Mycobiology

Research Note

Proteomic Analysis of *Coprinopsis cinerea* under Conditions of Horizontal and Perpendicular Gravity

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Abstract *Coprinopsis cinerea* was employed to investigate the fungal response to gravity. Mycelium growth revealed a consistent growth pattern, irrespective of the direction of gravity (i.e., horizontal vs. perpendicular). However, the fruiting body grew in the direction opposite to that of gravity once the primordia had formed. For the proteomic analysis, only curved-stem samples were used. Fifty-one proteins were identified and classified into 13 groups according to function. The major functional groups were hydrolases and transferases (16%), signal transduction (15%), oxidoreductases and isomerases (11%), carbohydrate metabolism (9%), and transport (5%). To the best of our knowledge, this is the first report on a proteomic approach to evaluate the molecular response of *C. cinerea* to gravity.

Keywords Fruiting body, Gravity, Inky mushroom, Proteome

Coprinopsis cinerea is a model basidiomycete fungus used for many basic research studies, including research of fungal developmental stages. As with other model organisms, *C. cinerea* is easy to maintain, has a short life cycle, and can be induced to develop fruit bodies within the laboratory. *C. cinerea* requires only 2 wk to develop mature fruit bodies [1-3]. Once mycelia have reached full growth stage, *C. cinerea* first develops hyphal knots and thereafter produces primordia followed by the development of fruit bodies. Stalk structures of the fruit body are empty and shaped as thin cylinders. Young fruit bodies contain many glycogen and insoluble proteins as nutrient sources used in the growth and development of the mature fruit bodies [4-6]. Traditionally, the *Coprinopsis* genus has been referred to as an ink cap mushroom, since disintegration of the mushroom results in the development of a black ink when the fruit body has reached full maturation [7-9]. This unique characteristic of *C. cinerea* differs in comparison to other mushrooms, which normally scatter spores by air or raindrops.

All creatures on Earth are affected by and respond to environmental factors, especially gravity. Most biological research involving gravity has to date made use of plant model systems. Parts of the plants that grow above ground show negative gravitropism when compared to the root, which shows positive gravitropism. Gravitropism can be split into three processes, namely gravity perception, signal transduction, and asymmetric growth response [10]. Plants have been shown to recognize gravity through starch-filled cells in the root tissue. The starch-filled hypothesis suggests that plants recognize gravity by the starch particles moving in the direction of the gravity. Starch granule movement is considered to initiate the signal transduction processes in response to gravity [10, 11]. Plants have been reported to recognize gravity through the endodermis layer inside the plant cortex [11, 12]. Research regarding the gravity response and its mechanism of action in fungi has rarely been conducted over past few decades. Therefore, in the present

Mycobiology 2017 September, **45**(3): 226-231
https://doi.org/10.5941/MYCO.2017.45.3.226
pISSN 1229-8093 • eISSN 2092-9323

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Received March 12, 2017
Revised July 17, 2017
Accepted August 23, 2017

study, we employed *C. cinerea* as the model fungus and adopted a proteomics approach to observe the physical and molecular responses of the mushroom to gravity.

C. cinerea was maintained on yeast malt glucose media (yeast extract 3 g, malt extract 3 g, glucose 10 g, agar 20 g per 1 L, pH 6.2) and grown at 30°C. Mycelia were grown on plates for 5 days. For fruit body formation, cultures were incubated at room temperature for 3–4 days under a 12 hr light/dark cycle in either a horizontal or perpendicular orientation. Regardless of the growth conditions, fruit bodies grew in the direction opposite to that of gravity, unlike growth of the hyphae (Fig. 1A). Fruit bodies grown in a perpendicular orientation, grew in a skyward direction and developed bent stalks (Fig. 1B). This demonstrates that the mycelium of *C. cinerea* did not recognize gravity, whereas the fruiting body actively responded to gravity.

Proteomic analyses were undertaken to identify protein expression under each growth condition. To extract protein, only banded-stalks (less than 1 cm) were harvested from mushrooms grown under horizontal and perpendicular conditions. A total of three biological replicates were employed for the two-dimensional gel electrophoresis (2-DE) experiments and for each experiment, 10–12 stalks were harvested for protein extraction. Total protein was extracted using the trichloroacetic acid/acetone/phenol protein extraction protocol, as previously described [13].

