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Identification of differentially expressed genes in fruiting body mutants of *Grifola frondosa*

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Abstract

The edible mushroom maitake (*Grifola frondosa*) is mass produced year-round in mushroom production facilities. During the cultivation process, spontaneous fruiting body mutants appear occasionally. The primary goal of this study was to identify candidate genes responsible for fruiting body differentiation using these spontaneous mutants. Therefore, we performed a comparative analysis of the genes expressed during fruiting body differentiation in the stock culture (Gf-N 2) of the M 51 strain of *G. frondosa* and its fruiting body mutants, Gf-A 1 and Gf-A 4.

In a microarray experiment, we identified 49 genes in Gf-A 1 and 69 genes in Gf-A 4 that were differentially expressed compared to their expression levels in Gf-N 2. From this experiment, we identified 24 genes that were up-regulated in both Gf-A 1 and Gf-A 4. This result suggests that these differentially expressed genes would otherwise be suppressed during normal fruiting body differentiation. We focused on *Gf.CRZ 1*, a homolog of *Crz 1* in *Saccharomyces cerevisiae* which activates transcription of stress-responsive genes, as it was the only gene encoding a transcription factor among the 24 genes with similar expression patterns between these mutants. *Gf.CRZ 1* might affect the expression of the other 23 genes that are up-regulated in both mutants via a transcriptional regulatory mechanism. However, *Gf.CRZ 1* expression was 211- and 31-fold higher in Gf-A 1 and Gf-A 4, respectively, than in Gf-N 2. The differences in the expression levels of these genes likely contribute to the distinctive phenotypes characteristic of each mutant.

Key words : Edible mushroom • fruiting body differentiation • maitake mushroom • mutant • microarray

1. Introduction

Grifola frondosa (maitake mushroom) is a delicious edible mushroom with high nutritional value, of which 44,452 t were produced in 2011 in Japan (MAFF, 2011). *G. frondosa* is mass produced year-round in mushroom production facilities. However, fruiting body mutations appear spontaneously during production and can reduce the yield and marketability of mushrooms. Fruiting body mutations can arise due to either genetic variation or environmental factors. If genes responsible for fruiting body differentiation are identified, these genes can be used as markers to discriminate and further analyze fruiting body mutants. Therefore, transcriptomic and genomic sequences of *G. frondosa* have been determined and a molecular genetics database for edible mushroom production has been constructed (Kurahashi et al. 2012; Sato et al. 2013). We have also previously reported 4 genes that are related to fruiting body differentiation: *Gf.HSP 9* (Kurahashi et al. 2013 a), *Pe. pleurotolysin A, Pe. ostreolysin* (Kurahashi et al. 2013 b), and *Gf.BMR 1* (unpublished observations).

In this study, we performed microarray-based transcriptome analysis of a stock culture, Gf-N 2, isolated from the M 51 strain of *G. frondosa*, and identified 2 fruiting body mutants, Gf-A 1 and Gf-A 4, to

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explore genes responsible for fruiting body differentiation.

2. Materials and Methods

2.1 Strain and culture conditions

Gf-N 2, the culture isolated from *G. frondosa* dikaryotic strain M 51, and its fruiting body mutants, Gf-A 1 and Gf-A 4, were used in this study. Gf-A 1 develops primordia that do not differentiate into fruiting bodies, while Gf-A 4 develops fruiting bodies but no expanded pilei. The processes of fruiting body development in each strain are shown in Figure 1. These strains were cultivated according to previously published methods (Kurahashi et al. 2012).

2.2 Isolation of total RNA and Microarray experiment

Total RNAs were prepared from 4 different growth stages during the 86 d cultivation period: at 80, 82, 84, and 86 d after starter culture inoculation (Fig. 1). Total RNA isolation and microarray experiments were performed according to previously published methods (Kurahashi et al. 2012).

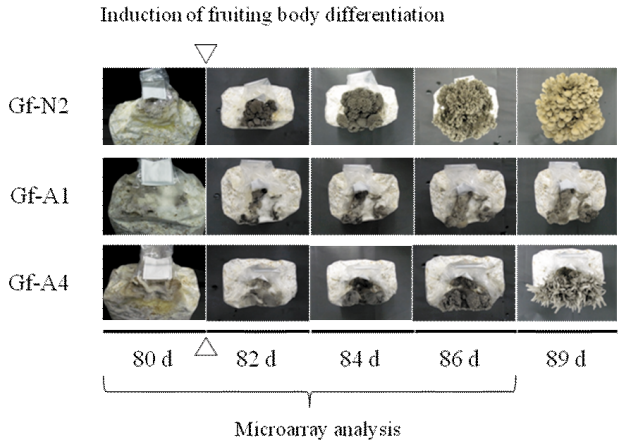


Fig. 1 - Fruiting body development in the culture isolated from the *Grifola frondosa* M 51 strain, Gf-N 2, and the fruiting body mutants, GfA 1 and Gf-A 4. Days showed after inoculation of starter culture

2.3 Annotation

From microarray experiments, we identified genes differentially expressed in strains Gf-A 1 and Gf-A 4 compared to in the strain Gf-N 2 normal fruiting bodies. Annotation of differentially expressed genes was performed according to previously published methods (Kurahashi et al. 2012).

