, Waltham, MA). cDNA from mycelium was labeled with Cy3 and cDNA from fruiting body was labeled with Cy5 by 3 DNA Array 50 kit (Genisphere, Hatfield, PA). The labeled genes were hybridized to NimbleGen expression customized arrays that had 135,000 probes (12 probes for each gene) for 10,405 target genes, with a 60 mer probe length and feature size of $13 \times 13\,\mu$l. The first hybridization was performed for 16 h and second hybridization was followed after washing and fixation. After hybridization, the sample was centrifuged at 1000 *g* for 3 min. Results of the array were scanned by Scan–array 5000 microarray scanner (Packard BioScience, Meriden, CT) at 532 nm for Cy3 and 635 nm for Cy5. Gene PIX program (Axon Instruments, Burlingame, CA) was used to analyze the scanned images and normalized. The results were analyzed by Significance analysis of microarray (SAM) and genes with increased or decreased expression levels were identified.

2.4. Real-time PCR

Based on the microarray results, DEGs in mutant mushrooms, either upregulated or downregulated, were validated in the mycelia of *F. velutipes*. Primers for real-time PCR were designed based on these genes using Primer version 3 (Whitehead Institute for Biomedical Science) [25,26]. The sequences of primers are listed in Supplementary Data 1 and 2. For the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. The GAPDH forward primer was 5′-GCCGTCAACAACAA CATCATTCCTT-3′, and the GAPDH reverse primer was 5′-CGGAAACATCGAGGGTAGGGACA-3′. A Takara real-time PCR system was used (Takara, Otsu, Japan). Cycling conditions were as follows: initial denaturation at 95 degree for 10 min followed by 40 cycles of denaturation at 95 degree for 10 s, and annealing and elongation at 60 degree for 15 s.

2.5. Protein sample preparation

The mycelia of WT and mutant *F. velutipes* were washed twice in ice-cold phosphate-buffered saline. The washed samples were then homogenized with liquid nitrogen. The homogenized powder was lysed in the sample buffer containing 7 M urea, 1 mM benzamidine, and 2 M thiourea with 4% (w/v) 3-[(3-cholamidopropy) dimethyammonio]-1-propane-sulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), and 2% (v/v) pharmalyte [27]. Samples were vortexed for 1 h at room temperature to extract the proteins. After centrifugation at 15,000 *g* for 1 h at 15 degree, the insoluble material was discarded, and the soluble fraction was used for two–dimensional gel electrophoresis. Protein loading was normalized, based on the result from the Bradford assay [28].

2.6. 2D-PAGE

To separate the proteins by isoelectric point and molecular weight, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to identify differentially expressed proteins [29]. IPG dry strips were equilibrated for 12–16 h with 7 M urea, loaded with 200 μg of sample and 2 M thiourea containing 2% CHAPS, 1% DTT, and 1% pharmalyte. Isoelectric focusing (IEF) was performed at 20 degree using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences, Amersham, UK), according to the manufacturer's instructions [30]. For IEF, the voltage was linearly increased from 150 to 3500 V over 3 h for sample entry, followed by a constant 3500 V, focusing completely after 96 kVh. Before the second dimension, strips were incubated for 10 min in an equilibration buffer (50 mM Tris–Cl, pH 6.8, containing 6 M urea, 2% sodium dodecyl sulfate [SDS], and 30% glycerol), first with 1% DTT and then with 2.5% iodoacetamide. Then, equilibrated trips were inserted in SDS–PAGE gels (20–24 cm, 10–16%). SDS–PAGE was performed using the Hoefer DALT 2 D system (Amersham Biosciences), following the manufacturer's instructions. The 2 D gels were run at 20 degree for 1.7 kVh. The 2D gels were silver stained as described by Oakley et al. [31,32].

2.7. Image analysis

Quantitative analysis of digitized images was performed using PDQuest version 7.0 (Bio-Rad, Hercules, CA) according to the protocol provided by the manufacturer. The total valid spot intensity was normalized for the quantity of each spot. Protein spots were selected based on significant expression variation greater than two-fold compared with the WT samples [33].

