Potential molecular mechanisms for fruiting body formation of Cordyceps illustrated in the case of Cordyceps sinensis

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ABSTRACT
The fruiting body formation mechanisms of Cordyceps sinensis are still unclear. To explore the mechanisms, proteins potentially related to the fruiting body formation, proteins from fruiting bodies, and mycelia of Cordyceps species were assessed by using two-dimensional fluorescence difference gel electrophoresis, and the differential expression proteins were identified by matrix-assisted laser desorption/ionisation tandem time of flight mass spectrometry. The results showed that 198 differential expression proteins (252 protein spots) were identified during the fruiting body formation of Cordyceps species, and 24 of them involved in fruiting body development in both C. sinensis and other microorganisms. Especially, enolase and malate dehydrogenase were first found to play an important role in fruiting body development in macro-fungus. The results implied that cAMP signal pathway involved in fruiting body development of C. sinensis, meanwhile glycometabolism, protein metabolism, energy metabolism, and cell reconstruction were more active during fruiting body development. It has become evident that fruiting body formation of C. sinensis is a highly complex differentiation process and requires precise integration of a number of fundamental biological processes. Although the fruiting body formation mechanisms for all these activities remain to be further elucidated, the possible mechanism provides insights into the culture of C. sinensis.

Introduction

Cordyceps is a large genus of entomogenous fungi with more than 400 species found world-wide, and the most famous and valuable species is Cordyceps sinensis (Berk.) Sacc. (Li et al. 2006a). Wild C. sinensis is also known as “Dong Chong Xia Cao” in Chinese or “Yartsa gunbu” in Tibetan, which means “Winter Worm Summer Grass” because of their appearance in different seasons (Paterson 2008) (Figure 1). C. sinensis has multiple beneficial effects on hepatic, renal, cardiovascular, immunologic, and nervous systems (Wang and Shiao 2000; Paterson 2008), and been used as highly prized herbal medicine and healthy food. Natural C. sinensis is found only in the soil of a prairie at an elevation of 3 000 to 5 000 m mainly in Tibet, Qinghai, Gansu, Sichuan, and Yunnan provinces in China. The worldwide demand for natural C. sinensis has been increasing continuously. With the reckless exploration, the annual harvest has been decreasing rapidly and resulting in serious habitat destruction (Li et al. 2011). The price of C. sinensis reached USD 13,000 per kg in 2008–2009 (Au et al. 2011), and the top quality C. sinensis rocketed up to USD 32,000 per kg in Hong Kong and San Francisco in late 2006 (Winkler 2008). Therefore, the cultured C. sinensis becomes an urgent need and inevitable trend. After several decades of efforts, 572 fungal strains of more than 37 genera have been isolated from natural C. sinensis (Zhang, Zhang et al. 2010, Zhang, Sun et al. 2010). Generally, the fungus of Hirsutella sinensis X.J. Liu, Y.L. Guo, Y.X. Yu & W. Zeng is recognised as the anamorph of C. sinensis (Chen et al. 2004; Li et al. 2006a; Zhong et al. 2010). Up to date, the molecular mechanisms, which are critical for cultivation of C. sinensis, of fruiting body development of C. sinensis are still unknown.

Proteins usually play important biological roles in regulating metabolic processes, signal transduction,
small molecule or ion transportation, cell replication, and apoptosis (Gauci et al. 2011). The identification of differentially expressed proteins during fruiting body development could improve better understanding of *C. sinensis* formation. Proteomics is aimed at the large-scale and systematic characterisation of the entire protein complement of a cell line, tissue, or organism at a particular time, under a particular set of conditions (Graves and Haystead 2002; Beranova-Giorgianni 2003; Giepmans et al. 2006). Proteomics can be used as an important tool in helping to elucidate mechanisms of biological processes in a high-throughput mode. Classical two-dimensional electrophoresis (2DE) for protein isolation coupled with protein spot identification by mass spectrometry is the most widely adopted approach in proteomics studies (De Roos and McArdle 2008), but traditional 2DE is time-consuming, labour-intensive, limited sensitivity and prone to experimental errors, so this approach requires several replicate runs to overcome the gel-to-gel variations (Minden 2007; Chevalier 2010). In order to overcome the limitations of 2DE, a modified 2DE technique called fluorescence difference gel electrophoresis (DIGE) has been developed for direct quantitative measurements among differentially labelled samples using cyanine fluorescent dyes prior to gel electrophoresis and it is more accurate, sensitive, confident, reproducible and not limited by the distortion from gel-to-gel variation (Van Den Bergh and Arckens 2004; De Roos and McArdle 2008; Muroi et al. 2010).

To date, little has been known for the fruiting body formation mechanism and proteome of *C. sinensis* (Jin 2005; Kao 2006). The objective of this study is to unveil the fruiting body formation mechanism of *C. sinensis* as well as its related species based on differential protein expression of the fruiting body, sclerotium of *C. sinensis* and mycelium of *H. sinensis*, mature (late stage), immature (early stage) fruiting bodies and mycelium of *Paecilomyces militaris* (Kob.) Brown & Smith ex Liang, anamorph of *C. militaris* (L.: Fr.) Link (Liu et al. 2002), as well as fruiting body and mycelium of *Isaria farinose* (Holm ex S.F. Gray) Fr., anamorph of *C. memorabilis* (Ces.) Sacc. (Zimmermann 2008).

**Materials and methods**

**Natural *C. sinensis*, fungal strains and materials**

Natural fresh *C. sinensis*, including the fruiting body and sclerotium, were collected from Huzhu County, Qinghai Province of China. The fungal strain of *H. sinensis* was purchased by the Institute of Microbiology of Chinese Academy of Sciences, China. Fungal strain of *P. militaris* (anamorph of *C. militaris*) was gift from Zhangjiagang City Zanglian Biotechnology Co., Ltd., Jiangsu
Province, China; and strain *Isaria farinosa* (anamorph of *C. memorabilis*) was isolated from infected caterpillar provided by Qinghai Academy of Animal and Veterinary Science, China, which was identified by the Institute of Microbiology of Chinese Academy of Sciences, China. Golden rabbit Thai fragrant rice purchased from San Miu Supermarket Limited in Macao, China; foxtail millet (*Setaria italica*) obtained from Yilan County Seed Company, Heilongjiang Province of China; silkworm larvae and silkworm pupa powder bought from Sericulture and Farm Produce Processing Research Institute, Guangzhou, China; mould liquid medium purchased from Guangdong Huankai Microbial Sci. & Tech. Co., Ltd., China.

**Fungal culture conditions and media**

The fungi were cultured as the method described in our previous report (Feng et al. 2009) with modification. In brief, the fungal strains in tube slant were implanted into improved mould liquid medium (16.6 g mould liquid medium, the extract of 200.0 g fresh potato, and 2.0 g yeast extract in 1 l with Milli-Q water, pH 5.6). Conical flasks (500 ml) containing 150 ml of medium were inoculated with purified colony and incubated in an C24KC refrigerated incubator shaker (New Brunswick Scientific, USA) under 150 RPM at 16°C for *H. sinensis* or 22°C for *P. militaris* and *I. farinosa* until plentiful mycelia balls presented. The mycelia were harvested by centrifugation, washed twice with sterile PBS buffer, and stored at 4ºC after lyophilisation. The fruiting body of *C. militaris*, *C. memorabilis* was cultured in improved rice medium (290 g Golden rabbit Thai fragrant rice, 290 g foxtail millet (*Setaria italica*), 50 g silkworm pupa powder, 16.6 g mould liquid medium and 1 l distilled water) sterilised at 121ºC for 30 min. Each cultivation bottle was inoculated with 8 ml of liquid seed and incubated in the dark at 22°C for *C. militaris* and *C. memorabilis* with humidity levels of 70%, respectively. When the mycelia completely colonised the jar, the jar was exposed to fluorescent lamp (about 200 lx). After 3 to 5 days, the temperature was set a cycle of 22°C for 12 h, and 12°C for 12 h under a 12 h light/dark cycle condition to promote primordia formation. Once a large number of primordia were produced, the temperature was kept at 22°C, and the relative humidity was kept at around 85% under a 12 h light/dark cycle condition for the formation of the fruiting body. The early and late stage fruiting bodies of *C. militaris* were cultured in fifth instar larvae of silkworm. In brief, the 5th instar silkworm larva was surface sterilised with medical povidone-iodine swabs and then 0.3 ml of mycelial homogenate was injected under aseptic conditions. The inoculated larvae were fed with fresh mulberry leaves at 22°C in the dark with 70% relative humidity. After the larva grew into stiff silkworm, the cultivation conditions were the same as the fruiting body of *C. militaris* grown on solid medium. When the fruiting body grew to approximate 2 cm (early stage) or produced spores (late stage), they were harvested and stored at −80°C.

**Extraction of proteins**

The investigated materials (mycelia, worm or the fruiting body) were ground to a fine powder in liquid nitrogen using a mortar and pestle, added lysis buffer (containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 0.5% (v/v) IPG buffer pH 3–10, and 1 mM PMSF, from GE Healthcare) based on the modified Handbook 80–6429-60AC (GE Healthcare), and continued to grind to homogenate. The homogenate was transferred to a 1.5 ml Eppendorf tube and frozen in liquid nitrogen for 3 min, and then it was thawed in 37°C water for 3 min. For fully extracting the intracellular proteins, this step was repeated three times. After centrifugation at 28,113 × g for 30 min at 4°C, the supernatant was transferred to new tubes.

**Clean-up proteins**

Proteins were purified with a 2-D Clean-Up Kit (GE Healthcare) according to the manufacturer’s instructions, revision 80–6486-60/Rev. CO/11–02. Briefly, proteins solution (200 μl) was mixed well with 600 μl of precipitant and incubated for 15 min on ice, then 600 μl co-precipitant was added and centrifuged at 28,113 × g for 5 min at 4°C. Added co-precipitant 4 times the size of the pellet after removing the supernatant, next
centrifuged at 28,113 × g for 5 min. Pipetted enough Milli-Q water to disperse the pellet, and added 1 ml of pre-chilled wash buffer and 5 μl wash additive at −20°C for at least 30 min, vortexed for 20–30 s once every 10 min. The mixture was further centrifuged at 28,113 × g for 5 min at 4°C, and the supernatant was discarded and the pellet was allowed to dry briefly. The pellet was solubilised in lysis buffer without DTT and IPG buffer. Lastly, the protein solution was centrifuged at 28,113 × g for 20 min at 4ºC, and the supernatant was collected and stored at −80ºC.

