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Analysis of Gene Expression Profiles during cultivation of *Grifola frondosa*

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Abstract

The edible mushroom *Grifola frondosa* (Maitake mushroom) is mass-produced industrially, yet little is known about the molecular mechanism of fruiting body development. Study of this mechanism is necessary to improve breeding efficiency and the cultivation process. Therefore, we sequenced the transcriptome of *G. frondosa* by using pooled RNA from 13 different stages. In total, 26,893 contigs were assembled and 10,150 open reading frames were predicted from 101 Mb of sequence. Subsequently, gene expression analyses of the 8 stages of fruiting body development of *G. frondosa* were performed using a microarray. We found that 56 genes were regulated by induction of primordia development and 187 genes were regulated by induction of fruiting body differentiation. This study provides information not only about fruiting of *G. frondosa* but also the mechanisms of fruiting body development common to basidiomycetes.

Key words : *Grifola frondosa*; transcriptome; second-generation sequencing; primordia development; fruiting body differentiation

(1) Introduction

Grifola frondosa (Maitake mushroom) is one of the cultivated edible basidiomycete mushrooms of economic significance. Some of the constituent parts of *G. frondosa* have been reported to have various potential antitumor, antiviral, antidiabetic, hematopoietic, and neurotrophic biological properties[1)-5)]. Molecular biological information is necessary not only to expand knowledge of medical and nutritional properties and of the mechanism of fruiting body development, but also to improve cultivation for mushroom production.

Research on other basidiomycete mushrooms has revealed related genes controlling fruiting body development in *Agaricus bisporus* (common mushroom)[6)-9)], *Coprinopsis cinerea*[10)-11)], *Flammulina velutipes* (winter mushroom)[12)-14)], *Laccaria bicolor*[15)], *Lentinula edodes* (Shiitake mushroom)[16)-19)], *Pleurotus ostreatus* (Oyster mushroom)[20),21)], and *Schizophyllum commune*[22),23)]. In addition, genome sequences of *A. bisporus, C. cinerea*[24)], *L. bicolor*[15)], *P. ostreatus, and S. commune*[25)] have been released by the Fungal Genomics Program of the Joint Genome Institute (http://www.jgi.doe.gov/). However, because the molecular mechanisms of fruiting in the aforementioned mushrooms are not identical, it is possible that *G. frondosa* has a unique mechanism of fruiting body development. It is therefore important to study the molecular biology of *G. frondosa* as a representative of various other commercial mushrooms to reveal the mechanisms of fruiting body development common to basidiomycetes.

In this study, we used the Roche 454 FLX system for mRNA sequencing, then using the data obtained, we created a microarray for gene expression analysis. Sequence data were generated because the

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Abbreviations: GO, Gene Ontology; qRT-PCR, quantitative real-time PCR

genome sequences of non-model mushrooms, including *G. frondosa*, were not yet available and a small number of randomly selected cDNAs cannot properly represent their respective transcriptome. In the past few years, with the advent of the second-generation sequencer, the cost of mRNA sequencing has been dramatically lowered, and transcriptome analysis is now possible to perform even in non-model organisms[26)-28)]. '

Transcriptome profiling of genes expressed during fruiting body development in *G. frondosa* are presented here for the first time.

(2) Material and Method

Strains and culture conditions. The *G. frondosa* strain used in this study was M51. *G. frondosa* was inoculated on sterilized sawdust medium (30% sawdust, 5% corn bran, 65% water) contained in plastic bags and was cultured in the dark for 75 days at 25℃. Thee culture was then incubated for 7 days at 16℃ and 95% humidity under a white fluorescent lamp with a 12 h dark/12 h light cycle. After the incubation, the fruiting body was induced by opening of the top of plastic bag; mature fruiting bodies were formed after 10 days.

454 sequencing and assembly. Total RNAs were prepared from 13 different developmental stages, and the total RNAs from all stages were pooled in equal amounts for each sequencing sample. The vegetative mycelia and fruiting bodies from each stage were freshly harvested and then frozen immediately under liquid nitrogen, and kept at -80℃ until RNA preparation. These samples were pulverized using mortar and pestle under liquid nitrogen, and total RNA was prepared using an RNeasy Plant Mini Kit (Qiagen, Frankfurt, Germany), according to the manufacturer's instructions. Quantity and quality of total RNA was analyzed using a spectrophotometer (Thermo Fisher Scientific, MA) and 2100 Bioanalyzer (Agilent Technologies, CA). Quality passed cDNA was synthesized using a SMART™ PCR cDNA Synthesis Kit (Clontech Laboratories, CA). Each cDNA sample was purified using Qiagen QIAquick PCR purification spin columns. Normalization was performed using the TRIMMER cDNA normalization kit (Evrogen, Moscow, Russia). Normalized, double-stranded cDNA was used for 454 sequencing (Roche, USA). Reads were then assembled using Newbler assembler ver. 1.1. (including custom service; Genaris, Inc., Yokohama, Japan)

Annotation. Assembled contigs were annotated against the non-redundant protein database using the BLASTx program (NCBI). BLASTx searches were performed with an e-value $\leq 1 \times 10^{-3}$ and a bit score >40. Then assembled contigs were annotated with Gene Ontology (GO) terms, Enzyme Commission (EC) number, and protein motifs by Blast2GO V2.4.0[29)]. Ontologies were categorized with respect to molecular function, biological process, and cellular component.

Microarray experiment. A custom 8 x 15K microarray (Agilent Technologies) containing 10,150 probes representing 10,150 predicted open reading frames (ORFs) of *G. frondosa* were designed using the eArray service (Agilent Technologies). Total RNAs were prepared from 8 different developmental stages over the 90-day cultivation period: at 20 days (early spawn run) and 74 days (late spawn run) after inoculation (abbreviated as S20 and S74); at 1, 3, and 6 days (primordia) after the first induction (P1, P3, and P6); and at 1 day, 3 days, and 8 days (fruiting body differentiation) after the second induction (Fig. 1). Following cRNA amplification and labeling, hybridization was performed using a Quick Amp Labeling Kit and Gene Expression Hybridization Kit (Agilent Technologies). In total, 36 hybridizations (8 conditions x four biological replicates per condition) were performed. Microarrays were scanned with a DNA Microarray Scanner (Agilent Technologies). The data were analyzed using GeneSpring GX 10 (Agilent Technologies) (including custom service; Bio Matrix Research Inc., Chiba, Japan).

(3) Results and Discussion

454 sequencing

Roche 454 generated 514,000 reads from *G. frondosa* and these reads were assembled into 26,893 contigs (Table 1). Average lengths of contig were 932 bp and the maximum lengths were 3.64 kbp. From all of the assembled contigs, 10,150 ORFs were predicted. *G. frondosa* 10,210 (38%) has significant BLAST matches in each all number of contigs. The

Table 1. Summary of sequencing results in transcripotme

Number of Reads	514,000
Number of Nucleotides (Mb)	101
Number of Contigs	26,893
Number of Large Contigs (≥0.5 kb)	6,839
Mean Length of Contig (kb)	0.89
Maximum Length of Contig (kb)	3.64

contigs to which GO terms were assigned were fewer, *G. frondosa* 5,975 (22%). These results show that knowledge of molecular biology in the mushroom is still insufficient.

Global analysis of expressed genes in G. frondosa cultivation.