2.8. In-gel digestion and mass spectrometry

Protein spots were enzymatically digested in-gel as previously described by Shevchenko et al., using modified porcine trypsin [34]. Gel pieces were washed with 50% acetonitrile to remove SDS. They were then salted and stained, and dried to remove the solvent. This was followed by rehydration with trypsin (8–10 ng/μl) and incubation for 8–10 h at 37 degree. The proteolytic reaction was terminated by adding 5 μl of 0.5% trifluoroacetic acid [35]. Tryptic peptides were recovered by combining the aqueous phase from several extractions of gel pieces with 50% aqueous acetonitrile. Next, the peptide mixture was desalted using C18ZipTips (Millipore, Billerica, MA) and acetonitrile. Finally, an aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile and the mixture was spotted onto a target plate [35]. Protein analyses were performed using Ettan matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) (Amersham Biosciences). First, peptides were evaporated with an N_2 laser at 337 nm using a delayed extraction approach. Then, they were accelerated with 20 Kv injection pulse for the time of flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by the Rockefeller University (http://129.85.19.192/profound_bin/ WebProFound.exe), was used for protein identification by peptide mass fingerprinting. Spectra were calibrated with trypsin auto-digestion ion peak m/z (842.510, 2211.1046) as internal standards [36–38].

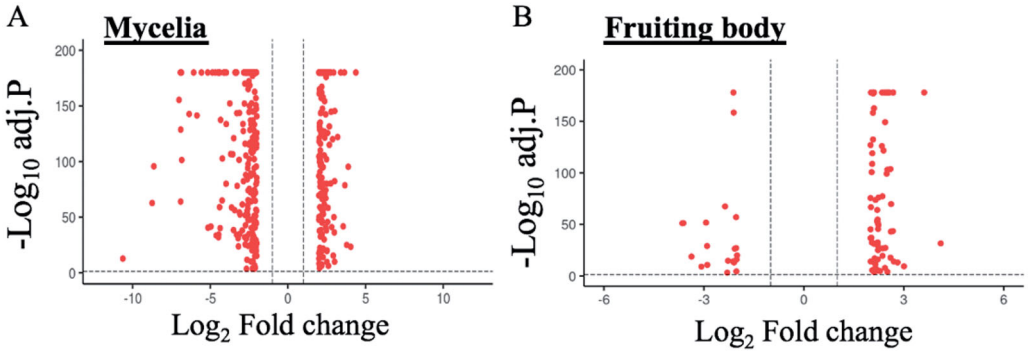

Figure 1. Differentially expressed genes in the mycelia and fruiting body. (A) Scatter plots of the DEGs in the mycelia, comparing the WT and mutant. $N = 371$ (upregulated, 156; downregulated, 215), $\log_2FC > 2$, p value < 0.05; (B) Scatter plots of the DEGs in the fruiting body, comparing the WT and mutant. $N = 161$ (upregulated, 135; downregulated, 26), $\log_2FC > 2$, p value < 0.05.

2.9. Visualization methods of DEGs

The DEGs that were up or down-regulated from mycelia and fruiting bodies were compared and visualize in Venn diagram. Scattered plots of DEGs in mycelia or fruiting bodies were visualized using "EnhancedVolcano" package version 1.10.0 (https://bioconductor.org/packages/release/bioc/html/EnhancedVolcano.html) within R program version 4.1.1 (The R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. RNA profiling of WT vs. mutant F. velutipes

To identify DEGs in clogging mutant *F. velutipes*, DNA microarray was performed, comparing WT and mutant mushrooms. The results showed that a total of 371 genes were differential expressed ($\log_2$ fold change, FC > 2; $p < 0.05$) from the mycelia of the mutant mushroom, including 156 upregulated genes and 215 downregulated genes respectively (Figure 1(A); Supplementary Data 1). In addition, a total of 161 DEGs were identified in the fruiting bodies (FC > 2; $p < 0.05$): 135 upregulated genes and 26 downregulated genes (Figure 1(B); Supplementary Data 1). The DEGs from mycelia and fruiting bodies were compared and visualized in a venn diagram (Figure 2; Table 1).