Prior to quantification, pH of protein samples was adjusted to 8.5 by using 1 M NaOH, as monitored by the pH Test Strip (4.5–10.0, Sigma). Finally, protein concentrations were determined with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) using BSA (2 mg ml⁻¹) as the standard.

Labelling of proteins with CyDye

All steps were operated in dark room. The proteome samples were labelled for DIGE analysis using Cy2, Cy3 and Cy5 CyDye™ DIGE Fluor minimal dye (GE Healthcare), respectively, according to the manufacturer manual (GE Healthcare). Cy2 was used to label an internal standard which was pooled equal amounts of each of all samples. Each 50 μg protein sample was labelled at a ratio of 400 pmol of dye on ice for 30 min, and the labelling reaction was terminated by adding 1 μl of 10 mM lysine and left on ice for 15 min. The three labelled samples were mixed into a single tube, and then both of extra 300 μg paired protein samples in a gel were added to the same tube, thus total of 750 μg protein samples were mixed in the tube and later could be used as preparative gel for spots picking. Equal volumes of 2× sample rehydration buffer (7 M urea, 2 M thiourea, 2% DTT, 4% CHAPS, 1% pH 3–10 NL IPG buffer (GE Healthcare), and 0.004% bromophenol blue) was added to the protein samples. Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.5% IPG buffer, and 0.004% bromophenol blue from Bio-Rad) was added to reach volumes to 450 μl for rehydration.

Two-dimensional electrophoresis

The mixture was transferred to IPGbox (GE Healthcare), and ReadyStrip IPG Strips (24 cm, pH 5–8 from Bio-Rad) was put on the mixture with the gel side down. The gel was covered with DryStrip Cover Fluid (GE Healthcare), and rehydrated for 18 h at 20°C. After rehydration, the IPG strip was transferred to Ettan IPGphor Manifold of Ettan IPGphor 3 Isoelectric Focusing Unit (GE Healthcare). The first dimension isoelectric focusing (IEF) separation of 2DE was performed at 20°C with following sequential steps: 50 V rapid for 4 h; 150 V gradient for 2 h; 250 V gradient for 2 h; 500 V gradient for 2 h; 1,000 V gradient for 3.5 h; 5,000 V rapid for 1.5 h; 8,000 V rapid for 2 h; 10,000 V rapid for 70,000 Vh. After IEF, the strips were equilibrated in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and 0.01% bromophenol blue with the addition of 2% DTT for 15 min in the dark. Subsequently, the strips were equilibrated with the same buffer with 2.5% (w/v) iodoacetamide instead of DTT for 15 min in the dark. Prior to preparation of SDS-polyacrylamide gels, the longer low fluorescent glass plate was painted with PlusOne™ Repel-Silane ES (GE Healthcare) to assure gel release; while the shorter glass plate was painted with 4 ml PlusOne™ Bind-Silane (GE Healthcare) in 1 ml of acidic ethanol (0.5% acetic acid in 95% ethanol) to covalently attach the polyacrylamide gel to glass surface. SDS-PAGE was performed as second dimensional separation in 12.5% acrylamide gels in an Ettan™ DALT Six-Large Vertical System (GE Healthcare). The electrophoresis was performed at 15 mA per gel for 40 min, then 30 mA per gel until the bromophenol blue line reached the bottom of the gel at 10°C in the dark.

Imaging and analysis

Cy2-, Cy3- and Cy5-labelled samples were acquired in an Ettan DIGE Imager (GE Healthcare) according to the manufacturer’s instructions. The images were checked for intensity during the acquisition process using ImageQuant™ TL software (GE Healthcare), and analysed by using DeCyder™ 2-D Differential Analysis Software v7.0 (GE Healthcare). After analysis, the gels were stained in 0.1% Coomassie Brilliant Blue-R250 solution in 50% ethanol and 10% acetic acid for 2.5 h, and destained in 25% ethanol and 8% acetic acid for 1.5 h. Then, they were washed with Milli-Q water and scanned on a UMAX PowerLook 2100XL scanner (UMAX Technologies). Protein spots at least
2-fold differences in expression level were automatically and accurately excised into 96-cell plates using Ettan Spot Picker (GE Healthcare). All parameters were set according to the manufacturer’s protocol.

**In-gel digestion and protein identification by MALDI TOF/TOF MS**

Each excised protein slice was washed with 100 μl Milli-Q water (twice, for 2 × 5 min) on ice, and destained with 80 μl of 50 mM ammonium bicarbonate (Sigma)/acetonitrile (ACN, Sigma) (1:1) for 30 min at 37°C. The gel piece was incubated in 70 μl of acetonitrile until it was white and sticky. After removing solution, the gel piece was rehydrated in 2 μl of trypsin solution (2 μg Promega’s Sequencing Grade Modified Trypsin mixed with 140 μl of 25 mM ammonium bicarbonate containing 10% acetonitrile) on ice for 20 min. Excess trypsin was removed, the gel piece was covered with 20 μl of 25 mM ammonium bicarbonate containing 10% acetonitrile, and trypsinised at 37°C for 16 h. After digestion, the peptides were transferred into a new PCR tube, and the pellet was extracted with 30 μl of 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich) in 67% acetonitrile at 37°C for 30 min, subsequently was ultrasonicated for 20 min at room temperature. This step was repeated again. Total extracts and the first digested peptides were mixed, vacuum-dried, and dissolved in 2 μl of 0.1% TFA in 30% acetonitrile. After in-gel digestion, 0.4 μl of the supernatant was spotted onto a MALDI plate (Opti-TOF™ 384-well Insert, Applied Biosystems), and 0.4 μl of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA) were added to the peptide and allowed to air-dry at room temperature. Tryptic peptides of 0.5 μl were analysed using a 4800 plus MALDI TOF/TOF Analyser (Applied Biosystems) with positive ion reflection mode, and standards (ABI 4700 Calibration Mixture, Applied Biosystems) were conducted to calibrate the spectrum to a mass tolerance within 0.1 Da. The parameters for database searching were peptide tolerance of 80–150 ppm; MS/MS tolerance of 0.2–0.3 Da; one missed cleavage; variable modifications of carbamidomethyl (Cys), oxidised (Met). GPS Explorer™ software v3.6 (Applied Biosystems) was used to search files in the National Centre for Biotechnology non-redundant (NCBInr) all species database, fungus database and insect database. All the identified proteins have MASCOT report total protein score C.I.% or total ion C.I.% greater than 95% and identification probability score at p < 0.05.

**Results and discussion**

**Proteins in fruiting bodies, sclerotia and mycelia of Cordyceps species**

**Differential proteins in the fruiting body/sclerotia and fruiting body/mycelia from natural C. sinensis**

Natural worm without infection of fungus of Cordyceps is difficult to obtain for the proteomics investigation due to specific life cycle of C. sinensis and the habitat. Therefore, the fruiting body and sclerotia (dead larvae) from natural C. sinensis were used, and 2100 spots were detected in both the fruiting body and sclerotium (Figure 2). The number was much more than 18 spots in natural C. sinensis (Jin 2005). Among the detected spots, 639 (30.4%) and 626 (29.8%) spots were up-regulated and down-regulated, respectively, in fruiting bodies of natural C. sinensis (Threshold mode: 2.0-fold). Among the detected proteins, only 62 proteins in 70 spots (36.8%) out of 190 picked spots (Figure 3) were successfully identified. The most likely reason for low ratio identification attributed to the limited genome or proteome database of C. sinensis, which is the greatest challenge for the study on proteome of C. sinensis. Indeed, no spot was identified except one protein, which was found in the fruiting body of natural C. sinensis rather than mycelia of Hirutella sinensis, had high similarity to protein of hypothetical protein AN8043.2, putative fimbrial usher or UDP-N-acetylglucosamine pyrophosphorylase according to 10 amino acids sequence of its N-terminal (Jin 2005). Our results confirmed that UDP-N-acetylglucosamine pyrophosphorylase compared to sclerotium was up-regulated in the fruiting body of C. sinensis. Besides, only serine protease among the identified proteins was reported in an entomopathogenic fungus CS2 from C. sinensis (Zhang et al. 2008). The other proteins were firstly identified in C. sinensis. For the identified proteins, 6 proteins, such as serine protease, GAF domain protein, predicted similar to
Figure 2. 2-D DIGE images of proteins from Cordyceps materials labeled with Cy3 (green in online version) or Cy5 (red in online version) and their merge (color in online version) and statistical analysis of differentially expressed spots. A. Fruiting body (red in online version) versus sclerotia (dead larvae, green in online version) of *C. sinensis*. B. Fruiting body (red in online version) of *C. sinensis* versus mycelia (green in online version) of *H. sinensis*. C. Later (red in online version) versus early (green in online version) stage fruiting body of *C. militaris*. D. Fruiting body (red in online version) versus mycelia (green in online version) of *P. militaris*. E. Fruiting body (red in online version) versus mycelia (green in online version) of *I. farinosa*.

For gel image, pH, 5 to 8 linear from left to right; mass, ~100 kDa to ~10 kDa from top to bottom.
Actin-5C isoform 1, actin 6, beta actins, heat shock 70 kd protein cognate 1, were down-regulated, while 53 proteins, including acetaldehyde dehydrogenase, enolase, tubulins, eukaryotic initiation factor 4A, elongation factor 2, elongation factor 3, cobalamin-independent methionine synthase, fructose-bisphosphate aldolase, inorganic pyrophosphatase, UTP-glucose-1-phosphate uridylyltransferase, vacuolar ATP synthase catalytic subunit A, malate dehydrogenase, O-acetylhomoserine sulfhydrylase, mannose-1-phosphate guanyltransferase, septin, rab GDP-dissociation inhibitor, T-complex protein 1 subunit zeta, heat shock 70 kDa protein, putative Hsp70 chaperones, etc. were up-regulated in the fruiting body of *C. sinensis* (Table 1).