2.4 Cloning and sequence analysis

Sequences of the zinc-finger transcription factor *Gf.CRZ 1 (gfr 01 g 0814)* (Accession no. AB 853027) and aquaporin *Gf.AQP 1 (gfr 01 g 1188)* (Accession no. AB 853029) were obtained by 3'- and 5'-RACE based on partial cDNA sequences from our in-house transcriptome database using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Primer sequences for RACE PCR are listed in Table 1. Full-length cDNAs of *Gf.CRZ 1* and *Gf.AQP 1* were then isolated by PCR based on the sequences of the 3'- and 5'-RACE products. The full-length cDNA sequence of formate dehydrogenase *Gf.FDH 1 (gfr 01 g 0023)* (Accession no. AB 853028) was isolated by PCR based on the partial cDNA sequence identified in the in-house transcriptome database. These primer sequences for full-length cDNA isolation are also listed in Table 1. Amino acid sequences were analyzed using blastp (Altschul et al. 1997) with an e-value $\leq 1 \times 10^{-3}$ and a bit score >40. Queries for conserved protein motifs were run using PROSITE at the Swiss Institute of Bioinformatics (Sigrist et al. 2002).

Table 1 Primer sequences used in this study.

Primer name	Sequence (5' to 3')
Gf.CRZ1-QPCR-F1	ACACCTTCTCTTAGAATGCTGACG
Gf.CRZ1-QPCR-R1	TCCTGTTGTCCCAATCATCTTCTC
Gf.FDH1-QPCR-F1	TCGCGGACTTATCAATGCTG
Gf.FDH1-QPCR-R1	CAGCGATGGCTTCCTTGTC
Gf.AQP1-QPCR-F1	CGCAATGTAAGCTCCCAGGAC
Gf.AQP1-QPCR-R1	TCTCATCGTCTGCTCCTCCAC
Gf.GAPDH-QPCR-F2	CAACCTTGACGAATACGACTC
Gf.GAPDH-QPCR-R2	CCTCGACGATACCGAAGTTG
Gf.CRZ1-5'RACE	CACAGCACTGGAAGACATGATGTTG
Gf.CRZ1-3'RACE	AATTCTCACCCCGTGATCAC
Gf.FDH1-5'RACE	GAAGTGCTTGAGGAGATCAGCATTG
Gf.FDH1-3'RACE	ACTACTCCGGTACGACTCTGGATG
Gf.AQP1-full-F1	TCGAACCACTCGTACATTCAGG
Gf.AQP1-full-R1	TTTCCCCGTGAGCACAAATTCC

2.5 Quantitative RT-PCR

Gf.CRZ 1, Gf.FDH 1, and *Gf.AQP 1* exhibited the greatest number of enriched gene ontology (GO) terms from among the 24 genes expressed in common between the mutants Gf-A 1 and Gf-A 4. The expression levels of these genes determined by microarray analysis were then confirmed by quantitative RT-PCR. Quantitative RT-PCR was performed according to previously published methods (2013 b). The

sequences of primer pairs for quantitative RT-PCR are listed in Table 1.

3. Results

3.1 Differentially expressed genes in Gf-A1

We performed microarray experiments to screen for genes related to development of the fruiting body in *G. frondosa*. Genes that were differentially expressed in Gf-A1 and Gf-A4 relative to Gf-N2 were identified. In one fruiting body mutant, Gf-A1, there were 49 genes with greater than 2-fold differential expression relative to Gf-N2 (Table 2). Among these,

Table 2 Comparison of differentially expressed genes in the Gf-A1 mutant compared to Gf-N2

No.	Gene ID	Putative gene product	Fold-change relative to Gf-N2			
			80 d	82 d	84 d	86 d
1	gfr01g0814	Cys2His2 zinc-finger protein (Gf.CRZ1)	142	211	199	144
2	gfr01g1161	hypothetical protein	7	-	3	4
3	gfr01g7819	alpha beta-hydrolase	6	169	319	226
4	gfr01g3268	alcohol oxidase	5	5	56	185
5	gfr01g6743	hypothetical protein	5	-	-	-
6	gfr01g1016	alcohol oxidase	5	5	56	111
7	gfr01g2975	glycerol kinase	4	7	5	4
8	gfr01g1146	cyanamide hydratase	4	71	124	129
9	gfr01g9127	perforin	3	39	46	36
10	gfr01g9332	isoprenylcysteine carboxyl methyltransferase (ICMT) family	3	8	19	18
11	gfr01g6012	cytochrome p450	3	12	24	19
12	gfr01g3974	cytochrome p450	3	10	26	42
13	gfr01g9585	hexose transporter	2	29	39	32
14	gfr01g0023	NAD-dependent formate dehydrogenase (Gf.FDH1)	2	13	51	140
15	gfr01g0918	formate nitrite transporter	2	6	16	30
16	gfr01g9987	mfs general substrate transporter	2	8	6	6
17	gfr01g6998	general substrate transporter	2	6	9	8
18	gfr01g1188	aquaporin-like protein (Gf.AQP1)	2	7	11	20
19	gfr01g1125	hypothetical protein	2	7	12	14
20	gfr01g2510	glycoside hydrolase family 29 protein	-	16	60	67
21	gfr01g0163	d-lactonohydrolase-like protein	-	9	37	45
22	gfr01g0921	mfs general substrate transporter	-	17	24	30
23	gfr01g2250	hypothetical protein	-	8	21	29
24	gfr01g1631	unknown function (DUF1768)	-	6	14	27
25	gfr01g4139	ER retention-related protein	-	14	16	26
26	gfr01g3684	glycoside hydrolase family 16 protein	-	38	31	26
27	gfr01g3839	fructosamine kinase	-	10	18	20
28	gfr01g4887	glycoside hydrolase family 31 protein	-	6	11	17
29	gfr01g7631	DnaJ domain-containing protein	-	6	7	17
30	gfr01g2241	hypothetical protein	-	6	5	17
31	gfr01g0550	general substrate transporter	-	6	16	15
32	gfr01g9356	CsbD domain-containing protein	-	13	40	15
33	gfr01g7422	alpha-galactosidase	-	7	18	13
34	gfr01g2573	glycoside hydrolase family 3 protein	-	11	11	12
35	gfr01g0984	general substrate transporter	-	6	12	12
36	gfr01g9343	mfs general substrate transporter	-	6	8	12
37	gfr01g3053	peptidase s28	-	13	13	10
38	gfr01g9968	pectin lyase-like protein	-	-	-	10
39	gfr01g0020	mfs general substrate transporter	-	6	8	9
40	gfr01g10018	urod-like protein	-	5	13	8