Since more genes were identified in the mycelia than in the fruiting bodies, DEGs with significant fold changes in the myclia were selected and validated by real-time PCR. Among the upregulated genes, the gene with FC > 2 were analyzed (Supplementary Table 1; Supplementary Data 2). We also validated the downregulated genes with FC< -2 (Supplementary Table 2; Supplementary Data 2). The data showed that 6 genes including cytochrome p450 and hydrophobin were differentially expressed between WT and mutant *F. velutipes*, consistently to the DNA microarray results (Supplementary Data 1; Table 2). Other four genes

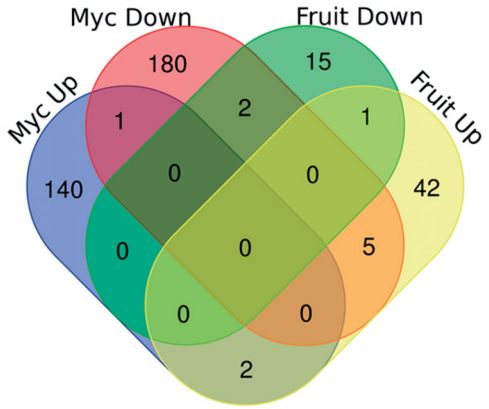

Figure 2. Summary of DNA microarray. Venn diagram summarizing DEGs identified through the DNA microarray analysis.

were selected from genes identified as being downregulated in the mutant [39,40]. The representative ones and their differential expressions are summarized in Table 2. It is noted that cytochrome p450 and hydrophobin have been reported to play important roles in both the growth and development of fruits [41]. Therefore, our mRNA analysis results suggest that cytochrome p450 and hydrophobin could be implicated with the clogging phenomenon in *F. velutipes*.

3.2. Protein profiling of WT vs. mutant F. velutipes

Total proteins were collected from both WT and mutant *F. velutipes* and analyzed using 2 D-PAGE. Representative gels of 2D-PAGE were shown in Supplementary Figure 1. After gel electrophoresis, gel images were obtained, and image analysis was performed using the PDQuest image analysis software. The first 2D-gel included 1313 and 1008 spots, the second 2D-gel 1764 and 1789 protein spots, and the third 2D-gel 1211 and 1345 from normal and clogging mutant mycelium mushrooms (Supplementary Figure 1; Figure 3). Different protein spots between the WT and mutant samples

Table 1. Commonly expressed DEGs in Mycelia (Myc) or fruiting bodies (Fruit).

Gene expression	Gene ID	Annotation
Fruit up *vs.* Myc up	EIW64561	ATP-dependent DNA helicase
	ADX07302	Putative peroxiredoxin Q
Fruit down *vs.* Myc down	XP_001876254	Aspartic peptidase A1
	EFY95978	Metal dependent phosphohydrolase
Fruit up *vs.* Myc down	XP_002340131	G-protein beta WD-40 repeats containing protein
	ADX07315	Putative peroxysomal citrate synthase
	CCA74312	Related to secreted protein-Streptomyces sviceus
	BAK09406	Cytochrome P450
	ADX07333	Putative hydrophobin

Table 2. Differentially expressed genes validated by real-time PCR.

Gene name	Primers	Phenotype	Relative expression
Cytochrome p450	Forward: TTGAAAAGGTCGAGGGAGGTGATG	WT	1
	Reverse: TGAGGGATATTGGGGGAAGATGGT	Mutant	0.3404972
Hydrophobin	Forward: CCGCTGGCCAGTGCAATGTTA	WT	1
	Reverse: GATGACCGTGATGGGAGAGCAATC	Mutant	0.52931
C4-dicarboxylate transporter/malic acid transport protein	Forward: TCTGGGCTATTGGGCATTGACAT	WT	1
	Reverse: TGAGTATAAGCCCCCGCCAGAA	Mutant	0.402826
Glycerate-and formate-dehydrogenase	Forward: ATCGCCTCCGCCGTAGAAAAG	WT	1
	Reverse: TGACGAGGGGCCCGAGTAGTT	Mutant	0.699457
Malate dehydrogenase	Forward: CTGGTGCCGTCGTCCTCAGTG	WT	1
	Reverse: ATCCCGCGCCGAAGAATAGAATA	Mutant	0.728301
Endo-beta-1,3-glucanase	Forward: CCGGATGATCAATGCCTGTGGT	WT	1
	Reverse: GAAAGCGGGGAATCGGTTATGAG	Mutant	0.4896595

were screened and measured for their intensity. Most protein spots were concentrated between 10 and 96 kDa, with pI ranging from pH 6 – 9 (Figure 3). A total of 79 spots showed significantly increased or decreased protein expression and these were selected for mass spectrometry (MS) analysis (Supplementary Table 3).