Though *Hirsutella sinensis* is usually considered as the anamorph of *C. sinensis* (Chen et al. 2004; Li et al. 2006a; Zhong et al. 2010), cultivation of its fruiting body is still very difficult. Alternatively, proteomic comparison of the fruiting body from natural *C. sinensis* and mycelia of *H. sinensis* was determined to explore the potential proteins related to the formation of the fruiting body. There were 1983 protein spots detected in both the fruiting body of natural *C. sinensis* and mycelia of *H. sinensis*, which was also much more than previous reports, 188 spots in mycelia of *H. sinensis* (Jin 2005) and 630 spots in mycelia of an isolated fungal strain of *C. sinensis* (Kao 2006). Among the detected spots, 559 (28.2%) and 401 (20.2%) spots, respectively, were up-regulated and down-regulated in fruiting bodies of natural *C. sinensis* (Threshold mode: 2.0-fold) (Figure 2), and 64 proteins in 69 protein spots (35.9%) out of 192 picked spots (Figure 3) were successfully identified (Table 1). There was no spot was identified in previous report (Kao 2006).

**Differential proteins in late/early stages of the fruiting body and fruiting body/mycelia of *C. militaris***

*C. militaris* is a major species of *Cordyceps* widely used in the market. The fruiting body of *Paecilomyces militaris*, anamorph of *C. militaris*, is easily formed in cultured media. It is great help to know the proteins expression during fruiting body formation based on the investigation of proteins in mycelia, early and late stages of the fruiting body of *C. militaris*. As a results, 2175 protein spots were detected in both early and late stage fruiting bodies of *C. militaris*, 295 (13.6%) and 234 (10.8%) out of the detected spots were up-regulated and down-regulated in the late stage fruiting body, respectively (Threshold mode: 2.0-fold) (Figure 2). Among 95 picked spots (Figure 3), 40 proteins in 48 protein spots (50.5%) were
successfully identified (Table 1). On the other hand, there were 2227 protein spots detected in both the fruiting body and mycelia of *P. militaris*, and 523 (23.5%) and 449 (20.2%) of detected spots were up-regulated and down-regulated, respectively, in the fruiting body of *C. militaris* (Threshold mode: 2.0-fold) (Figure 2). Finally, 33 proteins in 40 protein spots (44.4%) out of 90 picked spots (Figure 3) were successfully identified, which included 18 up-regulated and 22 down-regulated proteins in the late stage fruiting body of *C. militaris* (Table 1).

**Differential proteins between the fruiting body and mycelia of *C. memorabilis***

*C. memorabilis* is one of the species of *Cordyceps* genus. The fungus, *Isaria farinose*, anamorph of *C. memorabilis*, could form the fruiting body under laboratory conditions. Therefore, comparison of proteins in the fruiting body and mycelia of *I. farinose* is also helpful to well understand the molecular mechanism of formation of natural *C. sinensis*. By DIGE analysis, 2275 protein spots were detected in both the fruiting body and mycelia of *I. farinosa*. Among the detected spots, 554 (23.9%) and 430 (18.9%) spots were, respectively, up-regulated and down-regulated in the fruiting body (Threshold mode: 2.0-fold) (Figure 2), and 23 proteins in 25 protein spots (29.8%) out of 84 picked spots (Figure 3) were successfully identified (Table 1).

Totally, 115 differential expression proteins in 134 protein spots were found in both fruiting bodies and mycelia of three species of *Cordyceps* (*C. sinensis*, *C. memorabilis*, and *C. militaris*). It was worth to note that enolase/putative enolase up-regulated, while ATP synthase down-regulated coincidentally in all fruiting bodies of *Cordyceps*.

For natural *C. sinensis*, acetaldehyde dehydrogenase, beta-tubulin, elongation factor 2, enolase, malate dehydrogenase, heat shock 70 kDa protein and hypothetical protein FG09893.1 were simultaneously up-regulated in both fruiting bodies of *C. sinensis* versus sclerotia and fruiting bodies of *C. sinensis* versus mycelia of *H. sinensis*. The reasons may arise from different samples (natural sclerotia and cultured mycelia) and a small probability of picking the same spot in different gels under blind screening (the picked spot must possess simultaneously higher differential expression and intact three-dimensional separation map).

**Biological activities of proteins during fruiting body formation of *C. sinensis***

Fruiting body formation of filamentous fungi is one of the most complex developmental processes. It not only requires the aggregation of hyphae to form three-dimensional structures, and leads to the differentiation of a number of fruiting bodies-specific cell types not present in the vegetative mycelium (Nowrousian et al. 2007), but also requires precise integration of a number of fundamental biological processes under special environmental conditions and is controlled by many developmentally regulated genes (Pöggeler et al. 2006).

**Camp signal pathway in fruiting body formation of *C. sinensis***

Two cytoplasmic signalling branches, the cAMP-dependent protein kinase (PKA) and mitogen-activated protein kinase (MAPK) pathway, regulate gene expression that finally leads to fruiting body formation. Indeed, MAPK genes are required for fruiting in *Aspergillus nidulans*, *Neurospora crassa*, and *Lentinula edodes*, where MAPK kinase (Demek et al. 1997), MEK kinase (MEKK) and MAPK involve in the
Table 1. Differentially expressed proteins during fruiting body formation of *Cordyceps* were identified by MALDI-TOF/TOF MS.

| No. | Protein Name | Group | ID | Volume | Accession | MP | Protein Score | Total Ion Score |
|-----|--------------|-------|----|--------|-----------|----|---------------|----------------|
| 1   | Heat shock protein 90 | A/A/C/C/D | 106/740/64/39/39 | –2.05/1.33/3.39/4.19/-2.04 | gi|194,716,766 | 79.3 | 4.90 | 21/146/10/93/90/99.995 | 99.999/99.999/99.999/99.999/99.999 |
| 2   | Hypothetical protein | A/A/B | 152/154/155/75 | 5.21/4.94/3.24/-2.29 | gi|46,107,948 | 60.3 | 4.94 | 7/32/12/144 | 60.68/55.888/100/100 | 65/107/110/115 |
| 3   | Cobalamin-independent methionine synthase (Epicibie festucae) | A/A/C/C | 94/158/36/72 | 9.51/6.49/2.49/2.38 | gi|34,500,101 | 77.3 | 6.31 | 129/113/173/118/86 | 100/99.996/99.982/100 | 25/115/26/0/100 |
| 4   | HST7, NEBCR Heat shock 70 kDa protein (HSST7) (Gibberella zeae PH-1) | A/A/C | 64/77/85 | 15.52/12.79/-2.49 | gi|146,107,910 | 71.1 | 5.00 | 367/343/375 | 100/100/100 | 258/256/271 |
| 5   | Hypothetical protein | A/B/B | 67/154/160 | 14.68/4.21/19.63 | gi|85,136,755 | 52.4 | 5.25 | 16/150/176 | 100/100/100 | 106/102/105 |
| 6   | Malate dehydrogenase, mitochondrial precursor (Neurospora crassa OR34A) | A/B/C | 145/51/20 | 7.19/20.14/2.78 | gi|85,105,459 | 34.3 | 5.56 | 136/182/162 | 100/100/100 | 90/124/187 |
| 7   | UTP-glucose-1-phosphat uridlytransferase (Neurospora crassa OR34A) | A/A | 93/192 | 9.88/2.37 | gi|64,427,705 | 58.2 | 6.99 | 167/157 | 100/100 | 89/63 |
| 8   | HSP70 chaperone (HsA), putative (Tabellinomyces stipatus ATCC 15090) | A/A | 95/102 | 9.25/8.07 | gi|242,798,753 | 52.7 | 5.94 | 253/194 | 100/100 | 217/152 |
| 9   | Beta actin (Mamestra briseis) | A/A | 175/176 | –6.03/7.60 | gi|35,927,213 | 41.8 | 5.23 | 431/372 | 100/100 | 253/195 |
| 10  | Enolase BAC2349-Penicilium chestriogenum (Penicillium chstrijogenum Wisconsin 54-1235) | A/B | 91/177 | 10.21/0.23 | gi|255,938,796 | 47.2 | 5.26 | 157/185 | 100/100 | 95/131 |
| 11  | MPJ, TRIFEc Reckline: Full = Mannose-1-phosphate guanylyltransferase | A/B | 203/132 | 5.46/2.65 | gi|74,582,903 | 40.3 | 6.23 | 90/160 | 99.26/100 | 18/105 |
| 12  | Heat shock 70 kDa protein cognate 1 (Magnumothripes optatus 30-13) | A/C | 184/65 | –3.18/3.93 | gi|45,605,667 | 57.0 | 4.95 | 115/151 | 100/100 | 58/47 |
| 13  | Rab GDP-dissociation inhibitor (Neurospora crassa OR34A) | A/C | 54/67 | 19.26/2.56 | gi|85,105,909 | 51.4 | 5.33 | 97/137 | 99.991/100 | 45/117 |
| 14  | Hypothetical protein | A/E | 53/71 | 19.96/2.39 | gi|66,125,109 | 46.9 | 6.52 | 82/88 | 95.689/98.637 | 63/49 |
| 15  | Tubulin beta chain, ATHname Full = Beta-tubulin | A | 45 | 44.92 | gi|135,480 | 50.0 | 4.36 | 367 | 100 | 99 |
| 16  | Hypothetical protein | A | 58 | 17.48 | gi|255,144,188 | 196.6 | 6.00 | 83 | 96.31 |
| 17  | Heat shock protein 90 (Methanobium omelceni) | A | 59 | 17.38 | gi|85,766,397 | 80.1 | 4.98 | 149 | 100 | 35 |
| 18  | Bifunctional inhibition factor 4A (Sclerotinia sclerotiorum) | A | 60 | 17.09 | gi|56,107,455 | 44.9 | 5.14 | 150 | 100 | 64 |
| 19  | Hypothetical protein (Podospora anserina 5 mat +) | A | 63 | 16.56 | gi|71,690,144 | 54.0 | 5.77 | 57 | 83.850 | 48 |
| 20  | Acetaldehyde dehydrogenase (Ophiocordyceps heteropoda) | A | 72 | 13.80 | gi|18,596,330 | 32.0 | 7.75 | 147 | 100 | 86 |
| 21  | Beta-tubulin (Ophiocordyceps heteropoda) | A | 74 | 13.61 | gi|59,894,499 | 36.3 | 5.62 | 171 | 100 | 99 |
| 22  | UDP-arabinosepyrophosphorylase (Neurospora crassa OR34A) | A | 75 | 13.25 | gi|85,111,786 | 53.6 | 5.19 | 124 | 100 | 87 |
| 23  | Vacuolar ATP synthase catalytic subunit A (Neurospora crassa OR34A) | A | 76 | 12.97 | gi|85,103,674 | 67.1 | 5.32 | 187 | 100 | 108 |