We performed microarray experiment to screen for genes related to the cultivation process for *G. frondosa*. Generally, fruiting bodies are induced by physical stimuli and environmental changes. The cultivation process for *G. frondosa* occurs in three parts separated by two induction periods: spawn run, primordia development, and fruiting body differentiation (Fig. 1). The first induction consists of a decrease in temperature to induce primordia development. The second induction that stimulates fruiting body differentiation consists of increasing the ambient humidity and opening of the top of plastic culture bag. These two inductions are both necessary steps in the *G. frondosa* cultivation process. Most gene expression studies in other mushrooms were performed with very few time-series analysis of fruiting body developmental stages, which has produced the irregular results. Therefore, we analyzed with microarrays all genes expressed in all 8 stages of the *G. frondosa* cultivation process, especially focusing on the stages before and after each induction.

Genes involved in spawn run.

Spawn run is a necessary step in mushroom production because prior to fruiting body development, sufficient mycelium must be formed. We searched for genes involved in the spawn running stage and identified 99 highly expressed genes in this stage but not in the primordia developmental stage or fruiting body differentiation stage (Table 2). Manganese peroxidase (gfr01g3527) and laccase (gfr01g8219), which are commonly classified as FOLymes30), degrade lignin. Xing et al. reported that laccase activity attained maximum level during the spawn running stage[31], a result consistent with gene expression data obtained in the present study. Some esterases (gfr01g2331, gfr01g0801, gfr01g3009, gfr01g259), glycoside hydrolase family 3 (gfr01g4454, gfr01g0540, gfr01g7279), glycoside hydrolase family 5 (gfr01g3920, gfr01g1533, gfr01g6485), glycoside hydrolase family 10 (gfr01g4198), glycoside hydrolase family 28 (gfr01g1143, gfr01g0048), which are classified as carbohydrate-active enzymes (CAZymes), contribute to degradation of polysaccharides such as hemicelluloses, cellulose, and pectin. *G. frondosa* is a white rot fungus, which is able to degrade woody substrates in sawdust medium. These fungal oxidative lignin enzymes (FOLymes) and CAZymes are important in processes to supply carbon sources for vegetative mycelium growth. For the same reason, proteases such as carboxypeptidase (gfr01g7506) may play a role in the supply of nitrogen.

The gfr01g9225 was found to encode a hydrophobin (HGFI) specifically expressed in mycelium growth. Hydrophobins are small proteins that are expressed only by filamentous fungi and are related to the morphology of mycelium and fruiting

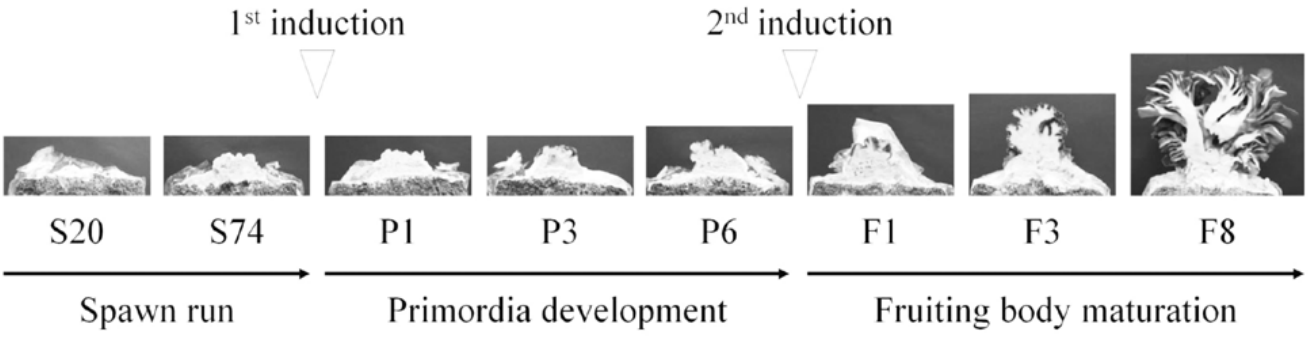

Fig. 1. Analysis Points for Microarray Experiment in Cultivation Process of *Grifola frondosa*.

body through hydrophobic interactions[32]. In *L. edodes*, the Le.hyd 2 hydrophobin was also specifically expressed during mycelium growth[33]. Le.hyd 2 might allow the dikaryotic mycelia to attach to the hydrophobic surface of the substrate. HGFI is a class I hydrophobin that not only fulfils a function in the mycelium development, but also in the expansion process of the new mycelium[34),35]. Another *G. frondosa* novel hydrophobin gfr 01 g 9225 might perform the same function.

Thus, genes highly expressed during the spawn running stage were observed that were related to the degradation of woody high molecular weight substrates and vegetative mycelium growth. These findings are useful for the study of the culture medium, conditions, and timespans in mushroom production. In addition, information related to FOLymes and CAZymes is also useful to improve the efficiency of production of bio-energy, such as bio-ethanol, from woody biomass.

Table 2. Highly expressed genes in spawn run

No.	Gene ID	Putative Gene Products	Organism	E-Value	Similarity
1	gfr01g7272	ycac protein	*Tuber melanosporum*	5.64E-83	82
2	gfr01g3009	fungal cellulose binding domain	*Coprinopsis cinerea*	9.75E-75	72
3	gfr01g3544	unknown	*Coprinopsis cinerea*	1.14E-19	79
4	gfr01g9225	hydrophobin	*Phlebiopsis gigantea*	1.13E-22	75
5	gfr01g1447	salicylate hydroxylase	*Postia placenta*	9.42E-43	80
6	gfr01g8148	voltage-gated potassium channel beta-2 subunit	*Coprinopsis cinerea*	3.23E-103	87
7	gfr01g1533	glycoside hydrolase family 5 protein	*Postia placenta*	1.83E-49	84
8	gfr01g2377	glycoside hydrolase family 13 protein	*Schizophyllum commune*	1.76E-20	75
9	gfr01g4198	endo-1,4 -beta-xylanase	*Postia placenta*	1.69E-47	79
10	gfr01g1296	unknown	*Postia placenta*	3.18E-110	82
11	gfr01g1847	unknown	*Laccaria bicolor*	2.31E-78	74
12	gfr01g1732	alpha-l-arabinofuranosidase domain protein	*Leucoagaricus gongylophorus*	1.30E-68	59
13	gfr01g8456	unknown	-	-	-
14	gfr01g2670	kynurenine alpha-aminoadipate aminotransferase	*Coprinopsis cinerea*	3.24E-38	69
15	gfr01g9637	glycoside hydrolase family 18 protein	*Postia placenta*	1.15E-44	79
16	gfr01g6668	manganese superoxide dismutase	*Phanerochaete chrysosporium*	1.41E-100	94
17	gfr01g7506	carboxypeptidase cpds	*Schizophyllum commune*	2.43E-71	72
18	gfr01g1885	glycoside hydrolase family 78 protein	*Postia placenta*	9.32E-14	70
19	gfr01g1900	histidine acid phosphatase	*Postia placenta*	3.01E-37	73
20	gfr01g8219	laccase	*Trametes versicolor*	1.02E-55	80
21	gfr01g9003	unknown	-	-	-
22	gfr01g6839	unknown	*Laccaria bicolor*	8.46E-15	95
23	gfr01g2762	class i alpha-mannosidase 1a	*Postia placenta*	7.20E-38	81
24	gfr01g5308	unknown	-	-	-
25	gfr01g5990	unknown	-	-	-
26	gfr01g4543	pq loop repeat protein	*Moniliophthora perniciosa*	3.13E-17	86
27	gfr01g3488	unknown	*Postia placenta*	3.06E-30	81
28	gfr01g5564	unknown	*Postia placenta*	2.44E-30	82
29	gfr01g8426	acetoin dehydrogenase	*Postia placenta*	2.49E-22	70
30	gfr01g9662	unknown	-	-	-
31	gfr01g5485	unknown	-	-	-
32	gfr01g2811	unknown	-	-	-
33	gfr01g3527	manganese peroxidase	*Ceriporiopsis rivulosa*	3.84E-15	95
34	gfr01g2153	glycosyltransferase family 2 protein	*Laccaria bicolor*	2.98E-15	89
35	gfr01g1355	unknown	-	-	-
36	gfr01g3920	cellulase	*Postia placenta*	1.60E-13	80
37	gfr01g5772	2,4-dichlorophenol 6-monooxygenase	*Penicillium marneffei*	8.23E-10	51
38	gfr01g1981	unknown	*Postia placenta*	5.62E-14	85
39	gfr01g7012	acyl- oxidase	*Aspergillus oryzae*	1.60E-05	63