3.3. Identification of differentially expressed proteins by mass spectrometry

The differential protein spots from the 2D-PAGE analyses were subjected to MALDI-TOF MS analysis, and the resulting tandem MS/MS scans with the protein spots were searched against the NCBInr Fungi database using the Mascot Server. Although some preoteins have not been annotate, the results were determined using a cutoff value of $p < 0.05$ and categorized into upregulated and downregulated protein groups. Among the upregulated proteins in mutant mushrooms, 14 protein spots were identified, including heat shock protein 70 and 90 kDa, glyceraldehyde-3-phosphate dehydrogenase, phosphopyruvate hydratase, transaldolase, 20S proteasome subunit, actin and enolase (Table 3). We identified three proteins in the downregulated protein samples, including HSP70-domain-containing protein, elongation factor 2, and pyruvate kinase (Table 4).

4. Discussion

This study compared WT and mutant *F. velutipes* for gene expression using genomics and proteomics analyses. The DNA microarray and real-time PCR analyses

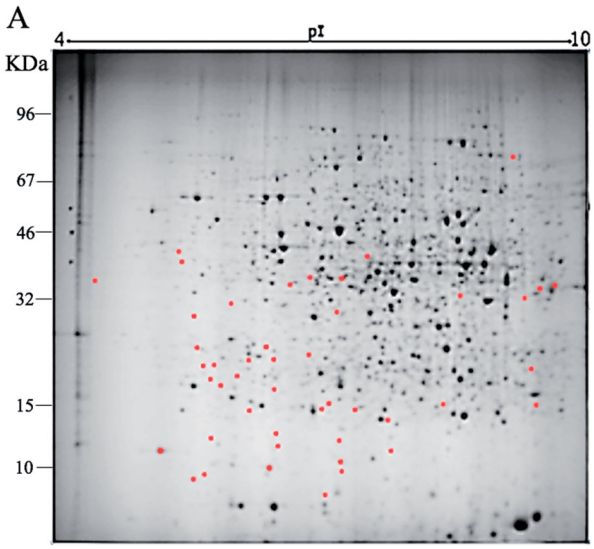

Figure 3. *In silico* identification of differentially expressed genes. (A) 2D-PAGE image showing proteins that are differentially expressed in the mycelia of mutant mushroom and were subjected to MALDI-MS-MS analysis. Marked with red dots ($n = 79$). Significant expression variation > 2.

showed that cytochrome p450 and hydrophobin were among the most significantly downregulated genes in mutant *F. velutipes* than in the WT (Table 1; Supplementary Table 2; Supplementary Data 1 and 2). As these two proteins participate in the construction of mushroom fruiting bodies [40], deregulation of these two proteins in the mutant mushrooms might lead to a poor quality of fruiting bodies. The expression of other genes such as C4-dicarboxylate transporter/malic acid transport protein, glycerate- and formate-dehydrogenase, malate dehydrogenase, and endo-beta-1,3-glucanase was also decreased in mutant *F. velutipes*. These genes suggest that altered energy and glucose

Table 3. Identification of upregulated proteins between the WT and mutant *Flammulina velutipes* by MALDI-TOF mass spectrometry.