(Continued)
| No. | Protein Name | Group | Accession | Mr (kDa) | Protein Score | Total Ion Score | Total Ion C.I. % |
|-----|--------------|-------|-----------|----------|---------------|-----------------|-----------------|
| 24  | Elongation factor 3 | A     | gi|85,107,753 | 117.0 | 5.83 | 82 | 95.689 | 18 | 0 |
| 25  | Fructose-bisphosphate aldolase | A     | gi|9,837,587  | 13.4  | 6.82 | 97 | 99.847 | 74 | 0 |
| 26  | Conserved hypothetical protein | A     | gi|15,606,158 | 59.8  | 5.23 | 72 | 99.465 | 17 | 0 |
| 27  | Hsp70 chaperone BiP/Kar2, putative | A     | gi|77,689,612 | 45.6  | 5.25 | 94 | 99.673 | 30 | 0 |
| 28  | Conserved hypothetical protein | A     | gi|12,506,989 | 64.5  | 5.61 | 30 | 98.957 | 30 | 0 |
| 29  | Insect origin recognition complex subunit, putative | A     | gi|22,070,172 | 20.8  | 9.51 | 82 | 95.273 | 30 | 0 |
| 30  | Hypothetical protein | A     | gi|16,200,812 | 45.6  | 5.25 | 94 | 99.673 | 30 | 0 |
| 31  | Hypothetical protein | A     | gi|21,689,612 | 45.6  | 5.25 | 94 | 99.673 | 30 | 0 |
| 32  | Conserved hypothetical protein | A     | gi|116,200,814 | 56.8  | 5.78 | 190 | 100 | 80 | 99.969 |
| 33  | Hypothetical protein | A     | gi|39,975,025  | 63.2  | 5.18 | 52 | 44.002 | 41 | 97.943 |
| 34  | Hypothetical protein | A     | gi|19,975,025  | 63.2  | 5.18 | 52 | 44.002 | 41 | 97.943 |
| 35  | Hypothetical protein | A     | gi|19,975,025  | 63.2  | 5.18 | 52 | 44.002 | 41 | 97.943 |
| 36  | Hypothetical protein | A     | gi|15,606,158 | 59.8  | 5.23 | 72 | 99.465 | 17 | 0 |
| 37  | Hypothetical protein | A     | gi|15,606,158 | 59.8  | 5.23 | 72 | 99.465 | 17 | 0 |
| 38  | Hypothetical protein | A     | gi|15,606,158 | 59.8  | 5.23 | 72 | 99.465 | 17 | 0 |
| 41  | Hypothetical protein | A     | gi|39,975,437  | 32.6  | 5.28 | 145 | 107 | 100 | 98.746 |
| 42  | Hypothetical protein | A     | gi|39,975,437  | 32.6  | 5.28 | 145 | 107 | 100 | 98.746 |
| 43  | Hypothetical protein | A     | gi|39,975,437  | 32.6  | 5.28 | 145 | 107 | 100 | 98.746 |
| 44  | Hypothetical protein | A     | gi|39,975,437  | 32.6  | 5.28 | 145 | 107 | 100 | 98.746 |
| 45  | Hypothetical protein | A     | gi|39,975,437  | 32.6  | 5.28 | 145 | 107 | 100 | 98.746 |
| 46  | Hypothetical protein | A     | gi|39,975,437  | 32.6  | 5.28 | 145 | 107 | 100 | 98.746 |
| No. | Protein Name | Group | Protein | Volume | Accession | Mr | Protein | Protein Score | Total Ion | Total Ion |
|-----|--------------|-------|---------|--------|-----------|----|---------|--------------|-----------|-----------|
| 47  | YAU01587p (Yammin (yapalhica)) | A     | 119     | 4.34   | gi|50,556,104 | 35.7 | 5.50    | 157          | 100       | 129      |
| 48  | Hypothetical protein (Podspora anserina 5 mat +) | A     | 197     | 4.22   | gi|77,681,451 | 75.4 | 8.96    | 72           | 99.512    | 100      |
| 49  | 70 KDa heat shock protein (Podospora anserina) | A     | 180     | 4.20   | gi|3,122,921  | 73.7 | 5.52    | 137          | 100       | 82       |
| 50  | Hypothetical protein (Podspora anserina 5 mat +) | A     | 144     | 4.01   | gi|77,681,445 | 41.3 | 5.91    | 64           | 96.384    | 99.985   |
| 51  | Chlorite binding enzyme (Streptomyces grifaei240) | A     | 196     | 3.98   | gi|67,837,750 | 69.4 | 5.97    | 84           | 96.948    | 100      |
| 52  | Conserved hypothetical protein (Mycosynothe grifaei240) | A     | 168     | 3.61   | gi|45,96,104  | 54.1 | 6.94    | 148          | 96        | 100      |
| 53  | Hypothetical protein (Podspora anserina 5 mat +) | A     | 194     | 3.37   | gi|77,688,652 | 64.1 | 8.67    | 75           | 99.719    | 96.641   |
| 54  | Hypothetical protein FG03242.1 (Gibberellae zoe PH-1) | A     | 143     | 3.36   | gi|64,121,687 | 27.9 | 5.81    | 148          | 100       | 129      |
| 55  | Molybdoprotein biosynthesis protein (Sulfitobacter sp. NAS-14.1) | A     | 187     | 2.36   | gi|83,954,363 | 33.6 | 5.64    | 69           | 0         | 63       |
| 56  | Proteosome component P吕1 (Neurospora crassa OR1A) | A     | 36      | 2.07   | gi|64,427,141 | 21.2 | 5.11    | 161          | 100       | 108      |
| 57  | GAF domain protein (Campylobacterium bacterium GD-1) | A     | 182     | -3.72  | gi|23,4,488,844 | 73.4 | 5.46    | 92           | 99.549    | 100      |
| 58  | Predicted similar to Actin-5C isoform 1 (Apis mellifera) | A     | 129     | -7.07  | gi|48,137,684 | 41.8 | 5.30    | 244          | 100       | 145      |
| 59  | Beta actin (Pseudomonas aeruginosa) | A     | 18      | -13.09 | gi|3,452,279 | 13.4 | 5.46    | 287          | 100       | 136      |
| 60  | Actin 6 (Andes argenti) | A     | 173     | -17.77 | gi|71,383,976 | 41.8 | 5.22    | 481          | 100       | 336      |
| 61  | Actin (Amblyomyxa americana) | A     | 174     | -24.85 | gi|94,47,724 | 21.1 | 5.27    | 278          | 100       | 148      |
| 62  | Serine peptidase (Oplochladosc anemisa) | B/B/B/C/D | 121 | -28.96 | gi|61,897,707 | 40.3 | 6.66    | 171          | 100       | 138      |
| 63  | Hypothetical protein (Podspora anserina 5 mat +) | B/B/B/C/D | 190/191/192/81/97 | -3.27/-4.22/-4.73/-3.87 | gi|71,696,268 | 72.9 | 5.88    | 182/190/144/153/135 | 100/100/100/100/100 | 84/120/70/67/35 |
| 64  | GTP-binding nucleoside protein Ram, putative (Aspergillus clavatus NRRL 1) | B/B | 28/139 | 3.26/3.31 | gi|19,396,242 | 23.6 | 6.44    | 209/140 | 100/100 | 103/52 |
| 65  | Hypothetical protein FG03454.1 (Gibberellae zoe PH-1) | B/B | 128/142 | -4.16/-2.37 | gi|66,122,153 | 45.3 | 6.78    | 147/107 | 100/99.985 | 53/22 |
| 66  | Hypothetical protein (Podspora anserina 5 mat +) | B/C | 184/62 | -4.58/-3.82 | gi|77,681,395 | 43.7 | 5.13    | 237/208 | 100/100 | 91/118 |
| 67  | Acetaldehyde dehydrogenase (Corynebacterium) | B/E | 153/93 | 6.78/-3.68 | gi|18,596,338 | 31.9 | 8.22    | 108/253 | 99.988/100 | 49/105 |
| 68  | FDH, NAD+ Reductase: Full = Formate dehydrogenase | B/E | 144/11 | -4.94/14.53 | gi|72,469 | 40.9 | 5.93    | 61/62 | 93.2/64/94.774 | 42/54 |
| 69  | Hypothetical protein (Podspora anserina 5 mat +) | B | 34 | 18.28 | gi|77,691,500 | 18.1 | 4.39    | 63 | 95.752 | 48 |
| 70  | Maga nashi protein (Neurospora crassa OR1A) | B | 108 | 54.37 | gi|85,083,322 | 18.1 | 6.10    | 90 | 99.159 | 25 |