41	gfr01g6753	glycoside hydrolase family 28 protein	-	8	16	8
42	gfr01g2843	unknown function	-	9	14	8
43	gfr01g0540	glycoside hydrolase family 3 protein	-	6	8	8
44	gfr01g1687	ptr2-domain-containing protein	-	6	8	7
45	gfr01g6965	general substrate transporter	-	7	8	7
46	gfr01g7523	vitamin b6 biosynthesis protein	-	5	9	7
47	gfr01g5906	glycoside hydrolase family 92 protein	-	5	8	6
48	gfr01g1177	mitochondrion protein	-	3	4	3
49	gfr01g6178	MFS general substrate transporter	-	-	2	3

blastp searches hit 32 genes with GO annotation, 5 genes without GO terms, and 12 genes for which no matches were identified (Fig. 2 A). In particular, genes for a Cys 2 His 2 zinc-finger protein (*gfr 01 g 0814*), an alpha beta-hydrolase (*gfr 01 g 7819*), 2 alcohol oxidases (*gfr 03 g 3268* and *gfr 01 g 1016*), a cyanamide hydratase (*gfr 01 g 1146*), and an NAD-dependent formate dehydrogenase (*gfr 01 g 0023*) underwent greater than 100-fold increases in expression relative to their levels of expression in Gf-N 2.

3.2 Differentially expressed genes in Gf-A 4

Sixty-nine genes were differentially expressed more than 2-fold increase in Gf-A 4 than in Gf-N 2 (Table 3). There were blastp hits for 37 of them that were annotated with Gene Ontology terms (GO), 9

Table 3 Comparison of differentially expressed genes in the Gf-A 4 mutant compared to Gf-N 2

No.	Gene ID	Putative gene product	Fold-change relative to Gf-N2			
			80 d	82 d	84 d	86 d
1	gfr01g1161	hypothetical protein	6	2	3	10
2	gfr01g0949	unknown function	5	6	5	5
3	gfr01g4454	glycoside hydrolase family 3 protein	4	9	14	-
4	gfr01g3268	alcohol oxidase	4	10	8	9
5	gfr01g1016	alcohol oxidase	4	10	5	5
6	gfr01g1146	cyanamide hydratase	4	27	18	8
7	gfr01g0814	Cys2His2 zinc-finger protein (Gf.CRZ1)	3	31	19	36
8	gfr01g2510	glycoside hydrolase family 29 protein	3	6	11	3
9	gfr01g9813	unknown function	3	3	13	15
10	gfr01g9127	perforin	3	14	9	6
11	gfr01g4930	subtilisin-like protein	3	7	3	7
12	gfr01g3691	FAD NAD-p-binding domain-containing protein (Gf.FDH1)	3	-	4	5
13	gfr01g2116	monooxygenase	3	6	3	4
14	gfr01g9332	isoprenylcysteine carboxyl methyltransferase (ICMT) family	2	4	5	4
15	gfr01g3530	unknown function	2	-	3	5
16	gfr01g0163	d-lactonohydrolase-like protein	2	5	15	7
17	gfr01g3345	protein kinase	2	3	7	3
18	gfr01g8870	methyltransferase	2	3	7	3
19	gfr01g3974	cytochrome p450	2	8	13	10
20	gfr01g5433	unknown function	2	-	2	11
21	gfr01g9356	CsbD domain-containing protein	-	7	7	-
22	gfr01g1381	enolase	-	-	5	-
23	gfr01g1234	glycoside hydrolase family 95 protein	-	5	5	-
24	gfr01g10018	urod-like protein	-	3	4	-
25	gfr01g1912	unknown function	-	7	4	-
26	gfr01g7422	alpha-galactosidase	-	5	4	-
27	gfr01g3980	glycoside hydrolase family 79 protein	-	5	3	-