SSP	M/N	Identification	Species similarity	Accession
0111R	5.7371	Uncharacterized protein SCHCODRAFT_75321	*Schizophyllum commune* H4-8	XP_003034427
0113R	4.959	HS70_TRIRU Heat shock 70 kDa protein	*Aspergillusnidulans* FGSCA4	XP_662733
8112R	3.2005	Glyceraldehyde-3-phosphate dehydrogenase	*Flammulinavelutipes*	AAQ08201
0513R	2.6972	Heat shock protein 70 kDa, partial	*Cladosporiumcladosporioides*	ACZ95780
0112R	2.106	rab GDP-dissociation inhibitor	*Trametesversicolor* FP-101664 SS1	EIW63882
1011R	2.0033	Heat shock 70-like protein	*Coccidioidesimmitis* RS	XP_001248723
3007R	1.8245	Predicted protein	*Laccaria bicolor* S238N-H82	XP_001877157
0011R	1.7949	Beta-tubulin	*Suillusbovinus*	AAM49060
0503R	1.6934	Heat shock protein 90-like protein	*Trametesversicolor* FP-101664 SS1	EIW51690
5001R	1.6162	Glyceraldehyde-3-phosphate dehydrogenase	*Flammulinavelutipes*	AAQ08201
1114R	1.5906	Actin 1	*Dichomitussqualens* LYAD-421 SS1	EJF55774
0507R	1.5366	Predicted protein	*Laccaria bicolor* S238N-H82	XP_001874825
3406R	1.5282	Phosphopyruvatehydratase	*Laccaria bicolor* S238N-H82	XP_001874138
5513R	1.44976	Mov34-domain-containing protein	*Fomitiporiamediterranea* MF3/22	EJD05421

Table 4. Identification of downregulated proteins by MALDI-TOF mass spectrometry.

SSP	M/N	Identification	Species similarity	accession
2906R	0.33	HSP70-domain-containing protein	*Coniophoraputeana* RWD-64-598 SS2	EIW83554
6804R	0.30	Elongation factor 2	*Schizosaccharomyces pombe*	BAA23590
8703R	0.33	Pyruvate kinase	*Trametesversicolor* FP-101664 SS1	EIW64358

metabolisms might be responsible for causing the clogging phenomenon in the mutant mushrooms.

In MALDI-TOF and bioinformatics analyses, unexpectedly, the DEGs identified through transcriptome analysis data were not detected. It is thought that the proteins for these genes have not been sequence-determined, small, or could be difficult to be solubilized for separation. For example, hydrophobin is about 10 kDa and highly hydrophobic, appearing difficult to be solubilized and MS-determined. Malic acid transport protein is located in the mitochondria membrane that is also proposedly less soluble. Instead, we identified 14 upregulated and 3 downregulated proteins in the mutant mushroom (Tables 3 and 4). Heat shock proteins were the most prominent among the upregulated proteins: homologous to heat shock protein 70 kDa (HS70) from *Aspergillus nidulans* FGSCA4, *Cladosporium cladosporioides*, and *Coccidioides immitis* RS; and heat shock protein 90 kDa (HS90) from *Trametes versicolor* FP-101664 SS1 (Table 3). Because heat shock protein expression is promoted in various stressful conditions, such as heat shock, cold, UV light, infection, inflammation, exercise, exposure of the cell to toxins, starvation, hypoxia, nitrogen deficiency, and dehydration, high levels of heat shock proteins could be evidence of more challenging mycelium environments or ineffective management/resolution of cellular stresses in the mutant *F. velutipes* with the clogging phenomenon than in the WT mushrooms [42]. In addition, actin and tubulin proteins are upregulated in the mutant mushroom (Table 3), suggesting that the over-production of skeletal proteins might be important for the clogging phenomenon. Another interesting protein, elongation factor 2, is an essential factor for protein synthesis. It promotes the GTP-dependent translocation of ribosomes in eukaryotes [43]. Pyruvate kinase also participates in translation, being involved in the last step of glycolysis, which produces ATP and pyruvate [44]. These proteins, elongation factor 2, pyruvate kinase, and HSP70-domain-containing proteins were identified to be downregulated in mutant mushrooms compared with the WT (Table 4). This suggests that the mutant mushrooms could have impaired translation and carbohydrate metabolism.

In this study, we suggest several deregulated genes in the clogging phenomenon of mushrooms, including cytochrome p450, hydrophobin, pyruvate kinase, and elongation factor 2 to be downregulated and heat-shock proteins and skeletal proteins to be increased in the clogging phenomenon of mushrooms. We suggest that the clogging phenomenon attributes to the deregulation of these genes in *F. velutipes*. Therefore, it might be possible to cure the clogging disease by restoring these genes for proper fruiting body formation in *F. velutipes*.

Ethical standards

Not applicable.

Disclosure statement

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

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