40	gfr01g0152	unknown	-	-	-
41	gfr01g3530	unknown	-	-	-
42	gfr01g6797	unknown	*Moniliophthora perniciosa*	2.37E-04	53
43	gfr01g0785	family methyltransferase	*Streptomyces hygroscopicus*	4.09E-41	75
44	gfr01g9915	unknown	-	-	-
45	gfr01g1439	unknown	*Postia placenta*	2.16E-18	67
46	gfr01g9623	unknown	*Postia placenta*	7.63E-12	75
47	gfr01g0048	pectin methylesterase	*Sclerotinia sclerotiorum*	6.35E-34	68
48	gfr01g7925	small heat shock protein	*Coprinopsis cinerea*	3.24E-38	68
49	gfr01g5549	unknown	-	-	-
50	gfr01g4137	unknown	-	-	-
51	gfr01g7982	unknown	-	-	-
52	gfr01g7819	unknown	*Laccaria bicolor*	7.93E-21	70
53	gfr01g9895	short-chain dehydrogenase reductase sdr	*Brevundimonas sp.*	5.08E-07	65
54	gfr01g6485	glycoside hydrolase family 5 protein	*Postia placenta*	8.44E-71	78
55	gfr01g7279	glycoside hydrolase family 3 protein	*Phanerochaete chrysosporium*	8.89E-47	84
56	gfr01g5611	unknown	*Postia placenta*	4.67E-13	59
57	gfr01g3551	alcohol dehydrogenase	*Postia placenta*	3.15E-74	86
58	gfr01g3345	unknown	*Moniliophthora perniciosa*	4.08E-14	71
59	gfr01g2886	unknown	-	-	-
60	gfr01g10064	unknown	-	-	-
61	gfr01g4454	glycoside hydrolase family 3 protein	*Moniliophthora perniciosa*	7.50E-40	63
62	gfr01g8961	unknown	*Postia placenta*	2.23E-79	75
63	gfr01g0782	ketol-acid reductoisomerase	*Schizophyllum commune*	2.05E-24	95
64	gfr01g2353	unknown	-	-	-
65	gfr01g1648	unknown	*Laccaria bicolor*	2.75E-12	61
66	gfr01g4845	aldo-keto reductase puatative	*Postia placenta*	2.15E-50	76
67	gfr01g3844	alpha beta	*Postia placenta*	2.27E-31	72
68	gfr01g9039	major royal jelly protein	*Schizophyllum commune*	6.52E-15	69
69	gfr01g1143	glycoside hydrolase family 28 protein	*Leucoagaricus gongylophorus*	1.09E-85	82
70	gfr01g6738	aldo keto reductase	*Coprinopsis cinerea*	7.11E-17	83
71	gfr01g2601	stress responsive protein	*Ajellomyces capsulatus*	4.95E-07	52
72	gfr01g4929	unknown	*Postia placenta*	2.11E-05	76
73	gfr01g3226	alpha-l-rhamnosidase	*Postia placenta*	3.92E-88	85
74	gfr01g8560	unknown	*Postia placenta*	2.85E-26	91
75	gfr01g9545	unknown	-	-	-
76	gfr01g1780	unknown	-	-	-
77	gfr01g1084	unknown	*Taiwanofungus camphoratus*	1.18E-71	81
78	gfr01g2625	unknown	*Postia placenta*	9.66E-27	64
79	gfr01g0801	carbohydrate esterase family 15 protein	*Leptosphaeria maculans*	9.89E-12	79
80	gfr01g2836	unknown	-	-	-
81	gfr01g6503	unknown	*Postia placenta*	1.11E-14	73
82	gfr01g0521	unknown	-	-	-
83	gfr01g1077	unknown	-	-	-
84	gfr01g5753	short chain dehydrogenase reductase family	*Moniliophthora perniciosa*	5.53E-45	65
85	gfr01g9238	retinol dehydrogenase	*Postia placenta*	3.47E-63	62
86	gfr01g6202	flavonol synthase	*Postia placenta*	1.59E-171	85
87	gfr01g2406	vacuolar sorting protein	*Schizophyllum commune*	3.36E-35	69
88	gfr01g6939	hydroquinone glucosyltransferase	*Postia placenta*	2.66E-55	71
89	gfr01g6941	unknown	*Postia placenta*	8.34E-10	59
90	gfr01g8295	cytochrome p450	*Taiwanofungus camphoratus*	1.82E-79	82
91	gfr01g0941	unknown	*Postia placenta*	7.85E-67	74
92	gfr01g7262	unknown	*Coprinopsis cinerea*	2.08E-16	51
93	gfr01g2088	unknown	-	-	-
94	gfr01g2331	esterase1	*Moniliophthora perniciosa*	4.07E-59	66
95	gfr01g0259	lipase	*Pleurotus sapidus*	4.03E-46	68
96	gfr01g3413	carboxylesterase	*Pleurotus sapidus*	3.94E-09	70
97	gfr01g0540	glycoside hydrolase family 3 protein	*Postia placenta*	2.48E-114	87

Genes involved in the induction of primordia development.

We screened for genes involved in the initiation of primordia development by comparing gene expression before and after the first induction. Ultimately, we identified 21 up-regulated genes and 34 down-regulated genes (Table 3) by the first induction step. The 21 up-regulated genes included a 12 kDa heat shock protein (HSP 9), some proteolysis-related proteins, a putative DNA binding protein, ribonucleotide reductase small and large subunits, and cytochrome p 450. In the yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, HSP 9 is undetectable during exponential growth in rich medium and is expressed at a low level when cells are grown in minimal medium, but is then strongly expressed upon entry into stationary phase[36),37)]. In general, the start of fruiting in basidiomycetes is triggered by conditions such as depletion of carbon and nitrogen sources in the medium. Increased expression of *G. frondosa* HSP 9 might have revealed a shift to fruiting from vegetative mycelium growth. Furthermore, in S. cerevisiae, HSP 9 expression is regulated by cAMP. In *Coprinus macrorhizus* and *L. edodes*, cAMP acts as a trigger substance in primordia development[38),39)]. It is then possible that this *G. frondosa* HSP 9 could also be regulated by cAMP, as part of a cascade of fruiting body development. Both gfr 01 g 0337 and gfr 01 g 1071 are encoded metalloproteases. The metalloprotease inhibitor talopeptin completely inhibited fruiting body development in *F. velutipes*[40)]. Thus, metalloproteases may also play an important role in fruiting body development in *G. frondosa*. Ribonucleotide reductase plays a critical role in regulating the total rate of DNA synthesis so that DNA-to-cell mass is maintained at a constant ratio during cell division. During primordia development, with cell number rapidly increasing, this enzyme supplies deoxyribonucleotides. Cytochrome P 450 has been reported to be associated with primordia development in some mushrooms including *C. cinerea*[41)], *A. bisporus*[6),42)], *L. edodes*[43)], *P. ostreatus*[44)], and *F. velutipes*[13)]. A mutation in the C. cinerea eln 2 gene, which encodes cytochrome P 450, blocked stipe elongation[45)]. Thus, P 450 may also play an important role in primordia development in *G. frondosa*.