28	gfr01g5893	amino acid transporter	-	5	3	-
29	gfr01g3053	peptidase s28	-	5	3	-
30	gfr01g0023	nad-dependent formate dehydrogenase	-	7	22	29
31	gfr01g0061	unknown function	-	11	17	23
32	gfr01g2915	unknown function	-	6	13	16
33	gfr01g9744	unknown function	-	-	3	15
34	gfr01g8759	glycoside hydrolase family 92 protein	-	5	4	10
35	gfr01g0277	catalase	-	3	4	10
36	gfr01g3671	unknown function	-	2	5	9
37	gfr01g9412	unknown function	-	3	5	8
38	gfr01g3544	peptidase family s41	-	2	-	6
39	gfr01g7895	carbohydrate esterase family 16 protein	-	2	2	6
40	gfr01g3082	peptidase family s41	-	2	-	6
41	gfr01g1188	aquaporin-like protein (Gf.AQP1)	-	4	4	6
42	gfr01g3692	unknown function	-	-	4	6
43	gfr01g8270	unknown function	-	4	3	5
44	gfr01g0478	unknown function	-	3	3	5
45	gfr01g9915	acetamidase	-	-	4	5
46	gfr01g2811	unknown function	-	2	-	5
47	gfr01g3159	unknown function	-	3	3	5
48	gfr01g1964	unknown function	-	2	-	5
49	gfr01g1631	unknown function (DUF1768)	-	4	7	5
50	gfr01g4919	unknown function	-	2	3	5
51	gfr01g4003	FAD NAD-binding domain-containing protein	-	3	3	5
52	gfr01g4460	unknown function	-	3	-	5
53	gfr01g2241	hypothetical protein	-	4	2	4
54	gfr01g0675	unknown function	-	4	6	4
55	gfr01g9040	unknown function	-	6	4	4
56	gfr01g4887	glycoside hydrolase family 31 protein	-	4	6	4
57	gfr01g2250	hypothetical protein	-	3	5	4
58	gfr01g6511	unknown function	-	3	5	4
59	gfr01g3012	alpha beta-hydrolase	-	4	5	4
60	gfr01g4139	ER retention-related protein	-	7	7	4
61	gfr01g7924	glycoside hydrolase family 2 protein	-	3	5	3
62	gfr01g3839	fructosamine kinase	-	6	7	3
63	gfr01g3465	unknown function	-	3	5	3
64	gfr01g8511	unknown function	-	2	6	3
65	gfr01g1125	hypothetical protein	-	4	7	3
66	gfr01g0114	unknown function	-	4	4	3
67	gfr01g4627	lectin 2a	-	-	5	3
68	gfr01g9968	pectin lyase-like protein	-	5	5	2
69	gfr01g7466	heat shock protein HSP20	-	-	6	2

genes with blastp hits without GO terms, and 23 genes with no blastp matches in the database (Fig. 2 A). However, the magnitude of differential expression of genes in Gf-A 4 was no greater than that in Gf-A 1. The gene with the greatest differential expression was *gfr01g0814* in both Gf-A 1 and Gf-A 4. However, the relative change in expression of *gfr01g0814* in Gf-A 4 (a 31-fold increase) was less than that observed in Gf-A 1 (a 211-fold increase). On the other hand, the number of genes with differential expression in Gf-A 4 (69 genes) was greater than that in Gf-A 1 (49 genes).

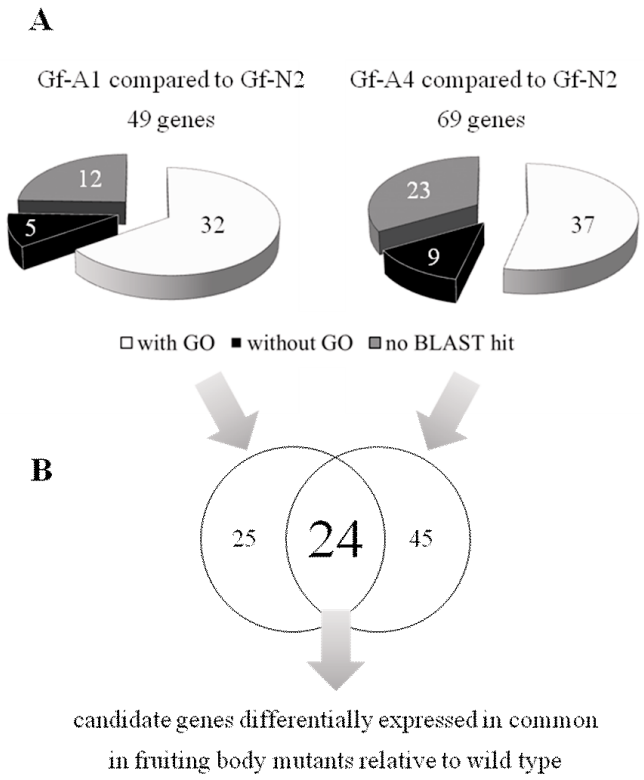


Fig. 2 - Screening genes related to fruiting body differentiation. A: Differentially expressed genes in Gf-A1 and Gf-A4 as compared to Gf-N2. B: Extracted genes common to Gf-A1 and Gf-A4

3.3 Common genes for fruiting body mutation

Twenty-four genes differentially expressed in common in Gf-A1 and Gf-A4 mutants were identified as genes in which fruiting body mutations might commonly occur (Fig. 2B, Table 4). Expression patterns for these genes are shown in Fig. 3. These genes could be divided into 4 groups (Groups A-D) based on their differential expression patterns during fruiting body development in Gf-A1. Genes in Group A, such as *gfr01g0814*, were strongly constitutively expressed in Gf-A1. Genes in Group B, such as *gfr01g1161*, were strongly expressed from 80 d to 86 d. Group C included 3 genes that had the same increased expression levels in Gf-A1 and Gf-A4 throughout fruiting body development. Finally, Group D included 19 other genes whose expression levels increased throughout fruiting body development, but decreased again in Gf-A4 closer to the expression levels observed in Gf-N2.