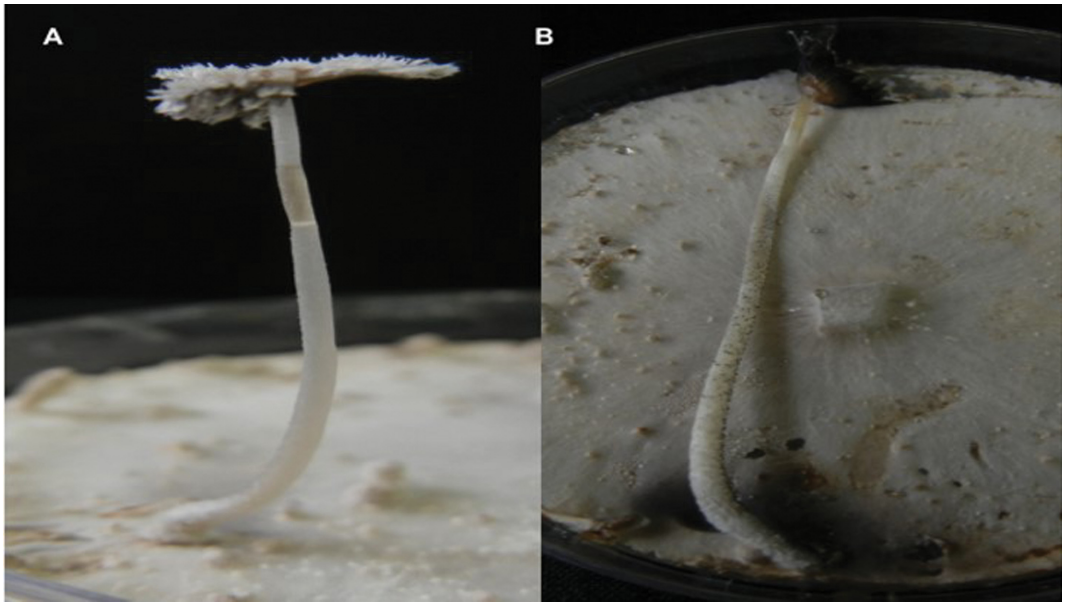

Fig. 1. Formation of fruiting bodies under different gravity growth conditions. A, Horizontal growth conditions; B, Perpendicular growth conditions.

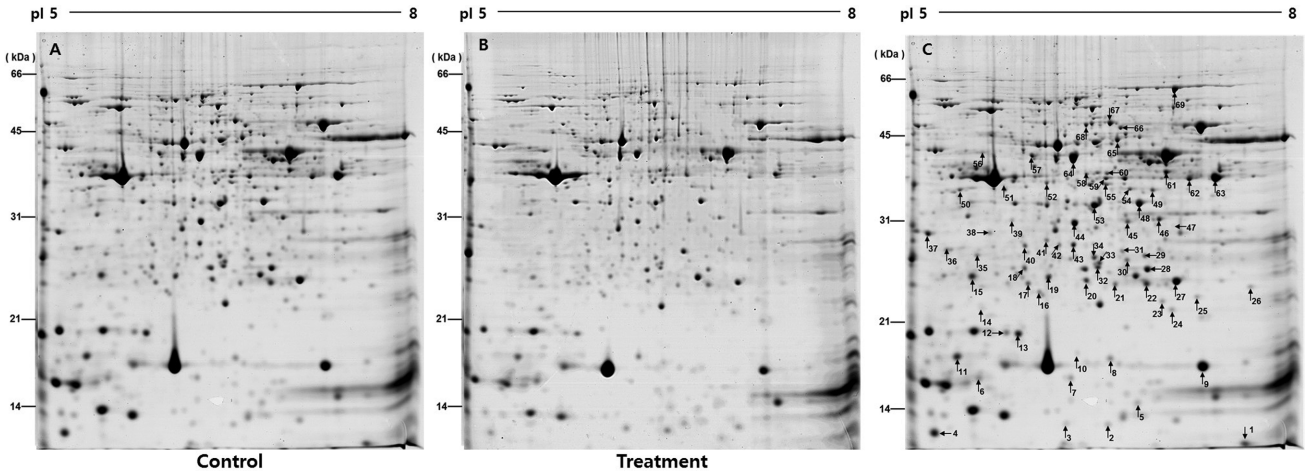

Fig. 2. Representative two-dimensional gel electrophoresis (2-DE) image of *Coprinopsis cinerea*. A, Horizontal growth conditions; B, Perpendicular growth conditions; C, Overlay of the horizontal and perpendicular 2-DE images to identify spots indicating differential protein expression. The numbers on the gel image indicate differentially expressed proteins.