Among the 35 down-regulated genes were lectin, some heat shock proteins, and some CAZymes. In *C. cinerea*, two fungal galectins, Cgl 1 and Cgl 2, are differentially regulated during fruiting body development[46)]. Cgl 2 expression was inhibited during the early stages of fruiting body development (hyphal knot formation) just as the gfr 01 g 1070-encoded lectin was reduced in expression during fruiting body development. This *G. frondosa* lectin might be unnecessary for primordia development and fruiting body differentiation, but is not necessary for hyphal growth. However, Nagata *et. al.*[47)] reported that the titer/mg protein of this lectin was highest in mature fruiting bodies. It will be necessary to compare the expression pattern of the gene with its protein. A total of four HSP 20 genes (gfr 01 g 7476, gfr 01 g 2265, gfr 01 g 9881, and gfr 01 g 7925) were found. The HSP 20 proteins are generally active as large oligomers consisting of multiple subunits, and are believed to be ATP-independent chaperones that prevent aggregation and are important in refolding in combination with other HSPs. These HSP 20 s might be necessary at higher temperatures during the spawn run rather than in the primordia developmental and fruiting body developmental stages. The gfr 01 g 4105 encoded a bacterialrhodopsin-like protein. The bacterialrhodopsins are retinal-binding proteins that serve ion transport and sensory functions in a light-dependent fashion in some halophilic bacteria[48)]. However, this *G. frondosa* bacterial rhodopsin-like protein gene seems not to be affected by light. This may be because the light intensity is high during the primordia developmental stage relative to the spawn running stage. In *Coriolus versicolor*, a bacterialrhodopsin-like protein was up-regulated by pentachlorophenol[49)]. This protein may degrade lignin and phenolic compounds in the sawdust medium. The gfr 01 g 1462 encoded a putative cellulase and other carbohydrate-active enzymes need to degrade cellulose contained in the medium during spawn run.

In the primordia developmental stage, genes related to degradation of woody substrates were down-regulated. On the other hand, cell proliferation-related genes, such as ribonucleotide reductase as described above, were observed in developing primordia. These gene expression states show the transition to sexual reproduction from vegetative mycelium growth.

Table 3. Up- and Down-regulated Genes by the First Induction

No.	Gene ID	Fold change	Putative Gene Products	Organism	E-Value	Similarity
			Up-regulated			
1	gfr01g0731	11.5	HSP9	*Coprinopsis cinerea*	1.26E-26	84
2	gfr01g9360	10.5	unknown	-	-	-
3	gfr01g0337	8.6	extracellular protease	*Grifola Frondosa*	1.80E-57	100
4	gfr01g6909	8.5	glutathione s-transferase	*Laccaria bicolor*	3.61E-75	76
5	gfr01g1071	8.2	deuterolysin m35 metalloprotease	*Schizophyllum commune*	6.35E-82	73
6	gfr01g3385	7.5	unknown	*Laccaria bicolor*	3.51E-13	56
7	gfr01g1186	7.3	aspartic peptidase a1	*Irpex Lacteus*	2.04E-42	88
8	gfr01g8035	6.8	unknown	-	-	-
9	gfr01g6884	6.4	unknown	-	-	-
10	gfr01g2318	6.1	phenylalanine ammonium lyase	*Schizophyllum commune*	4.85E-07	71
11	gfr01g9661	5.3	aldehyde dehydrogenase	*Schizophyllum commune*	5.75E-24	63
12	gfr01g7538	4.9	dna-binding protein	*Gibberella zeae*	3.32E-11	54
13	gfr01g6052	4.5	unknown	-	-	-
14	gfr01g3832	4.3	unknown	-	-	-
15	gfr01g7901	4.3	unknown	-	-	-
16	gfr01g3615	4.2	unknown	-	-	-
17	gfr01g5065	4.2	unknown	-	-	-
18	gfr01g2871	4.2	unknown	*Schizophyllum commune*	1.43E-22	61
19	gfr01g7673	4.2	ribonucleotide reductase small subunit	*Schizophyllum commune*	1.71E-52	97
20	gfr01g5524	4.1	ribonucleotide reductase large subunit	*Coprinopsis cinerea*	3.55E-20	93
21	gfr01g3104	4.1	cytochrome p450 61	*Nidula niveotomentosa*	3.27E-50	87
			Down-regulated			
1	gfr01g1070	10.9	lectin	-	-	-
2	gfr01g7476	10.9	HSP20	*Schizophyllum commune*	6.06E-42	70
3	gfr01g2265	9.0	HSP20	*Schizophyllum commune*	3.29E-35	81
4	gfr01g7859	7.1	class v chitinase	*Aspergillus clavatus*	3.07E-08	51
5	gfr01g6739	7.1	pyridoxal reductase	*Coprinopsis cinerea*	6.90E-09	74
6	gfr01g4439	6.9	unknown	-	-	-
7	gfr01g9881	6.1	HSP20	*Laccaria bicolor*	2.25E-33	63
8	gfr01g3931	6.1	glutamyl-trna amidotransferase subunit a	*Postia placenta*	1.18E-16	76
9	gfr01g4688	5.3	unknown	-	-	-
10	gfr01g7631	4.9	unknown	-	-	-
11	gfr01g4629	4.8	unknown	-	-	-
12	gfr01g7925	4.7	HSP20	*Coprinopsis cinerea*	3.18E-38	68
13	gfr01g1339	4.7	unknown	-	-	-
14	gfr01g5785	4.7	unknown	-	-	-
15	gfr01g5377	4.6	unknown	-	-	-
16	gfr01g2353	4.6	unknown	-	-	-
17	gfr01g9440	4.5	unknown	*Schizophyllum commune*	6.10E-65	71
18	gfr01g4770	4.5	unknown	-	-	-
19	gfr01g10147	4.5	unknown	*Conexibacter woesei*	2.07E-13	58
20	gfr01g5431	4.3	unknown	-	-	-
21	gfr01g4722	4.3	unknown	-	-	-
22	gfr01g10092	4.3	unknown	-	-	-
23	gfr01g4470	4.2	unknown	-	-	-
24	gfr01g2125	4.2	unknown	-	-	-
25	gfr01g6554	4.2	unknown	*Moniliophthora perniciosa*	5.12E-04	75
26	gfr01g4105	4.1	bacterialrhodopsin-like protein	*Trametes versicolor*	5.56E-66	78
27	gfr01g1635	4.1	macrolide-binding protein fkbp12	*Postia placenta*	6.49E-39	92

Genes involved in induction of fruiting body differentiation.