3.4 Gene structure and putative function of Gf.CRZ1

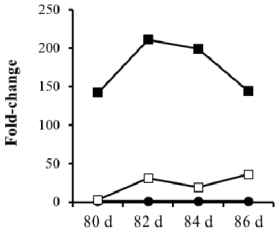
Gf.CRZ1, a homolog of *Crz1* in *Saccharomyces cerevisiae*, was the only gene encoding a transcription

Table 4 Genes differentially expressed in common in the Gf-A1 and Gf-A4 mutants compared to Gf-N2

No.	Gene ID	Putative gene products	Number of GOs	Group
1	gfr01g0814	Cys2His2 zinc-finger protein (Gf.CRZ1)	12	A
2	gfr01g1161	hypothetical protein	1	B
3	gfr01g0023	NAD-dependent formate dehydrogenase (Gf.FDH1)	9	C
4	gfr01g1188	aquaporin-like protein (Gf.AQP1)	7	C
5	gfr01g2241	hypothetical protein	0	C
6	gfr01g2510	glycoside hydrolase family 29 protein	6	D
7	gfr01g3268	alcohol oxidase	6	D
8	gfr01g10018	urod-like protein	4	D
9	gfr01g3974	cytochrome p450	3	D
10	gfr01g9968	pectin lyase-like protein	3	D
11	gfr01g7422	alpha-galactosidase	3	D
12	gfr01g9332	isoprenylcysteine carboxyl methyltransferase (ICMT) family	3	D
13	gfr01g3053	peptidase s28	2	D
14	gfr01g3839	fructosamine kinase	2	D
15	gfr01g4887	glycoside hydrolase family 31 protein	1	D
16	gfr01g0163	d-lactonohydrolase-like protein	1	D
17	gfr01g4139	ER retention-related protein	1	D
18	gfr01g1146	cyanamide hydratase	1	D
19	gfr01g1016	alcohol oxidase	1	D
20	gfr01g2250	hypothetical protein	0	D
21	gfr01g1125	hypothetical protein	0	D
22	gfr01g9127	perforin	0	D
23	gfr01g9356	CsbD domain-containing protein	0	D
24	gfr01g1631	unknown function (DUF1768)	0	D

Group A

gfr01g0814



Group B

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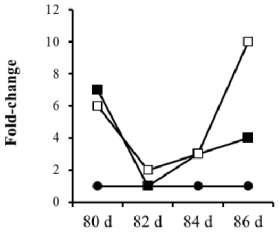

Group C

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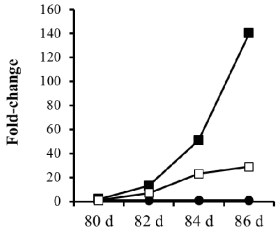

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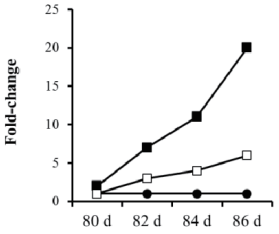

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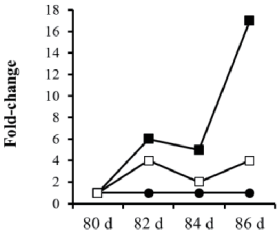

Group D

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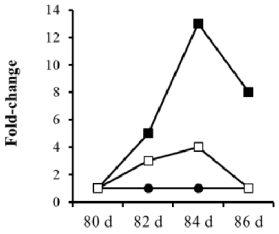

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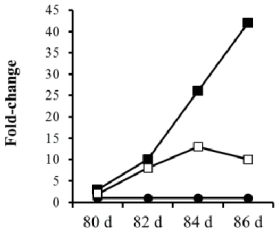

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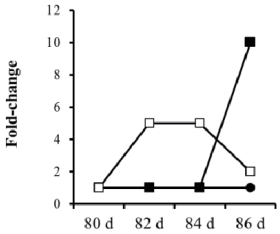

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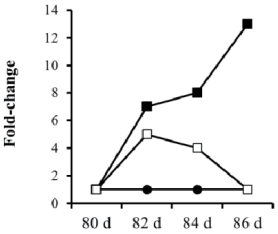

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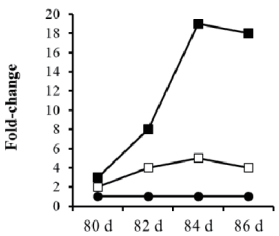

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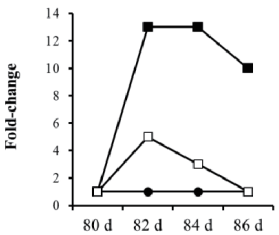

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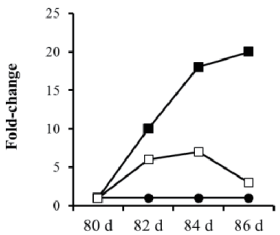