Table 1. Differentially expressed proteins identified under each gravity growth condition by 2-DE and MALDI-TOF MS

Spot ID[a]	Description	Organism	ID (Uniprot)	MW/Pi, Theor.	MP	Peptide hit	SC (%)	Fold change[b]
1	FK506-binding protein 1	*Coprinopsis cinerea* okayama7#130	gi|299755023	12,045/6.56	6	3	48	−3.30
2	Serine-type endopeptidase	*Coprinopsis cinerea* okayama7#130	gi|169846646	53,382/6.34	4	1	11	+1.75
7	Ubiquitin-conjugating enzyme E2	*Coprinopsis cinerea* okayama7#130	gi|299738848	18,635/7.01	7	1	35	−2.11
9	Nucleoside diphosphate kinase	*Coprinopsis cinerea* okayama7#130	gi|169853921	16,622/6.85	5	2	45	−1.80
10	Hypothetical protein CC1G_02494	*Coprinopsis cinerea* okayama7#130	gi|299742077	45,398/5.59	7	4	20	+2.18
11	Eukaryotic translation initiation factor 5A-2	*Coprinopsis cinerea* okayama7#130	gi|169855088	17,577/5.26	3	1	33	−2.03
12	Hypothetical protein CC1G_02672	*Coprinopsis cinerea* okayama7#130	gi|169867256	18,064/5.58	6	2	30	−2.07
13	Nascent polypeptide-associated complex subunit beta	*Coprinopsis cinerea* okayama7#130	gi|169851499	18,100/5.47	10	3	69	−2.59
14	Rho GDP-dissociation inhibitor	*Coprinopsis cinerea* okayama7#130	gi|299747878	23,516/5.45	12	6	61	+1.67
16	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	*Coprinopsis cinerea* okayama7#130	gi|299747867	23,768/5.71	10	4	63	−2.33
18	Hydroxyacylglutathione hydrolase	*Coprinopsis cinerea* okayama7#130	gi|299753690	28,473/5.47	6	3	31	−2.72
19	HHE domain-containing protein	*Coprinopsis cinerea* okayama7#130	gi|169857050	23,475/5.67	12	6	67	−2.76
20	ThiJ/PfpI	*Coprinopsis cinerea* okayama7#130	gi|299747566	24,367/5.85	10	5	51	−2.91
21	Glutathione S-transferase	*Coprinopsis cinerea*	A8P3K1	21,387/9.58	3	1	26	−1.61
22	GTP-binding nuclear protein RAN	*Coprinopsis cinerea* okayama7#130	gi|169852858	24,375/6.44	10	4	54	−2.29
24	Peptidylprolyl isomerase B	*Coprinopsis cinerea* okayama7#130	gi|299752075	34,671/6.02	8	3	24	−1.58
25	Hypothetical protein CC1G_08130	*Coprinopsis cinerea* okayama7#130	gi|169861885	22,740/6.90	3	2	28	−1.70
27	Glutathione S-transferase	*Coprinopsis cinerea* okayama7#130	gi|169863935	24,142/6.31	7	4	46	−2.27
29	Hypothetical protein CC1G_06890	*Coprinopsis cinerea* okayama7#130	gi|169847830	29,188/6.27	12	2	47	−3.00
32	Vacuolar H+ ATPase E1	*Coprinopsis cinerea*	A8NN56	26,084/6.25	7	0	23	−2.24
33	Proteasome subunit alpha type-3	*Coprinopsis cinerea* okayama7#130	gi|299747958	26,942/6.00	9	3	38	−1.93
34	Proteasome subunit alpha type 4	*Coprinopsis cinerea* okayama7#130	gi|299752286	28,694/5.99	10	5	52	−2.21
35	Isopentenyl-diphosphate Delta-isomerase	*Coprinopsis cinerea* okayama7#130	gi|169843960	28,797/5.21	7	2	34	−2.29
37	Ran/spi1 binding protein	*Coprinopsis cinerea* okayama7#130	gi|299747230	23,232/4.96	8	3	40	−2.77
38	Hypothetical protein CC1G_07378	*Coprinopsis cinerea* okayama7#130	gi|169847504	34,298/8.33	7	3	26	−1.50
39	Phytanoyl-CoA dioxygenase	*Coprinopsis cinerea* okayama7#130	gi|299752189	34,619/5.37	10	1	44	−1.67
40	Hypothetical protein CC1G_02958	*Coprinopsis cinerea* okayama7#130	gi|299743595	25,765/5.55	8	2	31	−2.42
42	Hypothetical protein CC1G_02958	*Coprinopsis cinerea* okayama7#130	gi|299743595	25,765/5.55	10	1	37	−1.76