(Continued)
| No. | Protein Name                                      | Group | Protein ID | Volume Ratio | Accession No. | Mr (kDa) | P I Score | Protein Score | Total Ion C. I. % | Total Ion Score | C. I. % |
|-----|--------------------------------------------------|-------|------------|--------------|---------------|---------|----------|--------------|----------------|----------------|--------|
| 71  | Mannitol-1-phosphate 5-dehydrogenase [Bacillus clausii KSM-K16] | B     | 61         | 13.91        | gi|56,964,690 | 41.3     | 5.22     | 98           | 99.887         | 78                | 99.968 |
| 72  | Hypothetical protein                             | B     | 114        | 12.85        | gi|45,605,837 | 25.7     | 9.51     | 63           | 95.752         | 100               | 100    |
| 73  | Hypothetical protein                             | B     | 22         | 8.29         | gi|46,121,809 | 22.2     | 5.28     | 128          | 100            | 77                | 99.29  |
| 74  | Heat shock 70 kDa protein                        | B     | 88         | 6.61         | gi|16,200,213 | 71.4     | 5.01     | 255          | 100            | 144               | 100    |
| 75  | Elongation factor 2; Short = EF-2                | B     | 114        | 12.85        | gi|189,045,117 | 71.4     | 5.01     | 255          | 100            | 144               | 100    |
| 76  | Hypothetical protein                             | B     | 22         | 8.29         | gi|46,121,809 | 22.2     | 5.28     | 128          | 100            | 77                | 99.29  |
| 77  | Serine protease [Calothrix thiolaeformis]        | B     | 29         | 5.73         | gi|300,250,850 | 24.8     | 7.86     | 52           | 96.705         | 42                 | 100    |
| 78  | Hypothetical protein heavy chain-binding protein homolog [Gibberella zeae PH-1] | B     | 25         | 4.87         | gi|46,135,911 | 74.5     | 5.08     | 87           | 98.398         | 33                 | 0      |
| 79  | Hypothetical protein [Podospora anserina S mat+] | B     | 133        | 4.46         | gi|71,694,850 | 38.2     | 6.67     | 58           | 85.066         | 47                 | 99.423 |
| 80  | Hypothetical protein [P. eryngii 1957]           | B     | 87         | 3.94         | gi|235,930,542 | 39.8     | 9.99     | 52           | 39.997         | 43                 | 99.749 |
| 81  | Cell division control protein 3 [Neurospora crassa OR1MA] | B     | 41         | 3.54         | gi|164,423,542 | 52.1     | 7.21     | 123          | 100            | 63                 | 99.13  |
| 82  | Hypothetical protein [Amp12]                     | B     | 103        | 3.51         | gi|219,449,381 | 42.9     | 5.83     | 82           | 99.999         | 28                 | 99.045 |
| 83  | Hypothetical protein                             | B     | 136        | 2.89         | gi|15,388,251 | 39.1     | 6.46     | 93           | 99.966         | 73                 | 99.912 |
| 84  | 5-Methylthioadenosine phosphorilase (Meu1), putative [Amp12] | B     | 37         | 2.85         | gi|19,396,242 | 33.9     | 5.95     | 55           | 70.612         | 50                 | 99.496 |
| 85  | Pseudoskene [Haloferax mediterranei]             | B     | 46         | 2.83         | gi|67,628,213 | 63.2     | 6.09     | 83           | 96.466         | 47                 | 0      |
| 86  | Hypothetical protein [Podospora anserina S mat+] | B     | 52         | 2.77         | gi|71,681,866 | 38.9     | 6.01     | 74           | 99.670         | 59                 | 99.951 |
| 87  | Beta-tubulin [M. oryzae 70-15]                   | B     | 181        | 2.67         | gi|39,974,499 | 49.9     | 4.80     | 85           | 99.973         | 22                 | 0      |
| 88  | Hypothetical protein [Gibberella zeae PH-1]     | B     | 135        | 2.67         | gi|46,134,853 | 36.6     | 6.18     | 93           | 99.641         | 39                 | 0      |
| 89  | Subtilisin-like serine protease PRHI [H. anosphaerica] | B     | 32         | 2.54         | gi|214,331,261 | 53.9     | 6.21     | 59           | 89.081         | 50                 | 99.548 |
| 90  | Guanine nucleotide-binding protein beta subunit-like protein [Chaetomium globosum CBS 148.51] | B     | 118        | 2.30         | gi|16,201,077 | 35.1     | 6.55     | 174          | 100            | 101                | 100    |
| 91  | Hypothetical protein [Podospora anserina S mat+] | B     | 113        | 2.16         | gi|28,854,632 | 41.6     | 5.45     | 277          | 100            | 102                | 100    |
| No. | Protein Name                          | Group | ID | Ratio | Accession     | Mr | Protein Score | Total Ion Score | C. I. % | Total Ion Score | C. I. % |
|-----|--------------------------------------|-------|----|-------|---------------|----|--------------|-----------------|--------|----------------|--------|
| 93  | Hypothetical protein FG08593.1 [Gibberella zeae PH-1] | B     | 36 | 2.07  | gi|46,128,431 | 25.9 | 6.70 | 103            | 99.96 | 57            | 94.689 |
| 94  | Actin                                | B     | 23 | 2.04  | gi|23,938,589 | 41.6 | 5.61 | 206            | 100   | 148           | 100    |
| 95  | Transaldolase [Magnaporthe grisea 70-15] | B     | 45 | 2.05  | gi|39,970,315 | 35.6 | 5.38 | 243            | 100   | 196           | 100    |
| 96  | Conserved hypothetical protein [Oxotremorium globosum CBS 14651] | B     | 73 | -2.06 | gi|16,205,583 | 37.8 | 5.76 | 192            | 100   | 72            | 99.848 |
| 97  | Conserved hypothetical protein [Magnaporthe oryzae 70-15] | B     | 47 | -2.12 | gi|39,973,499 | 34.2 | 5.62 | 160            | 100   | 100           | 100    |
| 98  | Hypothetical protein FG00505.1 [Gibberella zeae PH-1] | B     | 5  | -2.24 | gi|46,107,244 | 21.8 | 4.84 | 185            | 100   | 165           | 100    |
| 99  | UDP-glucose pyrophosphorylase [Phoma herbarum] | B     | 123 | -2.32 | gi|59,499,918 | 57.8 | 7.23 | 101            | 100   | 87            | 100    |
| 100 | Pc20g01500 [Penicillium chrysogenum Wisconsin 54-1255] | B     | 58 | -2.51 | gi|23,941,883 | 38.4 | 5.40 | 60             | 90.49 | 51            | 99.686 |
| 101 | Hypothetical protein [Entamoeba dispar SWN768] | B     | 161 | -2.64 | gi|67,387,459 | 57.7 | 7.16 | 92             | 99.59 | 42            | 0      |
| 102 | Ketolicidareductoisomerase, mitochondrial precursor [Neurospora crassa OR74A] | B     | 49 | -2.76 | gi|85,102,477 | 44.6 | 8.52 | 142            | 100   | 97            | 100    |
| 103 | Hypothetical protein [Magnaporthe oryzae 70-15] | B     | 180 | -2.87 | gi|39,968,579 | 16.9 | 9.34 | 46             | 0     | 41            | 98.288 |
| 104 | Conserved hypothetical protein [Magnaporthe oryzae 70-15] | B     | 146 | -2.96 | gi|46,606,056 | 59.4 | 6.06 | 88             | 99.98 | 27            | 20.665 |
| 105 | Putative RNA polymerase Rpb1, domain 2 [Uncolized marine cyanobacteria HF4000_ANIW93I24] | B     | 59 | -3.19 | gi|67,042,230 | 140.3 | 7.81 | 84             | 97.21 | 77            | 100    |
| 106 | Pc18g01770 [Penicillium chrysogenum Wisconsin 54-1255] | B     | 97 | -3.45 | gi|55,942,305 | 26.6 | 5.80 | 72             | 99.44 | 56            | 99.945 |
| 107 | Chloramphenicol mutase [Pyrenophora triticirepens Ps-SC-RP] | B     | 80 | -3.59 | gi|89,206,279 | 30.5 | 5.63 | 113            | 99.96 | 68            | 99.667 |
| 108 | ATB6_NEUCR ATP synthase beta chain, mitochondrial precursor [Gibberella zeae PH-1] | B     | 182 | -3.71 | gi|46,114,940 | 54.9 | 5.40 | 229            | 100   | 106           | 100    |
| 109 | ATP-citrat-lyase [Gibberella pulicaris] | B     | 152 | -3.71 | gi|75,899,697 | 53.0 | 5.57 | 195            | 100   | 149           | 100    |
| 110 | Putrescine aminopropyltransferase [Saccharomyces cerevisiae YM1889] | B     | 111 | -3.80 | gi|51,942,852 | 33.3 | 5.33 | 117            | 99.98 | 45            | 54.649 |
| 111 | Conserved hypothetical protein [Magnaporthe oryzae 70-15] | B     | 173 | -4.80 | gi|45,612,487 | 46.6 | 5.33 | 136            | 100   | 67            | 99.997 |
| 112 | Hypothetical protein [Magnaporthe oryzae 70-15] | B     | 129 | -5.04 | gi|45,612,637 | 46.4 | 7.01 | 87             | 99.91 | 34            | 90.103 |
| 113 | Pc20g08020 [Penicillium chrysogenum Wisconsin 54-1255] | B     | 55 | -5.82 | gi|23,946,115 | 226.4 | 7.77 | 48             | 0     | 39            | 95.150 |
| 114 | Thiodendrin peroxydase [Oxalapogus oceanicus] | B     | 93 | -6.95 | gi|18,152,531 | 21.4 | 5.95 | 96             | 99.87 | 36            | 0      |
### Table 1. (Continued).