A total of 187 genes exhibited great changes in expression level upon the second induction to induce fruiting body differentiation (Table 4). The second induction includes an increase in humidity and the physical stimulus of opening the top of the culture bag of *G. frondosa* cultures. Hydrophobins and genes involved in cell division were detected among 56 genes up-regulated by the second induction. Interestingly, gfr01g7313, encoding hydrophobin, was specifically expressed following the second induction. This particular hydrophobin may be required to initiate fruiting body differentiation. The other hydrophobins (gfr01g9225 and HGFI) identified in *G. frondosa* were specifically expressed during the spawn run (Fig. 2). The expression pattern of the gfr01g7313 hydrophobin was similar to Le.hydl[50] in *L. edodes* and Fv-

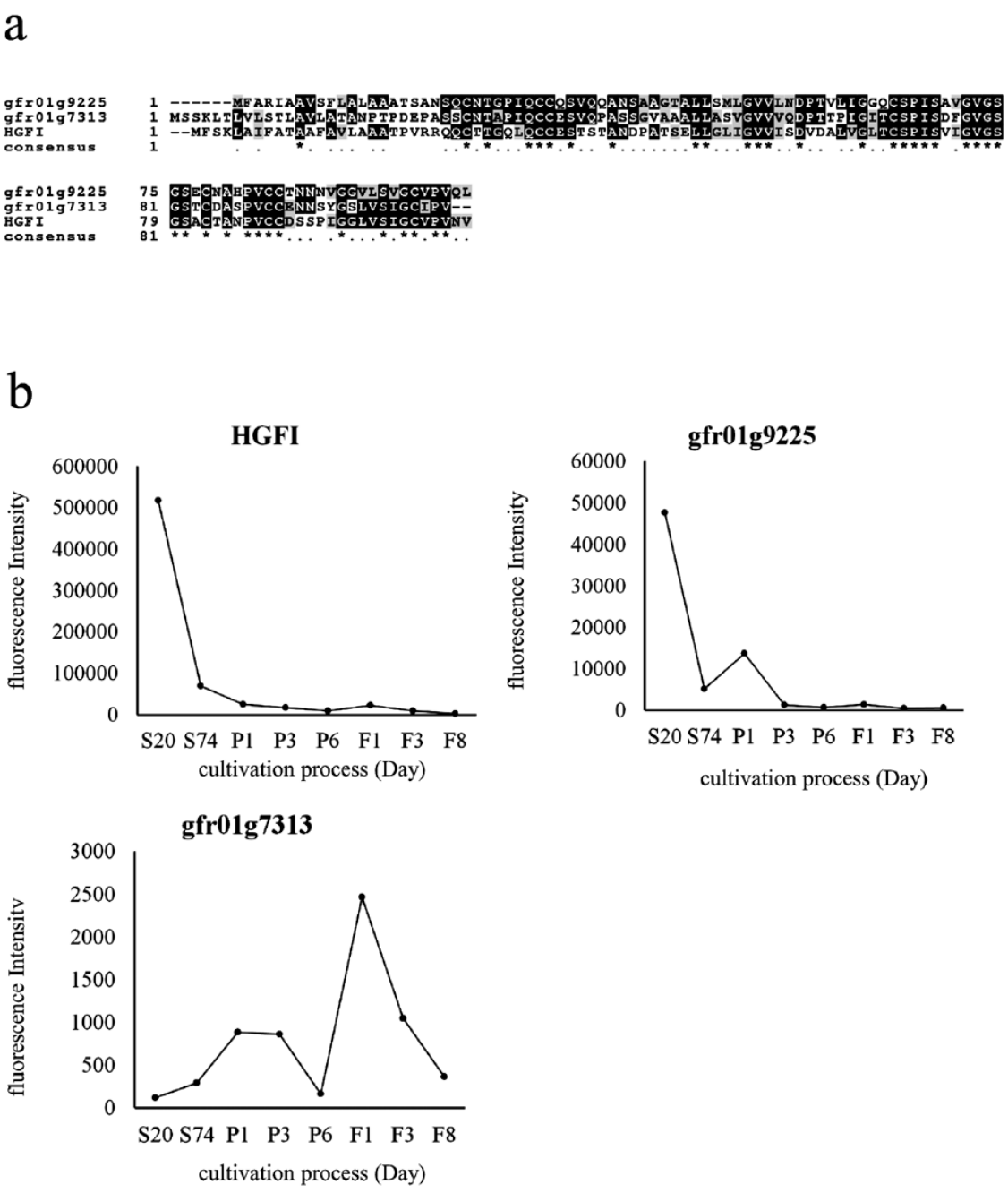

Fig. 2. Expression Pattern of Hydrophobins in Cultivation Process.

A, The alignment of amino acid sequence of hydrophobins in *G. frondosa*. B, The expression pattern of each hydrophobin. The Y axis represents raw signal value of microarray data. The X axis represents time course of cultivation process in *G. frondosa*. The data was shown an average of four biological replicates.

hyd1 in *F. velutipes*[51]. These results suggest that this hydrophobin has a common function among these species. During fruiting body differentiation there is rapid cell growth compared with the primordia development stage. The greatly increased expression of gfr01g2792, encoding putative G2 mitotic-specific cyclin, and gfr0g6318, encoding topoisomerase II, is consistent with the rapid cell proliferation in this stage. Ribonucleotide reductases continued to be strongly expressed as in the previous primordia developmental stage. In L. edodes, the Le.rnr2 gene was found to be most actively transcribed in the hymenophores of mature fruiting bodies[52]. A putative DNA-binding protein was encoded by gfr01g7538, which is a member of the DUF3140 superfamily (IPR021487) of proteins of unknown function. The expression level of this gene increased gradually from the primordia developmental stage to the fruiting body differentiation stage. This gene may be a transcription factor that functions to initiate enlargement during fruiting body formation.