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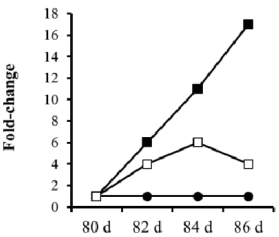


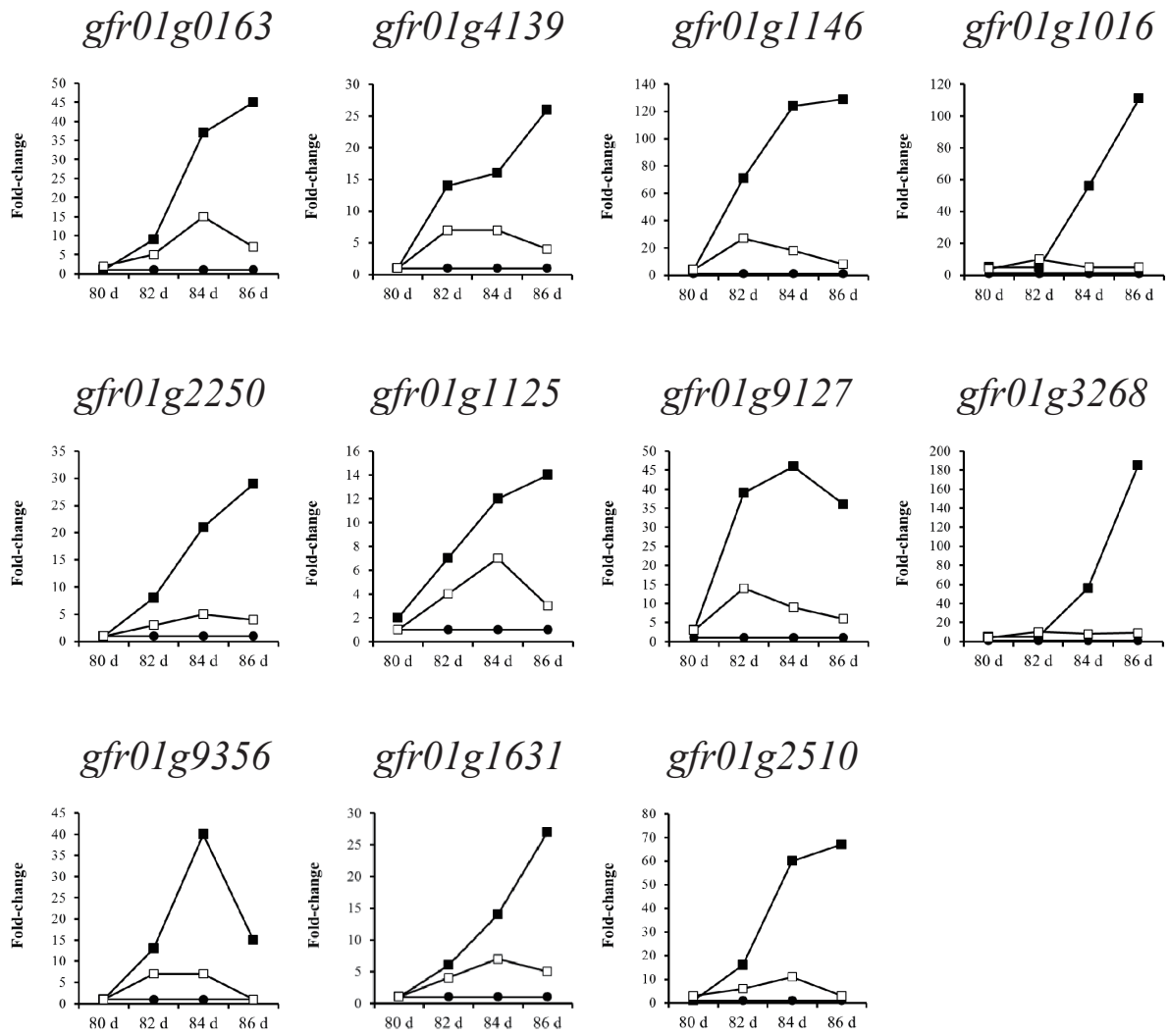


Fig. 3 - The expression pattern of 24 genes expressed in common between Gf-A1 and Gf-A4 revealed by microarray analysis. Time courses during cultivation in Gf-N2 (closed circles), Gf-A1 (closed squares), and Gf-A4 (open squares). Fold-change on each day relative to the expression level in Gf-N2 was designated as 1.

factor among the above-mentioned 24 genes whose expression changed in common relative to wild type in these mutants. Thus, we considered the possibility that Gf.CRZ1 might transcriptionally regulate and affect the expression levels of other genes related to the morphological mutations. *S. cerevisiae* Crz1p is required for the calcineurin-dependent induction of certain proteins, such as the yeast 1,3-beta-D-glucan synthase FKS2, which confers tolerance to high Ca^{2+}, Mn^{2+}, Na^{+}, and cell wall damage, respectively (Matheos et al. 1997). We determined the structure of this gene based on sequence information from the *G. frondosa* in-house genome database and after cloning of the full-length cDNA. The *Gf.CRZ1* gene is comprised of 5 exons and 4 introns (Fig. 4A), and encodes a protein sequence of 345 amino acids with 3 C2H2-type zinc-finger domains (I, II, and III) in its C-terminal region (Fig. 4B). Two of these zinc-finger domains (I and II) have a typical zinc-finger structure containing an α-helix and an antiparallel ß-sheet. However, the third zinc-finger domain (III) lacks an antiparallel ß sheet structure. The C-terminal structure of Gf.CRZ1 was the same as the C terminus of Crz1p in *S. cerevisiae*.