| No. | Protein Name                                      | Group | ID      | Volume Ratio | Accession   | MP | Protein | Protein Score | Total Ion | Score | C. I. % | Total Ion | Score | C. I. % |
|-----|--------------------------------------------------|-------|---------|--------------|-------------|----|---------|---------------|-----------|-------|---------|-----------|-------|---------|
| 115 | Conserved hypothetical protein (Magnaporthe oryzae 70–15) | B     | 50      | –7.20        | gi|19,927,291 | 29.6 | 6.14 | 72           | 99.386    | 29    | 27.759 |
| 116 | Hypothetical protein (Podospora arvensis 5 mat +) | B     | 120     | –7.66        | gi|71,677,424 | 35.0 | 6.55 | 124          | 100       | 39    | 97.853 |
| 117 | PX domain-containing protein (Toxoplasma gondii ME49) | B     | 109     | –8.24        | gi|27,82,101  | 267.2 | 7.71 | 85           | 97.611    |       |        |
| 118 | Hop70 chaperone (NovA) | B     | 164     | –9.73        | gi|42,798,748 | 64.7 | 5.33 | 299          | 100       | 268   | 100    |
| 119 | Unnamed protein product (Podospora arvensis 5 mat +) | B     | 127     | –11.01       | gi|70,940,277 | 52.7 | 8.30 | 117          | 100       | 56    | 99.919 |
| 120 | Hypothetical protein MGG_13201 (Magnaporthe oryzae 70–15) | B     | 183     | –15.99       | gi|45,608,296 | 16.5 | 9.79 | 64           | 96.778    |       |        |
| 121 | Hypothetical protein (Penicillium chrysogenum Wisconsin 54–1235) | B     | 19      | –18.07       | gi|255,940,706 | 15.8 | 9.75 | 66           | 97.666    |       |        |
| 122 | Putative endonuclease (Bravera bosiana) | C/C/D/E/E/E | 51/53/55/58/23/56/58/ | 2.45/3.36/2.21/6/30/6.13/3.44/3.39 | gi|10,592,112 | 47.2 | 5.07 | 253/159/471/292/166/125/103 | 100/100/100/100/100/100/99.902 | 182/117.3/8/12/97.5/7/7 | 100/100/100/100/100/100/81.499/91.839 |
| 123 | 6-phosphogluconate dehydrogenase (Aspergillus clavatus NBR1, 1) | C/D/D/D | 29/64/75.87 | 3.62/4.11/6.16/6.06 | gi|19,936,138 | 56.0 | 6.05 | 89/153/79/70 | 99.990/100/99.839/99.209 | 60/109/51/51 | 99.958/100/99.753/99.753 |
| 124 | Acetaldehyde dehydrogenase (Corynebacterium) | C/C/C/C | 28/42/26 | –2.62/2.05/3.95 | gi|18,956,536 | 31.9 | 8.22 | 410/264/202 | 100/100/100 | 240/112/74 | 100/100/99.917 |
| 125 | Guanine nucleotide-binding protein subunit beta-like protein (Neurospora crassa) | C/C | 18/22 | 2.69/2.69 | gi|3,023,852 | 35.1 | 6.79 | 145/126 | 100/100 | 104/70 | 100/99.998 |
| 126 | Actin (Genomemmomyces graminis) | C/C | 11/49 | 2.75/2.81 | gi|7,722,096 | 41.6 | 5.45 | 311/493 | 100/100 | 202/39 | 100/100 |
| 127 | Hop70 chaperone (NovA), putative (Aspergillus clavatus NBR1, 1) | C/D/D | 80/98 | –3.32/–3.17 | gi|18,404,708 | 66.9 | 5.19 | 306/182 | 100/100 | 285/156 | 100/100 |
| 128 | Hypothetical protein MGG_0620 (Magnaporthe oryzae 70–15) | C/D | 52/5 | –2.66/4.00 | gi|9,976,753 | 38.3 | 5.18 | 100/63 | 100/99.06 | 86/94 | 100/99.06 |
| 129 | Spermidine synthase (Loddermucy abigaliporus NRRL Y-42319) | C/C | 8 | 6.51 | gi|49,39,971 | 33.9 | 5.18 | 114 | 99.97 | 82 | 99.980 |
| 130 | Heat shock protein 70 (Parsococcisidios basilevis) | C | 4 | 5.25 | gi|4,538,023 | 70.8 | 5.05 | 259 | 100 | 237 | 100 |
| 131 | Heat shock protein 62 (Aspergillus terreus NIH2624) | C | 89 | 4.09 | gi|15,42,960 | 79.8 | 4.97 | 138 | 100 | 23 | 0 |
| 132 | P.g2g2.1330 (Penicillium chrysogenum Wisconsin 54–1235) | C | 6 | 3.99 | gi|5,950,526 | 93.8 | 8.65 | 63 | 96.036 |       |       |
| 133 | Conserved hypothetical protein (Magnaporthe oryzae 70–15) | C | 27 | 3.82 | gi|39,513,501 | 34.3 | 6.85 | 188 | 100 | 104 | 100 |
| 134 | Ribonuclease R (Sclerotina frigida Magnaporthe G14) | C | 54 | 3.71 | gi|14,564,516 | 92.8 | 8.70 | 84 | 97.018 |       |       |
| 135 | ATP synthase beta chain, mitochondrial precursor (Chlamydomonas reinhardtii CBS 148.35) | C | 58 | 3.53 | gi|16,204,743 | 55.6 | 5.10 | 216 | 100 | 135 | 100 |
| 136 | Hypothetical protein FG05315.1 (Gibberella zeae PH-1) | C | 21 | 3.30 | gi|46,12,875 | 44.8 | 6.19 | 100 | 99.923 | 35 | 0 |

(Continued)
| No. | Protein Name | Group | ID | Volume | Accession | Mr | Protein Score | C. I. % | Total Ion Score | Total Ion C. I. % |
|-----|--------------|-------|----|--------|-----------|----|---------------|--------|----------------|------------------|
| 137 | Heat shock 70 kDa protein | C | 45 | 3.22 | gi|54,285,900 | 66.9 | 5.44 | 155 | 100 | 127 | 100 |
| 138 | Hypothetical protein | C | 17 | 2.56 | gi|71,694,267 | 95.9 | 5.22 | 73 | 100 | 99.58 | |
| 139 | Inorganic diphosphatase, putative | C | 10 | 2.39 | gi|23,488,693 | 43.6 | 7.06 | 168 | 100 | 89 | 99.997 |
| 140 | Septin | C | 63 | 2.29 | gi|19,402,350 | 43.1 | 5.03 | 135 | 100 | 75 | 100 |
| 141 | Inorganic diphosphatase, putative | C | 50 | 2.00 | gi|168,041,049 | 10.9 | 5.57 | 84 | 100 | 96.804 | |
| 142 | Zinc finger homeodomain 4 (predicted) | C | 73 | −2.09 | gi|49,048,501 | 222.6 | 5.85 | 82 | 95.486 | |
| 143 | Beta-tubulin | C | 60 | −2.12 | gi|1,002,511 | 49.7 | 4.88 | 296 | 100 | 108 | 100 |
| 144 | Beta-tubulin | C | 82 | −2.20 | gi|12,222,974 | 56.5 | 10.73 | 63 | 95.24 | |
| 145 | Heat shock protein | C | 95 | −2.22 | gi|1,012,737 | 61.4 | 5.57 | 288 | 100 | 216 | 100 |
| 146 | Predicted protein | C | 66 | −2.24 | gi|56,372,872 | 462.0 | 6.09 | 87 | 98.573 | |
| 147 | Poly(A) RNA binding protein | C | 78 | −2.41 | gi|70,674,510 | 79.9 | 5.38 | 206 | 100 | 153 | 100 |
| 148 | Hypothetical protein | C | 77 | −2.43 | gi|96,011,279 | 709.9 | 5.91 | 98 | 99.867 | |
| 149 | Hypothetical protein | C | 38 | −2.47 | gi|56,399,827 | 17.4 | 5.95 | 90 | 99.179 | |
| 150 | Heat shock protein 70-2 | C | 84 | −2.62 | gi|38,325,813 | 71.2 | 5.07 | 262 | 100 | 124 | 100 |
| 151 | Beta-tubulin | C | 59 | −2.69 | gi|200,978,742 | 37.3 | 5.33 | 135 | 100 | 86.749 | |
| 152 | Predicted protein | C | 92 | −4.37 | gi|224,006,584 | 213.1 | 5.46 | 84 | 97.217 | |
| 153 | GLYC_NEUCR Serine hydroxymethyltransferase | C | 30 | −4.41 | gi|65,123,825 | 54.3 | 6.74 | 121 | 100 | |
| 154 | Chitin deacetylase, putative | D/D | 6/121 | 11.3/2/11.41 | gi|56,3,806,283 | 53.4 | 6.30 | 49/60 | 90.370 | 42/53 | 97.245/99.850 |
| 155 | Hypothetical protein | D/D | 60/62 | 8.38/6.38 | gi|39,974,293 | 44.8 | 5.89 | 80/62 | 99.070/95.123 | |
| 156 | Hypothetical protein | D/D | 79/80 | −2.56/−7.38 | gi|71,695,892 | 523 | 5.36 | 84/70 | 99.965/99.172 | |
| 157 | Glyceraldehyde-3-phosphate dehydrogenase | D/D | 67/68 | −2.88/−2.25 | gi|422,228 | 36.0 | 6.28 | 100/86 | 100/99.979 | 59/56 | 97.308/99.948 |
| 158 | Hypothetical protein | D | 150 | 3.00 | gi|145,608,294 | 35.6 | 5.45 | 88 | 99.988 | |
| 159 | Inosine-adenosine-guanosine-nucleoside hydrolase | D | 66 | 2.89 | gi|2,645,495 | 35.8 | 5.23 | 82 | 95.051 | |
| 160 | Glutathione synthetase | D | 133 | 2.44 | gi|288,933,984 | 35.5 | 5.20 | 73 | 99.523 | |
| No. | Protein Name                          | Group | Volume | Accession   | Mr | Protein Score | Total Ion | C. I. % | Total Ion | C. I. % |
|-----|--------------------------------------|-------|--------|-------------|----|---------------|-----------|--------|-----------|--------|
| 161 | Hypothetical protein                  | D     | 54     | 2.36        | gi| 145,610,056  | 160.7     | 6.32   | 98.103    | 19     |
| 162 | Tubulin alpha-B chain                 | D     | 52     | 2.15        | gi| 146,379,810  | 49.9      | 5.05   | 99.477    | 49     |
| 163 | Hypothetical protein                  | D     | 77     | 2.11        | gi| 39,977,965   | 54.4      | 6.40   | 98.775    | 18     |
| 164 | Citrate synthase                      | D     | 72     | -2.15       | gi| 30,314,377   | 52.0      | 8.30   | 100       | 224    |
| 165 | Vascular ATP synthase subunit B       | D     | 53     | -2.21       | gi| 39,942,328   | 56.7      | 5.33   | 99.997    | 18     |
| 166 | Fructose-1,6-bisphosphatase           | D     | 134    | -2.24       | gi| 18,400,142   | 38.8      | 5.30   | 99.998    | 83     |
| 167 | V-type proton ATPase catalytic subunit| D     | 130    | -2.26       | gi| 137,461      | 67.1      | 5.32   | 97.381    | 50     |
| 168 | Hypothetical protein                  | D     | 101    | -2.42       | gi| 71,687,995   | 88.8      | 6.57   | 99.977    | 99.405 |
| 169 | Beta glucosidase, putative            | D     | 47     | -2.53       | gi| 18,396,344   | 84.3      | 5.60   | 93.571    | 47     |
| 170 | Hypothetical protein                  | D     | 82     | -2.54       | gi| 16,192,255   | 43.4      | 5.78   | 98.698    | 88     |
| 171 | Predicted protein                     | D     | 118    | -2.62       | gi| 56,736,551   | 41.4      | 6.27   | 95.787    | 83     |
| 172 | Heat shock protein 60                 | D     | 110    | -2.77       | gi| 45,608,376   | 61.8      | 5.83   | 100       | 77     |
| 173 | Probable succinyl-CoA ligase          | D     | 71     | -3.49       | gi| 24,665,374   | 34.7      | 9.70   | 99.987    | 55     |
| 174 | Heat shock protein 70 (Hsp70)         | D     | 99     | -3.59       | gi| 18,403,457   | 72.5      | 5.81   | 99.941    | 82     |
| 175 | Adenylhomoocysteine                  | D     | 78     | -3.65       | gi| 39,940,170   | 48.9      | 5.94   | 99.907    | 35     |
| 176 | Myosin, heavy polypeptide 1, skeletal muscle | D     | 96     | -4.07       | gi| 57,742,222   | 222.7     | 5.54   | 99.754    | 95     |
| 177 | Hypothetical protein                  | D     | 94     | -4.31       | gi| 89,091,826   | 63.7      | 5.94   | 96.126    | 57     |
| 178 | Isoleucyl-tRNA synthetase             | D     | 48     | -4.39       | gi| 288,937,224  | 104.4     | 5.64   | 98.347    | 67     |
| 179 | Hypothetical protein                  | D     | 49     | -5.61       | gi| 71,688,418   | 47.6      | 8.94   | 98.883    | 69     |
| 180 | Hypothetical protein                  | D     | 2      | -9.20       | gi| 71,690,254   | 117.5     | 6.22   | 98.625    | 68     |
| 181 | Glyceraldehyde-3-phosphate dehydrogenase | E/E  | 42/55   | 5.14/-2.57  | gi| 50,651,022   | 36.1      | 6.54   | 174/189   | 100/100 |
| 182 | 212L (Invertebrate iridescent virus 6) | E     | 3      | 47.79       | gi| 50,741,924   | 43.0      | 5.88   | 99.605    | 63     |
| 183 | Unnamed protein product               | E     | 6      | 24.36       | gi| 50,301,991   | 33.2      | 5.24   | 99.179    | 39     |