Table 1. Continued

Spot ID[a]	Description	Organism	ID (Uniprot)	MW/Pi, Theor.	MP	Peptide hit	SC (%)	Fold change[b]
43	6-Phosphogluconolactonase	*Coprinopsis cinerea* okayama7#130	gi|169844398	28,531/5.70	13	3	61	−2.49
44	Inorganic diphosphatase	*Coprinopsis cinerea* okayama7#130	gi|169844935	33,738/5.80	14	6	60	−2.43
45	Purine-nucleoside phosphorylase	*Coprinopsis cinerea* okayama7#130	gi|169865712	35,221/6.21	11	5	43	−2.27
46	Glycerol dehydrogenase	*Coprinopsis cinerea* okayama7#130	gi|299741930	34,343/6.32	12	2	37	−2.46
47	Thiazole biosynthetic enzyme	*Coprinopsis cinerea* okayama7#130	gi|299743793	33,446/6.25	7	3	35	+1.91
48	L-malate dehydrogenase	*Coprinopsis cinerea* okayama7#130	gi|299741021	34,551/6.13	16	6	74	−1.67
49	Formate dehydrogenase	*Coprinopsis cinerea* okayama7#130	gi|299752079	41,132/6.32	18	7	55	−1.96
50	Adenosine kinase	*Coprinopsis cinerea* okayama7#130	gi|169845471	36,958/5.14	10	3	41	−4.57
51	Acyl-CoA oxidase	*Coprinopsis cinerea* okayama7#130	gi|169860487	45,960/6.32	16	5	51	+1.87
53	Fructose-bisphosphate aldolase	*Coprinopsis cinerea* okayama7#130	gi|169865684	39,273/5.73	10	1	36	−2.07
54	Septin ring protein	*Coprinopsis cinerea* okayama7#130	gi|169852480	36,130/6.09	18	9	64	−2.04
55	Sranslation initiation factor 3 subunit 3	*Coprinopsis cinerea* okayama7#130	gi|299755630	40,074/6.11	11	6	41	−1.53
56	Phosphatidylserine decarboxylase	*Coprinopsis cinerea* okayama7#130	gi|169864505	50,526/5.27	11	3	28	+1.08
57	Mannose-6-phosphate isomerase	*Coprinopsis cinerea* okayama7#130	gi|169868160	46,988/5.48	7	2	17	−1.92
58	Translation initiation factor 2B subunit I family protein	*Coprinopsis cinerea* okayama7#130	gi|299751092	42,862/6.14	12	6	40	−1.56
59	Aspartate aminotransferase	*Coprinopsis cinerea* okayama7#130	gi|299753536	45,930/5.97	15	10	49	−1.80
62	Acetyl-CoA acetyltransferase	*Coprinopsis cinerea* okayama7#130	gi|299747538	74,673/5.80	10	1	24	−2.39
63	Hypothetical protein CC1G_07378	*Coprinopsis cinerea* okayama7#130	gi|169847504	34,298/8.33	10	5	36	−3.27
65	Aldehyde dehydrogenase	*Coprinopsis cinerea* okayama7#130	gi|169856054	54,634/5.93	21	10	50	−1.59
66	Dihydrolipoyllysine-residue acetyltransferase	*Coprinopsis cinerea* okayama7#130	gi|169844197	48,173/819	16	3	38	−2.11
67	Pyruvate kinase	*Coprinopsis cinerea* okayama7#130	gi|169843774	578,89/5.99	27	9	57	−2.10
68	UDP-N-acetylglucosamine diphosphorylase	*Coprinopsis cinerea* okayama7#130	gi|169845016	546,44/5.81	23	5	47	−3.13
69	5-Methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	*Coprinopsis cinerea* okayama7#130	gi|169861261	846,62/6.22	35	6	56	−2.03

[a]2-DE, two-dimensional gel electrophoresis; MALDI-TOF/TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MW, molecular weight; MP, ratio of matched peptides; SC, sequence coverage.

[b]'+' Up-regulation, '–' down regulation; 1.5-fold change was set as the cut-off for differential protein expression.