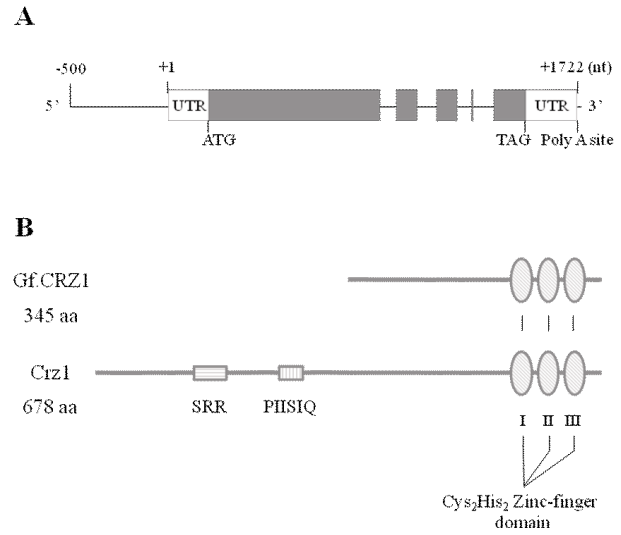
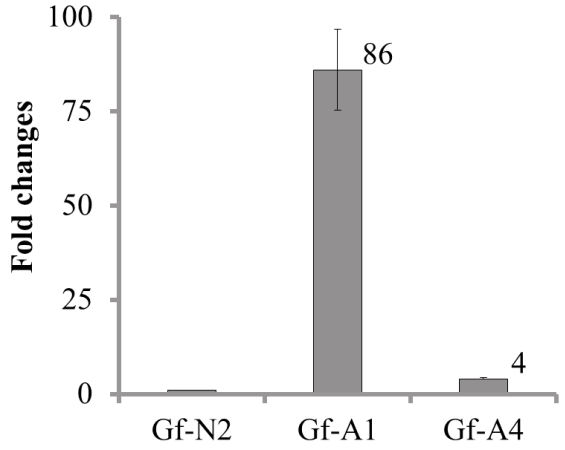


Fig. 4 - The structure of Gf.CRZ1. A: The gene structure of *Gf.CRZ1.* Open boxes indicate untranslated regions and closed boxes indicate exons in the open reading frame. B: The location of 3 zinc-finger domains (I, II, and III) within the primary structures of Gf.CRZ1 and Saccharomyces cerevisiae Crz1p. The SSR (serine-rich region) and calcineurin-docking motifs (PIISIQ) in Crz1p are also labeled.

3.5 Expression levels of Gf.CRZ1 in the starter culture

We measured expression levels of *Gf.CRZ1* in the starter culture of Gf-A1 to verify the potential as a selective marker for mutation. At the result, *Gf.CRZ1* expressed 86-fold and 4-fold in starter culture of Gf-A1 and Gf-A4 compared with Gf-N2 (Fig.5). This result suggests expression levels of *Gf.CRZ1* can be used as markers to discriminate mutant strain.



Fgi. 5 - Expression levels of Gf.CRZ1 in the starter culture of Gf-A1 and Gf-A4

4. Discussion

Industrial cultivation of *G. frondosa* is roughly divided into 3 stages: spawn run, primordia induction, and fruiting body development (Kurahashi et al. 2012). Occasionally, spontaneous mutants appear during production. The fruiting body morphological mutants, Gf-A1 and Gf-A4, were isolated as such spontaneous mutants. Gf-A1 and Gf-A4 showed similar phenotypes during the early stages of fruiting body differentiation.

We expected to identify genes related to fruiting body differentiation or gene responsible for morphological mutations when comparing transcript profiles of the normal fruiting body strain Gf-N2 and the mutants Gf-A1 and Gf-A4. Microarray experiments identified 49 genes that were differentially expressed in Gf-A1 (Table 2) and 69 genes that were differentially expressed in Gf-A4 (Table 3), as compared to Gf-N2. Among these differentially expressed genes, 24 were differentially expressed in common between the Gf-A1 and Gf-A4 mutants (Table 4). We selected 3 genes (*Gf.CRZ1, Gf.FDH1,* and *Gf.AQP1*) as those most enriched for GO terms to confirm expression level data from microarrays (data not shown). Because the expression patterns observed in the microarray data were confirmed by quantitative RT-PCR, the gene expression levels determined based on the microarray data were considered reliable. All differentially expressed genes, including the 24 genes in common between the mutants, were up-regulated relative to Gf-N2. This result suggested that the expression of these genes could be suppressed in during normal fruiting body differentiation in Gf-N2. However, there were greater differences in expression levels of each gene between Gf-A1 and Gf-A4. For example, the C2H2-type zinc-finger transcription factor encoded by the gene *Gf.CRZ1* (*gfr01g0814*) exhibited 211- and 31-fold differential expression in Gf-A1 and Gf-A4. We thought that the differences in phenotype between Gf-A1 and Gf-A4 might result from the different profiles of expressed genes and their expression levels in these mutants.