Table 4. Up- and Down-regulated Genes by the Second Induction

No.	Gene ID	Fold change	Putative Gene Products	Organism	E-Value	Similarity
			Up-regulated			
1	gfr01g6820	20.8	unknown	-	-	-
2	gfr01g10033	19.9	unknown	-	-	-
3	gfr01g8035	12.9	unknown	-	-	-
4	gfr01g2078	9.9	unknown	*Postia placenta*	1.09E-57	77
5	gfr01g7313	9.1	hydrophobin	*Coprinopsis cinerea*	8.00E-05	71
6	gfr01g2729	8.2	g2 mitotic-specific cyclin 3	*Laccaria bicolor*	3.11E-25	76
7	gfr01g9257	8.1	methylene-fatty-acyl-phospholipid synthase	*Moniliophthora perniciosa*	9.24E-22	92
8	gfr01g8071	8.1	unknown	-	-	-
9	gfr01g10031	7.5	unknown	-	-	-
10	gfr01g4066	7.5	unknown	-	-	-
11	gfr01g4374	7.4	unknown	-	-	-
12	gfr01g8946	7.3	unknown	-	-	-
13	gfr01g5469	7.3	unknown	*Coprinopsis cinerea*	1.99E-08	87
14	gfr01g5492	7.2	unknown	-	-	-
15	gfr01g7036	7.1	cytochrome c peroxidase	*Coprinopsis cinerea*	1.66E-63	92
16	gfr01g3615	7.1	unknown	-	-	-
17	gfr01g1782	6.9	unknown	-	-	-
18	gfr01g2546	6.4	hmg box-containing protein	*Laccaria bicolor*	9.00E-17	75
19	gfr01g8569	6.3	unknown	-	-	-
20	gfr01g5493	6.2	unknown	*Moniliophthora perniciosa*	1.26E-10	81
21	gfr01g3832	6.0	unknown	-	-	-
22	gfr01g9661	6.0	aldehyde dehydrogenase	*Schizophyllum commune*	5.75E-24	63
23	gfr01g2053	5.9	2-oxoisovalerate dehydrogenase subunit beta	*Puccinia graminis f. sp. Tritici*	7.85E-05	76
24	gfr01g3385	5.9	unknown	*Laccaria bicolor*	3.51E-13	56
25	gfr01g4155	5.5	peroxidase	*Coprinellus disseminatus*	5.07E-04	72
26	gfr01g5581	5.3	unknown	-	-	-
27	gfr01g5524	4.9	ribonucleotide reductase large subunit	*Coprinopsis cinerea*	3.55E-20	93
28	gfr01g7673	4.9	ribonucleotide reductase small subunit	*Schizophyllum commune*	1.71E-52	97
29	gfr01g1186	4.8	aspartic peptidase a1	*Irpex Lacteus*	2.04E-42	88
30	gfr01g6605	4.8	protein	*Laccaria bicolor*	9.34E-06	52
31	gfr01g9717	4.7	unknown	-	-	-
32	gfr01g6318	4.5	dna topoisomerase ii	*Coprinopsis cinerea*	2.52E-11	64
33	gfr01g9345	4.4	small nuclear ribonucleoprotein sm d2	*Postia placenta*	1.10E-14	100
34	gfr01g6346	4.4	oxaloacetate acetylhydrolase	*Schizophyllum commune*	3.80E-23	95
35	gfr01g4294	4.3	unknown	-	-	-
36	gfr01g6604	4.3	unknown	-	-	-
37	gfr01g7955	4.3	sarco-endoplasmic reticulum calcium atpase	*Coprinopsis cinerea*	2.56E-19	85
38	gfr01g0420	4.2	unknown	*Moniliophthora perniciosa*	1.02E-04	67
39	gfr01g1250	4.2	wd repeat-containing protein slp1	*Coprinopsis cinerea*	2.06E-21	68

40	gfr01g6345	4.2	oxaloacetate acetylhydrolase	*Coprinopsis cinerea*	1.07E-25	98
41	gfr01g3086	4.2	dihydrodipicolinate synthetase	*Laccaria bicolor*	5.16E-20	87
42	gfr01g3207	4.2	adenylyl-sulfate kinase	*Coprinopsis cinerea*	2.40E-86	87
43	gfr01g9563	4.1	o-methylsterigmatocystin oxidoreductase	*Ajellomyces capsulatus*	3.47E-21	74
44	gfr01g9971	4.1	hydroxymethylglutaryl- synthase	*Laccaria bicolor*	4.04E-33	73
45	gfr01g9607	4.1	unknown	-	-	-
46	gfr01g6474	4.1	unknown	-	-	-
47	gfr01g6336	4.1	unknown	*Schizophyllum commune*	3.65E-10	62
48	gfr01g6808	4.1	unknown	*Laccaria bicolor*	1.99E-11	58
49	gfr01g3145	4.1	unknown	*Laccaria bicolor*	2.16E-13	47
50	gfr01g5601	4.1	sphingolipid c9-methyltransferase	*Laccaria bicolor*	8.01E-34	80
51	gfr01g7836	4.1	unknown	-	-	-
52	gfr01g9606	4.1	unknown	-	-	-
53	gfr01g5311	4.0	unknown	*Bacillus sp. NRRL B-14911*	8.66E-04	47
54	gfr01g6197	4.0	unknown	-	-	-
55	gfr01g5215	4.0	unknown	-	-	-
56	gfr01g7538	4.0	dna-binding protein	*Gibberella zeae*	3.32E-11	54

Down-regulated

1	gfr01g2915	99.0	unknown	*Postia placenta*	1.01E-61	67
2	gfr01g7830	87.9	unknown	-	-	-
3	gfr01g9112	48.2	cerato-platanin	*Nectria haematococca*	2.56E-40	75
4	gfr01g7349	41.5	protease s8 tripeptidyl peptidase	*Postia placenta*	1.79E-70	70
5	gfr01g4180	41.4	cerato-platanin	*Postia placenta*	4.24E-06	68
6	gfr01g5121	40.4	unknown	*Postia placenta*	1.58E-16	70
7	gfr01g7829	29.5	unknown	*Coprinopsis cinerea*	4.18E-38	78
8	gfr01g2544	23.2	phosphopyruvate hydratase	*Laccaria bicolor*	3.28E-119	88
9	gfr01g2265	22.8	HSP20	*Schizophyllum commune*	3.29E-35	81
10	gfr01g8915	20.8	cerato-platanin	*Laccaria bicolor*	1.34E-20	85
11	gfr01g4821	17.2	protease s8 tripeptidyl peptidase	*Postia placenta*	8.36E-55	75
12	gfr01g4105	16.0	bacterialrhodopsin-like protein	*Trametes versicolor*	5.56E-66	78
13	gfr01g10144	15.0	unknown	-	-	-
14	gfr01g2570	12.5	unknown	-	-	-
15	gfr01g9444	11.9	unknown	*Nectria haematococca*	8.67E-04	72
16	gfr01g0061	11.9	unknown	*Ajellomyces dermatitidis*	1.04E-04	57
17	gfr01g6891	11.3	unknown	*Postia placenta*	5.48E-57	78
18	gfr01g5111	11.3	unknown	-	-	-
19	gfr01g7476	10.3	HSP20	*Schizophyllum commune*	6.06E-42	70
20	gfr01g7422	10.1	alpha-galactosidase	*Phanerochaete chrysosporium*	0.00E+00	85
21	gfr01g2844	9.4	tyrosinase	*Glomerella graminicola*	7.12E-10	46
22	gfr01g4608	9.4	unknown	-	-	-
23	gfr01g9794	9.3	acetoin reductase	*Moniliophthora perniciosa*	2.70E-41	68
24	gfr01g3766	9.1	unknown	-	-	-
25	gfr01g3943	9.0	unknown	-	-	-
26	gfr01g10147	8.8	unknown	*Conexibacter woesei*	2.07E-13	58
27	gfr01g7117	8.7	gmc oxidoreductase	*Schizophyllum commune*	1.36E-17	88
28	gfr01g3296	8.6	HSP20	*Schizophyllum commune*	1.30E-23	58
29	gfr01g8657	7.7	unknown	-	-	-
30	gfr01g3640	7.7	unknown	*Coprinopsis cinerea*	5.72E-08	57
31	gfr01g0675	7.5	unknown	-	-	-
32	gfr01g9239	7.5	cerato-platanin	*Taiwanofungus camphoratus*	1.89E-35	70
33	gfr01g5660	7.4	unknown	-	-	-
34	gfr01g9704	7.4	unknown	-	-	-
35	gfr01g9277	7.2	serine protease	*Grifola frondosa*	6.88E-56	100
36	gfr01g2108	7.1	gmc oxidoreductase	*Schizophyllum commune*	6.16E-26	76
37	gfr01g10135	7.1	unknown	*Conexibacter woesei*	7.11E-14	64
38	gfr01g1332	7.1	alcohol dehydrogenase	*Postia placenta*	4.38E-86	75
39	gfr01g9881	6.7	HSP20	*Laccaria bicolor*	2.25E-33	63
40	gfr01g9790	6.6	unknown	-	-	-
41	gfr01g0390	6.6	unknown	-	-	-
42	gfr01g3470	6.6	unknown	*Coprinopsis cinerea*	6.99E-06	43