Extracted protein concentrations were measured using a 2-DE Quant kit (Amersham Bioscience, San Francisco, CA, USA). For the 2-DE experiments, 200 μg of protein was loaded onto immobilized strips (17 cm, pH 5–8) and subjected to isoelectric focusing for 15 min at 250 V, 3 hr at 8,000 V, and 5 hr at 50,000 V. The equilibrated strips were subsequently separated on 12% polyacrylamide gels at 25 mA at room temperature and the gels stained with Coomassie brilliant blue. The *C. cinerea* fruiting bodies that were grown under either the horizontal or perpendicular conditions were evaluated as three independent, biological replicates. Each gel image was captured using a GS-800 Imaging Densitometer Scanner (Bio-Rad, Hercules, CA, USA) (Fig. 2). The 2-DE image of *C. cinerea* stalks grown in a horizontal orientation served as the control for quantitative analysis of stalks grown in a perpendicular orientation. With the differential expression threshold cut-off set as a change of 1.5-fold, a total of 69 differentially expressed protein spots were identified (Table 1). Irrespective of fold change, 10 proteins were up-regulated and 59 proteins down-regulated when comparing expression levels under perpendicular growth conditions to those under horizontal conditions. These 69 proteins were subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) for further identification (Table 1). With the cut-off for differential expression set as a change of 1.5-fold, 51 proteins were identified as being differentially expressed in the respective gravity states. Under perpendicular growth conditions, six proteins were up-regulated and 45 proteins were down-regulated compared with their expression levels under horizontal growth conditions.

Among the 69 proteins showing significantly different expression levels as a result of the gravity growth conditions, 51 were identified in a protein database. The identified proteins were subsequently classified into 13 groups according to their molecular function. The major functional groups were as follows: hydrolases and transferases (16%), signal transduction (15%), oxidoreductases and isomerases (11%), and carbohydrate metabolism (9%) (Fig. 3). We confirmed that the level of protein expression declined under perpendicular growth conditions, with those proteins showing differential expression under perpendicular growth conditions being primarily related to signal transduction processes. This finding suggests that gravity triggers a molecular response that either increases or decreases the levels of protein expression. Tyrosinases and serine-proteases are also known to be specifically expressed in different stalk and cap tissues during senescence [14]. During the sexual development of *C. cinerea*, xenobiotic detoxification-related proteins (cytochrome p450, glutathione S-transferase, transporter) and several types of proteases are known to be differentially expressed in specific tissues [15]. In the present study, glutathione S-transferase, several transporters, and serine-type peptidases were detected as differentially expressed under the different gravity growth conditions. This suggests that the response of *C. cinerea* to gravity may be related to the fruiting body development mechanism of the fungus.

In conclusion, we identified 51 proteins that were differentially expressed in *C. cinerea* under horizontal and perpendicular growth conditions. The functions of the identified proteins were related to hydrolases and transferases, signal transduction, oxidoreductases and isomerases, and carbohydrate metabolism. This study, which examined fungi gravitropism through a proteomic analysis of *C. cinerea*, will help contribute towards our understanding of fungal responses to gravity.

ACKNOWLEDGEMENTS

This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (Project No. 315004-5).

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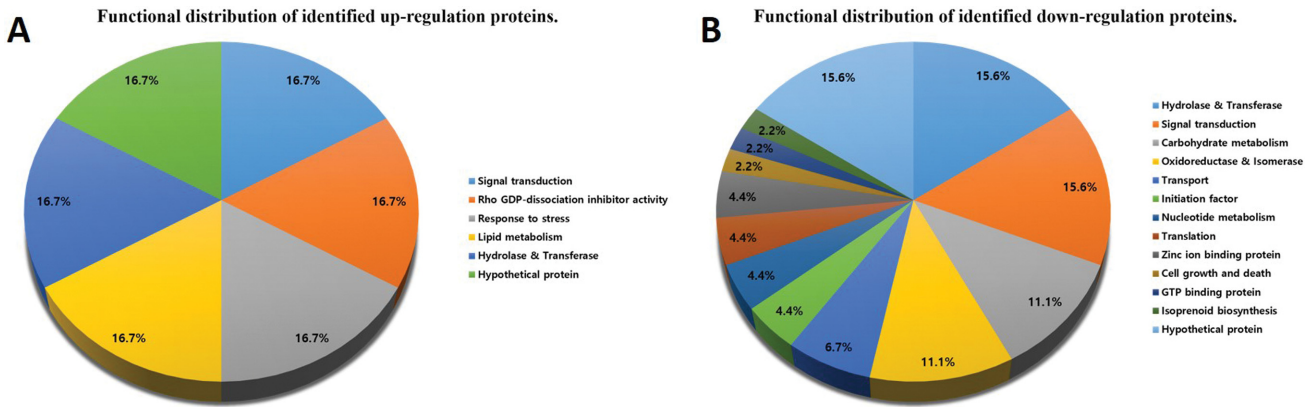

Fig. 3. Functional classification of proteins that were differentially expressed under each growth condition. A, up-regulated proteins; B, down-regulated proteins.

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