The 24 genes that were extracted from each list of differentially expressed genes (Fig. 2B, Table 4) as candidates for control of fruiting body development were divided into 4 groups (Groups A-D) based on their differential expression pattern during fruiting

body development in the mutant Gf-A 1. As mentioned above, we hypothesized that all of the genes in Groups A-D might need to be suppressed in order for normal fruiting body differentiation to occur. Moreover, at 86 d, the Group D genes in Gf-A 4 had recovered to wild-type expression levels relative to their earlier highly expressed state. The fruiting bodies of Gf-A 4 develop further than those of Gf-A 1 and differences in their ultimate fruiting body phenotypes finally become apparent after 89 d. Thus, suppression of these Group D genes might be necessary for complete fruiting body development. Unfortunately, it was not possible to determine whether these genes could be involved in fruiting body differentiation simply from their annotations. It will be necessary to dissect the function of these genes in fruiting body differentiation through a gene disruption assay using gene silencing or overexpression techniques.

Gf.CRZ1 was the only gene encoding a transcription factor among 24 genes expressed in common in these morphological mutants. Transcription factors regulate the transcription of target genes by binding to their promoter DNA sequences and enhancing or suppressing transcription. Thus, we focused on the transcription factor *Gf.CRZ1*, as it might affect the transcript levels of the other 23 genes commonly up regulated in Gf-A 1 and Gf-A 4. Gf.CRZ 1 is also a homolog of Crz 1p in *S. cerevisiae*. Crz 1p activates transcription of stress-responsive genes (Stathopoulos AM and Cyert MS 1997) via a calcineurin-mediated signaling pathway. Calcineurin is a conserved Ca^{2+} /calmodulin-dependent protein phosphatase that plays a critical role in Ca^{2+} signaling. Crz 1p is dephosphorylated by calcineurin and translocated to the nucleus from the cytosol (Stathopoulos-Gerontides et al. 1999). We wondered whether Gf.CRZ 1 might bind to similar target DNA sequences as Crz 1p does, because Gf.CRZ 1 possesses the same zinc-finger structure as found in Crz 1p. However, Gf.CRZ 1 lacks the serine-rich region (SRR) and calcineurin docking site that are required for calcineurin dephosphorylation (Fig. 4B). Dephosphorylated Crz 1 p also regulates expression of the mRNA for 1,3-beta-D-glucan synthase *FKS2* by binding a calcineurin-dependent response element in the *FKS2* promoter. However, there was no difference in the mRNA level of the *G. frondosa* homolog of *FKS2* between Gf-N 2 and Gf-A 1. Thus, Gf.CRZ 1 might not be under the control of a Ca^{2+}/calcineurin signaling pathway. In a subsequent study, we plan to explore whether Gf.CRZ 1 controls the 24 genes that are up-regulated in common between Gf-A 1 and Gf-A 4. Therefore, we have been developing an overexpression line, Gf-N 2/+*Gf.CRZ1* to elucidate the relationship between these genes.

Furthermore, we revealed *Gf.CRZ1* can be used as markers to discriminate Gf-A 1 and Gf-A 4 mutant strain in starter culture. However, we only have one marker in the presence. It needs to explorer a lot of markers for effective mutant strain screening.

This is the first report of a large-scale comparative analysis between a normal strain and fruiting body mutants in *G. frondosa*. This work provides lists of candidate genes for fruiting body differentiation. Continuation of this comparative analysis will provide more information for the understanding of fruiting body differentiation in *G. frondosa* and further contribute to the improvement of edible mushroom production.

Acknowledgment

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マイタケ子実体生育不全突然変異体における過剰発現遺伝子の同定

論文要旨

食用きのこであるマイタケ (*Grifola frondosa*)は，工業的に大規模模周年栽培が行われている．そのような所では，しばしば突然変異による子実体の生育不全が見られる．本研究は，これらの突然変異体の子実体分化過程で発現する遺伝子と正常な子実体分化過程で発現する遺伝子の質的・量的差異を分析することで，マイタケの子実体分化に発現を要する遺伝子を見出すことにある．そのために我々は，マイクロアレイを用いて，マイタケM51株の保存系統Gf-N2と子実体生育不全の程度が異なる突然変異体2系統（Gf-A1，Gf-A4）の子実体分化過程で発現する遺伝子の比較解析を行った．

その結果，Gf-N2に比較してGf-A1で49遺伝子，Gf-A4で69遺伝子が過剰に発現していた．さらに，24遺伝子が共通してGf-A1及びGf-A4で過剰発現していた．よって，本研究で用いた突然変異体の子実体生育不全は，これらの遺伝子が子実体分化過程で過剰に発現されることでもたらされた．これら遺伝子の過剰発現は，転写が抑制されるべきところで促進されたと考えられたので，共通する24遺伝子を調べたところ，酵母（*Saccharomyces cerevidiae*）のストレス耐性に関与する転写因子Crz1pのホモログとなるGf.CRZ1をコードする遺伝子が見いだされた．また，*Gf.CRZ1*の発現量は，Gf-N2と比較して子実体原基は形成されるがそれ以上分化しないGf-A1で211倍，子実体は生長するが傘が分化伸展しないGf-A4で31倍となっており，このような発現量の違いが最終的な子実体の表現型の違いに寄与したものと考えられた．