43	gfr01g1631	6.5	unknown	*Laccaria bicolor*	1.54E-48	70
44	gfr01g4320	6.5	unknown	*Schizophyllum commune*	3.80E-15	72
45	gfr01g4903	6.4	cyclohydrolase	*Coprinopsis cinerea*	6.75E-12	70
46	gfr01g8998	6.3	unknown	-	-	-
47	gfr01g9155	6.3	unknown	-	-	-
48	gfr01g4122	6.2	unknown	-	-	-
49	gfr01g6555	6.2	unknown	-	-	-
50	gfr01g6611	6.2	unknown	*Schizophyllum commune*	1.28E-54	74
51	gfr01g2709	6.2	unknown	-	-	-
52	gfr01g8437	6.1	alpha beta hydrolase fold protein	*Moniliophthora perniciosa*	8.40E-07	47
53	gfr01g4139	6.1	protein-er retention-related	*Laccaria bicolor*	1.69E-07	82
54	gfr01g4614	6.1	unknown	-	-	-
55	gfr01g2811	6.1	unknown	-	-	-
56	gfr01g0427	6.1	short-chain dehydrogenase	*Moniliophthora perniciosa*	2.84E-43	55
57	gfr01g8951	6.0	unknown	-	-	-
58	gfr01g7631	5.9	unknown	-	-	-
59	gfr01g4444	5.9	d-amino acid	*Schizophyllum commune*	3.40E-16	69
60	gfr01g3808	5.8	unknown	-	-	-
61	gfr01g0921	5.8	cycloheximide resistance	*Pyrenophora tritici*	2.75E-33	59
62	gfr01g5914	5.6	unknown	*Moniliophthora perniciosa*	1.31E-07	77
63	gfr01g1964	5.6	unknown	-	-	-
64	gfr01g0898	5.6	3-ketoacyl-acyl carrier protein reductase	*Laccaria bicolor*	7.32E-62	65
65	gfr01g8511	5.5	mitochondrial hypoxia responsive protein	*Postia placenta*	3.43E-48	82
66	gfr01g9356	5.3	unknown	*Postia placenta*	2.25E-04	88
67	gfr01g8466	5.3	unknown	-	-	-
68	gfr01g4887	5.3	glycoside hydrolase family 31 protein	*Postia placenta*	3.72E-23	67
69	gfr01g9656	5.2	unknown	*Laccaria bicolor*	1.20E-05	73
70	gfr01g3036	5.1	aldehyde dehydrogenase	*Postia placenta*	2.16E-47	82
71	gfr01g5405	5.1	unknown	-	-	-
72	gfr01g0949	5.1	unknown	-	-	-
73	gfr01g0360	5.1	unknown	-	-	-
74	gfr01g7466	5.1	HSP20	*Moniliophthora perniciosa*	1.84E-65	87
75	gfr01g1163	5.0	unknown	*Postia placenta*	1.61E-05	51
76	gfr01g6739	5.0	pyridoxal reductase	*Coprinopsis cinerea*	6.90E-09	74
77	gfr01g5563	4.9	unknown	-	-	-
78	gfr01g3980	4.9	glycoside hydrolase family 79 protein	*Laccaria bicolor*	2.79E-10	72
79	gfr01g0606	4.8	unknown	-	-	-
80	gfr01g5073	4.8	unknown	-	-	-
81	gfr01g2056	4.8	unknown	-	-	-
82	gfr01g7713	4.7	unknown	*Laccaria bicolor*	6.11E-11	82
83	gfr01g5616	4.7	unknown	-	-	-
84	gfr01g1735	4.7	glycosyl hydrolase family 88	*Postia placenta*	1.55E-159	83
85	gfr01g5893	4.7	apc amino acid permease	*Postia placenta*	2.54E-11	82
86	gfr01g1443	4.7	unknown	-	-	-
87	gfr01g2352	4.6	unknown	-	-	-
88	gfr01g0603	4.6	pectinesterase family protein	*Schizophyllum commune*	8.22E-18	77
89	gfr01g3954	4.6	gtp cyclohydrolase-2	*Schizophyllum commune*	9.78E-32	80
90	gfr01g10029	4.6	unknown	-	-	-
91	gfr01g7655	4.6	unknown	-	-	-
92	gfr01g1644	4.6	pleiotropic drug resistance abc transporter	*Coprinopsis cinerea*	5.68E-19	71
93	gfr01g2520	4.6	glycoside hydrolase family 18 protein	*Laccaria bicolor*	8.22E-26	85
94	gfr01g3140	4.5	unknown	*Laccaria bicolor*	1.83E-04	52
95	gfr01g1286	4.5	unknown	-	-	-
96	gfr01g8302	4.5	unknown	*Postia placenta*	1.62E-10	73
97	gfr01g10042	4.5	unknown	-	-	-
98	gfr01g4199	4.5	unknown	-	-	-
99	gfr01g1666	4.5	unknown	-	-	-
100	gfr01g8090	4.4	unknown	-	-	-
101	gfr01g4381	4.4	unknown	-	-	-
102	gfr01g8540	4.4	unknown	-	-	-
103	gfr01g0208	4.4	short-chain dehydrogenase reductase sdr	*Coprinopsis cinerea*	1.23E-26	79
104	gfr01g4129	4.4	rho gtpase activating protein 22	*Coprinopsis cinerea*	2.49E-56	61
105	gfr01g6512	4.3	serine family	*Aspergillus oryzae*	1.51E-16	47

106	gfr01g2894	4.3	unknown	-	-	-
107	gfr01g5589	4.3	unknown	-	-	-
108	gfr01g4332	4.3	unknown	-	-	-
109	gfr01g2280	4.3	response regulator receiver protein	*Laccaria bicolor*	1.31E-36	98
110	gfr01g1411	4.3	unknown	-	-	-
111	gfr01g4613	4.2	unknown	*Coprinopsis cinerea*	4.87E-15	61
112	gfr01g9344	4.2	unknown	-	-	-
113	gfr01g4688	4.2	unknown	-	-	-
114	gfr01g4470	4.2	unknown	-	-	-
115	gfr01g5374	4.2	unknown	-	-	-
116	gfr01g4739	4.2	unknown	-	-	-
117	gfr01g3880	4.2	unknown	-	-	-
118	gfr01g2692	4.2	unknown	-	-	-
119	gfr01g8553	4.2	thaumatin-like protein	*Lentinula edodes*	9.75E-112	79
120	gfr01g3226	4.2	alpha-l-rhamnosidase	*Postia placenta*	3.84E-88	85
121	gfr01g0950	4.1	universal stress protein	*Postia placenta*	4.91E-47	94
122	gfr01g7147	4.1	unknown	-	-	-
123	gfr01g1018	4.1	unknown	-	-	-
124	gfr01g4759	4.1	homoserine o-acetyltransferase	*Bradyrhizobium sp.*	6.43E-23	63
125	gfr01g0368	4.1	unknown	-	-	-
126	gfr01g1233	4.1	glycoside hydrolase family 95 protein	*Aspergillus niger*	5.61E-12	83
127	gfr01g7899	4.1	unknown	-	-	-
128	gfr01g10028	4.1	unknown	-	-	-
129	gfr01g7279	4.0	glycoside hydrolase family 3 protein	*Phanerochaete chrysosporium*	8.72E-47	84
130	gfr01g7773	4.0	unknown	-	-	-
131	gfr01g8789	4.0	unknown	-	-	-