(Continued)
| No. | Protein Name                                | Group | ID  | Ratio | Accession   | Mr   | Protein Score | Total Ion | Total Ion |
|-----|--------------------------------------------|-------|-----|-------|-------------|------|---------------|-----------|-----------|
| 184 | Translation elongation factor EF-Tu, putative (Aspergillus clavatus NRRL 1) | E     | 10  | 15.62 | gi|19,397,185 | 48.3 | 6.52 | 44 | 0 | 36 | 95,982 |
| 185 | Heat shock protein 70 kDa (Hypocreae lanii) | E     | 14  | 12.89 | gi|67,843,281 | 71.0 | 5.05 | 352 | 100 | 197 | 100 |
| 186 | Copper-zinc superoxide dismutase (Cordycps militaris) | E     | 22  | 8.16  | gi|26,000,295 | 15.7 | 6.28 | 77 | 99.810 | 53 | 99.819 |
| 187 | Cytochrome P450 (Aspergillus clavatus NRRL 1) | E     | 28  | 7.10  | gi|19,396,129 | 60.0 | 9.00 | 64 | 96.626 |
| 188 | Protein disulphide isomerase (Hypocreae lanii) | E     | 51  | 4.17  | gi|3,288,650 | 54.6 | 4.83 | 82 | 94.697 | 60 | 97,205 |
| 189 | P235.100 (Pencillium chrysogenum Wisconsin 54-123) | E     | 60  | 3.07  | gi|23,914,987 | 89.7 | 6.36 | 68 | 98.687 |
| 190 | SPEE_NEBD10 RedName Full = Spermidine synthase (Penicillium chrysogenum) | E     | 67  | 2.37  | gi|8,134,725 | 33.1 | 5.54 | 72 | 99.414 | 18 | 0 |
| 191 | Hypothetical protein (Penicillium chrysogenum) | E     | 44  | 2.05  | gi|71,695,866 | 21.9 | 5.34 | 61 | 92.950 | 49 | 99.741 |
| 192 | Chain R, Isometrically Contracting Insect Asynchronous Flight Muscle | E     | 74  | −2.30 | gi|90,789,282 | 41.4 | 5.16 | 68 | 99.671 | 13 | 0 |
| 193 | Vacuolar ATP synthase catalytic subunit A, putative (Glabromycetes stipitatus FIC 16,300) | E     | 82  | −2.37 | gi|242,791,712 | 76.8 | 5.31 | 88 | 98.637 | 37 | 0 |
| 194 | Malate dehydrogenase, mitochondrial precursor (Clonostachys rosea CBS 146.51) | E     | 80  | −2.59 | gi|16,197,348 | 35.3 | 8.61 | 194 | 100 | 57 | 97.025 |
| 195 | Pol polyprotein (Human immunodeficiency virus type 1) | E     | 87  | −2.99 | gi|3,738,350 | 45.5 | 8.96 | 83 | 96.158 |
| 196 | Unnamed protein product (Penicillium chrysogenum) | E     | 90  | −3.41 | gi|71,696,384 | 59.3 | 9.24 | 118 | 99.999 | 18 | 0 |
| 197 | Cell division control protein 10 (Neurospora crassa OR74A) | E     | 91  | −6.33 | gi|85,076,041 | 38.6 | 7.21 | 111 | 99.994 | 61 | 96.391 |
| 198 | Chaperone protein DnaK, putative (Stigmatella aurantiaca DW2A-1) | E     | 54  | −9.77 | gi|15,379,880 | 45.6 | 9.34 | 87 | 98.519 |

A-E, the same as in Figure 2.
pathway (Szeto et al. 2007). However, no proteins related to the MAPK pathway were identified in this study. Similarly, orthologous MAPK genes were not transcribed (CCM_04200 vs. AN1017) or transcribed at low levels (CCM_01235 vs. NCU02393) by C. militaris (Zheng et al. 2011). The reasons may include: 1) Different higher fungi might depend on different signal pathways in fruiting body development; 2) Although the related MAPK proteins involved in fruiting body development of C. sinensis, they were not successfully identified for the limited Cordyces database.

In the two signalling cascades, either heterotrimeric G proteins or ras and ras-like proteins relay extracellular ligand-stimulated signals to the cytoplasm (Pöggeler et al. 2006). Indeed, two main upstream signalling regulators of adenyl cyclase, guanine nucleotide-binding protein (G proteins) beta subunit-like protein and GTP-binding protein Ran, increased in the fruiting body of C. sinensis (Table 2). G proteins can interact with adenyl cyclases and catalyse the formation of cAMP (D’Souza and Heitman, 2001; Kamerewerd et al. 2008). GTP-binding protein Ran belongs to the superfamily of Ras proteins and is crucial regulator of adenyl cyclase (Schlenstedt et al. 1997; Seewald et al. 2003). Rab GDP-dissociation inhibitor (RabGDI) is a key regulator of Rab/Ypt GTPases that controls the distribution of active GTP and inactive GDP-bound forms between membranes and cytosol (Rak et al. 2003). Actually, RabGDI was up-regulated in the fruiting body of C. sinensis (Table 2). The same result has also been observed in the fruiting body of mushroom L. edodes (Sakamoto et al. 2009). In addition, GAF domain protein with 3’, 5’-cyclic-AMP phosphodiesterase activity, downstream signalling regulator of adenyl cyclase, catalyses cAMP to AMP (De Oliveira et al. 2007) decreased in the fruiting body of C. sinensis. Finally, increased biosynthesis and decreased degradation of cAMP result in accumulation of cAMP in the fruiting body of C. sinensis. As a signalling factor, cAMP plays an important role in controlling fruit body formation (Kinoshita et al. 2002; Palmer and Horton 2006). It is closely related to the onset of fruiting body development in L. edodes (Miyazaki et al. 2005). The level of cAMP in dikaryotic mycelia of Schizophyllum commune reached peak before primordium formation, and then gradually increased until the final stage of fruit body formation (Kinoshita et al. 2002). Light causes an increase of cAMP level in fungi Coprinus macrorhizus and S. commune, and induces their fruiting body formation (Kinoshita et al. 2002). It has been confirmed that C. militaris fail to form the fruiting body without light. Moreover, cAMP also regulates the expression of a large number of genes required for fruiting body formation of Dictyostelium discoideum (Bishop et al. 2002). Therefore, the cAMP signal pathway should involve in fruiting body development of C. sinensis.

**Heat shock proteins responded to environmental stress**

In fungal kingdom, fruit body formation usually could not happen until some severe stressors occur. In nature, these stressors are heat and cold, fire and flood, or nutrient deficiency (Holliday and Cleaver 2008). A sudden change in temperature (heat shock or cold shock) or other adverse environmental conditions can stimulate living organisms to produce heat shock proteins (Hsps) for protection and cell repairmen activities. Some Hsps play important roles in all major growth-related processes including cell division, DNA synthesis, transcription, translation, protein folding and transportation, and membrane translocation (Chaffin et al. 1998). Generally, heat shock proteins Hsp70, Hsp70 chaperone and Hsp 90 in the fruiting body of C. sinensis had higher expression, and a similar change was also found in the mature fruiting body of C. militaris (Table 2). Except Cordyceps, in Podospora anserina, a gene encoding Hsp90 homolog involves in both sexual development and vegetative growth (Loubradou et al. 1997). Under certain environmental stresses, dikaryotic mycelia aggregate to form primordium, which marks the beginning of fruit body development (Chum, et al. 2008). Heat shock treatment accelerates the fruiting body formation and sporulation of Myxococcus xanthus because heat shock induces some proteins expression and perhaps involve in fruiting body formation and sporulation (Otani et al. 2001), which well explained why some fungal cultures cannot produce fruit bodies without temperature downshift or light illumination (Yoon et al. 2002). As far as we know, natural C. sinensis grows in Qinghai-Tibetan Plateau, where the temperature difference between day and night can reach about
A gene encoding Hsp90 homolog involves in both sexual development and vegetative growth of Podospora anserina (Loubadou et al. 1997).

Enolase, also known as phosphopyruvate hydratase, is a metalloenzyme responsible for the catalysis of the conversion of 2-phosphoglycerate to phosphoenolpyruvate, might involve the fruiting body formation of Cordyceps sinensis (De Groot et al. 1997).