Among the 131 genes down-regulated by the second induction were cerato-platanin, heat shock proteins, S8 proteases, GMC oxidoreductase, and carbohydrate-active enzymes. Four genes (gfr01g9112, gfr01g4180, gfr01g8915, and gfr01g9239) were presumed to encode cerato-platanin, which is a known phytotoxic protein. Cerato-platanin is localized in the cell wall of Ascomycete fungi and may be involved in plant infection53,54). Interestingly, the expression levels of all four cerato-platanin genes in *G. frondosa* increased before the second induction and then rapidly decreased (Fig. 3). Although the role of these genes in fruiting body differentiation is completely unknown, cerato-platanins are known to have properties similar to those of hydrophobins. These proteins may be involved in cell aggregation to form primordia. Two genes (gfr01g7349 and gfr01g8915) presumed to encode an S8/S53 peptidase, two genes (gfr01g7117 and gfr01g2108) presumed to encode an GMC oxidoreductase, a pectinesterase family protein (gfr01g0603), and many carbohydrate-active enzymes (GH3, GH18, GH31, GH79, GH88, GH95) greatly decreased in expression level after the second induction. These enzymes may be needed to obtain nutrients for the growth of mycelium cells.

The genes involved in spawn run were continuously expressed at low levels from the primordia developmental stage to the fruiting body differentiation stage. A novel cerato-platanin showed a unique expression pattern related to fruiting body development. Hydrophobins were also specifically expressed in fruiting body development, and these small hydrophobic proteins may play important roles in fruiting body development.

A mentioned above, 99 genes involved in spawn run, 55 genes involved in primordia development, and 187 genes involved in fruiting body differentiation were identified.

To conclude, in this study, we have identified genes associated with each part of the *G. frondosa* cultivation process: spawn run, primordia development, and fruiting body differentiation. These findings will not only help to improve the cultivation process for *G. frondosa* but also contribute to understanding the common mechanisms of basidiomycete fruiting body development. However, much work still needs to be done in the study of genetics in *G. frondosa*. The expected roles of the genes expressed during each cultivation process were described above; however, their exact functions in fruiting body

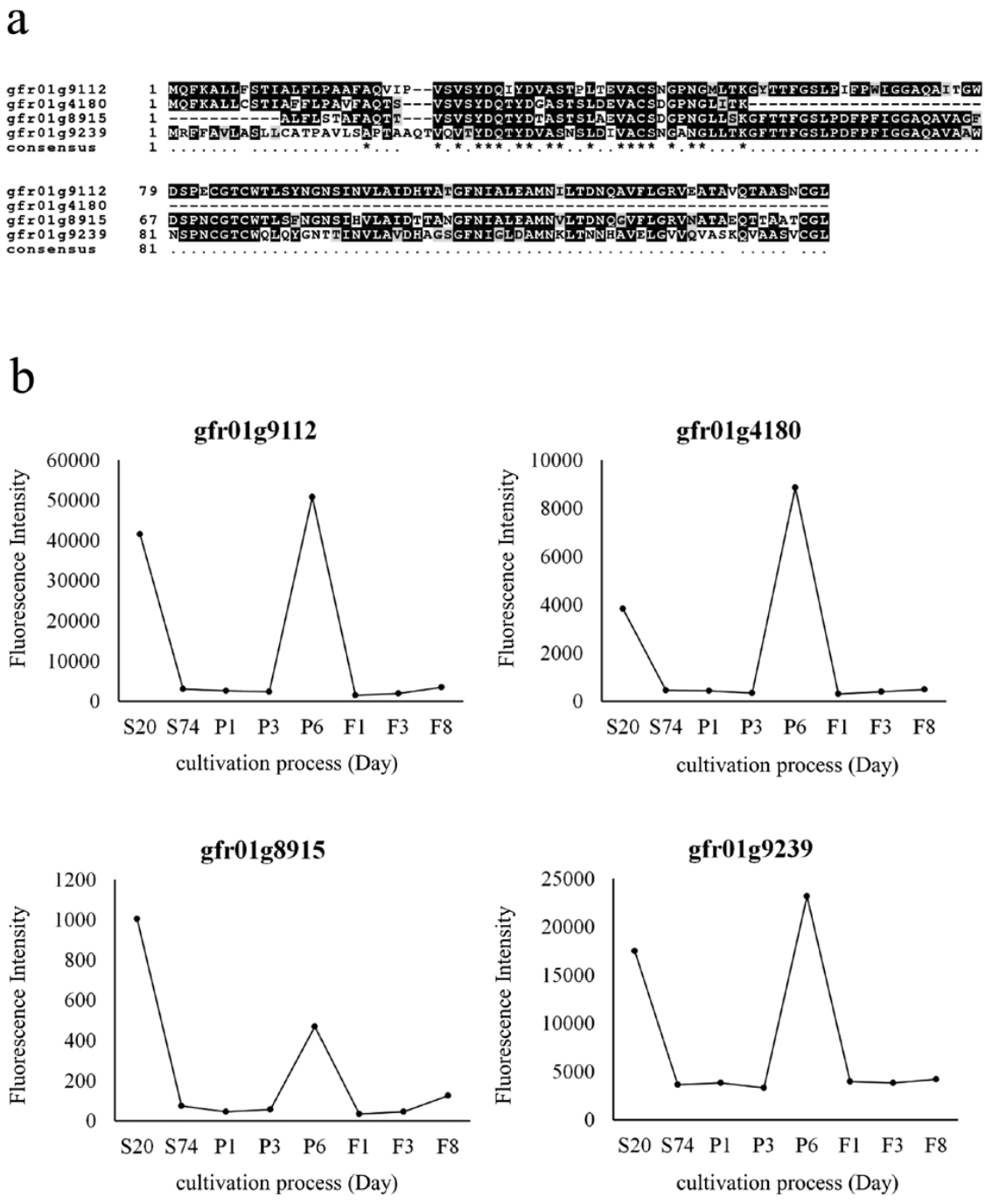

Fig. 3. Expression Pattern of Cerato-platanins in Cultivation Process.

A, The alignment of amino acid sequence of cerato-platanins in *G. frondosa*. B, The expression pattern of each cerato-platanin. The Y axis represents raw signal value of microarray data. The X axis represents time course of cultivation process in *G. frondosa*. The data was shown an average of four biological replicates.

development are still unclear. It will be necessary to dissect these developmental processes using functional analyses such as gene targeting, because approximately 60% of the listed genes identified in this study are of unknown function.

(4) **Acknowledgment**

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マイタケの栽培工程における発現遺伝子プロファイルの解析

論文要旨

マイタケは，大量生産技術が確立されているが，子実体生育メカニズムについては理解されていない．このメカニズム解明は，効率的な育種や栽培工程の改良に必須である．我々はRoche 454を用いてマイタケの栽培工程13か所にわたるトランスクリプトームの配列を決定した．総塩基長101 Mbpから26,893のコンティグが得られ，10,150のORFが予測された．次に，マイクロアレイでマイタケの栽培工程8か所の発現遺伝子解析を行った．その結果，我々は原基形成に関わる56遺伝子と子実体成熟に関わる187遺伝子を見出し，特に重要と思われる14遺伝子の抽出に成功した．本研究は，マイタケに関する情報ばかりでなく，担子菌共通のメカニズムの理解にも貢献する情報を提供できる．