Acetaldehyde dehydrogenase is induced by heat shock in Myxococcus xanthus, and is related to fruiting body formation of the mushroom Flammulina velutipes (Otani et al. 2001; Yoon et al. 2002).

GTP-binding nuclear protein Ran belongs to the superfamily of Ras proteins and is crucial regulator of adenyl cyclase (Otani et al. 1997; Otani et al. 2001).

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The glyceraldehyde-3-phosphate dehydrogenase gene GAPDH was expressed in both mycelia and fruiting bodies, suggesting that the GAPDH gene product is a heat shock protein which might be involved in the developmental phase of the Lentinus polychrous (Yu 2006; Li et al. 2007).

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Acetaldehyde dehydrogenase is induced by heat shock in Myxococcus xanthus, and is related to fruiting body formation of the mushroom Flammulina velutipes (Otani et al. 2001; Yoon et al. 2002).

GTP-binding nuclear protein Ran belongs to the superfamily of Ras proteins and is crucial regulator of actin-like cyclase (Schlenstedt et al. 1997; Seewald et al. 2003).
| No. | Protein Name                                                                 | Group | Role in fruiting body development                                                                                                                                                                                                 | Microorganism                          | References                      |
|-----|------------------------------------------------------------------------------|-------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|----------------------------------|
| 23  | Rab GDP-dissociation inhibitor                                               | A1, C1| Rab GDP-dissociation inhibitor, a key regulator of Rab/Ypt GTPases that controls the distribution of the active GTP and inactive GDP-bound forms between membranes and cytosol, is up-regulated in fruiting body of *Lentinula edodes*.                  | *Lentinula edodes*                     | Rak et al. 2003; Sakamoto et al. 2009 |
| 24  | Serine proteases                                                            | A1, B1| The serine proteases play an important role in the pathogenic fungus during the penetration and colonization of their hosts.                                                                                                        | *Cordyceps sinensis*                   | Li et al. 2006b; Zhang et al. 2008 |
| 25  | Formate dehydrogenase                                                        | B1, E1| The UTP-glucose-1-phosphate uridylytransferase is a developmentally regulated enzyme which involves in trehalose, cellulose, and glycogen synthesis in fungus *Dictyostelium discoideum*.                                      | *Dictyostelium discoideum*             | Fishel et al. 1982; Bishop et al. 2002 |
| 26  | UTP-glucose-1-phosphate uridylytransferase                                  | A1    | The mannitol-1-phosphate 5-dehydrogenase is proposed as the major enzyme for mannitol biosynthesis, and the increase of mannitol is related to the fruiting body initiation and development of *Agaricus bisporus*.                               | *Agaricus bisporus*                    | Kulkarni, 1990; Vélëz et al. 2007   |
| 27  | Chorismate mutase                                                           | B1    | The UDP-N-acetylglucosamine pyrophosphorylase is a major regulatory enzyme in amino sugar synthesis during cyst wall (encystment) formation of *Giardia*.                                                                                       | *Giardia*                              | Bulik et al. 2000                  |
| 28  | Chorismate mutase                                                           | B1    | The mutant strains of *Aspergillus nidulans* which have been knocked out the chorismate mutase gene *aroC*, decreases the capacity for fruit body formation and ascosporeogenesis.                                                  | *Aspergillus nidulans*                 | Krapmann, and Braus, 2003           |
| 29  | O-acetyltobosinephosphate sulphohydrolase (homocysteine synthase)            | A1    | The mago nashi protein participates fungi development and abundantly expresses in natural fruiting bodies of medicinal fungus *Antradia cinnamomea*.                                                                                      | *Tuber borchii*                        | Zeppa et al. 2010                  |
| 30  | UDP-N-acetylglucosamine pyrophosphorylase                                   | A1    | The UDP-N-acetylglucosamine pyrophosphorylase is a major regulatory enzyme in amino sugar synthesis during cyst wall (encystment) formation of *Giardia*.                                                                                       | *Giardia*                              | Bulik et al. 2000                  |
| 31  | Mago nashi protein                                                          | B1    | The mago nashi protein participates fungi development and abundantly expresses in natural fruiting bodies of medicinal fungus *Antradia cinnamomea*.                                                                                      | *Antradia cinnamomea*                  | Chu et al. 2009                   |
| 32  | T-complex protein 1 subunit zeta                                             | A1    | The T-complex protein is the developmentally specific gene product in mature fruiting body of *Lentinula edodes*.                                                                                                                  | *Lentinula edodes*                     | Miyazaki et al. 2005               |
| 33  | Inorganic pyrophosphatase or putative inorganic diphosphatase                | A1, C1| The T-complex protein is the developmentally specific gene product in mature fruiting body of *Lentinula edodes*.                                                                                                                  | *Lentinula edodes*                     | Miyazaki et al. 2005               |
| 34  | 6-phosphogluconate dehydrogenase                                            | C1, D1| Although their bioactivities of these hypothetical proteins are unknown, they might play important roles in the fruiting body development of *C. sinensis*.                                                                                                                             | *C. sinensis*                          |                                 |
| 35  | Hypothetical protein (gi|171,690,628)                              | B1, C1, D1| Although their bioactivities of these hypothetical proteins are unknown, they might play important roles in the fruiting body development of *C. sinensis*.                                                                                                                             | *C. sinensis*                          |                                 |
| 36  | Hypothetical protein (gi|171,683,195)                                | B1, C1| Although their bioactivities of these hypothetical proteins are unknown, they might play important roles in the fruiting body development of *C. sinensis*.                                                                                                                             | *C. sinensis*                          |                                 |
| 37  | Hypothetical protein FG08933.1                                              | A1, B1| Although their bioactivities of these hypothetical proteins are unknown, they might play important roles in the fruiting body development of *C. sinensis*.                                                                                                                             | *C. sinensis*                          |                                 |

↑ represents up-regulated, ↓ represents down-regulated, ↑↓ represents up-regulated and down-regulated.
20°C during fruiting body formation and development season. Therefore, it is reasonable to speculate that heat shock proteins (Hsps) highly express during fruiting body formation and development. On the other hand, Hsps are also immunodominant antigens and major targets of host immune response during different types of infection (Chaffin et al. 1998), which is helpful to better understand why some Hsps show higher expression in sclerotium. It could be presumed that the host larva produces Hsps when it is infected by hyphal or spore of fungus.

Proteins involved in carbohydrate metabolism

Carbohydrate catabolism not only provides energy for hyphal growth but also supplies carbon skeleton to other metabolisms (Deveau et al. 2008), which is significantly changed during fruiting body initiation and development of primordia into the mature fruiting body (Kulkarni, 1990).

Proteins involved in the glycolytic pathway and tricarboxylic acid cycle (TCA)

The fructose-bisphosphate aldolase, enolase and pyruvate kinase of the glycolytic pathway, as well as malate dehydrogenase of tricarboxylic acid cycle (TCA), were shown higher expression in the fruiting body of C. sinensis (Table 1). It was very intriguing that putative enolase was also up-regulated in fruiting bodies of C. memorabilis and C. militaris, and enhanced in the mature fruiting body of C. militaris (Table 2). These results suggest that enolase may play an important role during fruiting body formation and development of Cordyceps. It is consistent with that glycolysis and TCA cycles are the major pathways of glyco-metabolism in sporulating stage of fruiting body development in Pleurotus ostreatus (Chakraborty et al. 2003). In contrast to C. sinensis, malate dehydrogenase showed lower expression in the fruiting body of C. memorabilis and mature one of C. militaris, which may attribute to the different formation mechanisms of individual fungus because the fruiting body of both C. memorabilis and C. militaris could be produced under the same culture conditions, but C. sinensis failed to develop its fruiting body.

Proteins involved in the glyoxylate pathway

Pyruvate kinase (PK) and aldehyde dehydrogenase are putative indole receptor proteins involved in multicellular development which are essential for fruiting body formation in Stigmatella aurantiaca (Stamm et al. 2005). Acetaldehyde dehydrogenase of the glyoxylate pathway, which can be induced by heat shock in M. Xanthus (Otani et al. 2001), is related to fruiting body formation of mushroom Flammulina velutipes (Yoon et al. 2002). These enzymes increased in the fruiting body of C. sinensis and the mature fruiting body of C. militaris (Table 2) suggested these enzymes might involve in fruiting body development.

Proteins involved in the mannitol pathway

Mannitol-1-phosphate 5-dehydrogenase is proposed as main enzyme for mannitol biosynthesis (Vélez et al. 2007), and the enzyme abundance in the fruiting body of C. sinensis was near 13-fold higher than that in mycelia (Table 1). As a result, it may increase the content of mannitol in natural C. sinensis (Wang et al. 2009; Guan et al. 2010). Increased mannitol is related to fruiting body initiation and development of A. bisporus (Kulkarni, 1990), and the mannitol content in the fruiting body of A. bisporus is about 8–20 times higher than that in mycelia (Hammond and Nichols 1976; Wannet et al. 2000).

Proteins involved in the trehalose pathway

The trehalose pathway is clearly shown by enhanced expression of UTP-glucose-1-phosphate uridylyltransferase (Uridine diphosphoglucose pyrophosphorylase) in the fruiting body, which is a developmental regulation enzyme involving in trehalose, cellulose and glycogen synthesis in fungus D. discoideum (Fishel et al. 1982; Bishop et al. 2002). It is essential for fungus to complete its life cycle, and it increases 3-fold at the stage of fruiting body formation than that in vegetative growth and early stage of
Proteins involved in the mannose pathway

The content of mannose-1-phosphate guanyltransferase in the fruiting body of *C. sinensis* was higher than that in sclerotium, but lower than that in mycelium (Table 1). The overexpressed mannose-1-phosphate guanyltransferase promotes increase of GDP-mannose in fungus *Trichoderma reesei*. GDP-mannose was effectively utilised by mannosyltransferases and resulted in hypermannosylation of secreted proteins in both N and O glycosylation, which indicated that GDP-mannose might play a major regulatory role in protein glycosylation in *T. reesei* (Zakrzewska et al. 2003).

Proteins involved in energy metabolism

Fruiting body developmental programme needs more energy than simple vegetative growth (Busch and Braus 2007). ATP synthase, which can be induced by heat shock (Otani et al. 2001), is high expressed in fruiting body development and maturation of *A. bisporus* (De Groot et al. 1997). Obviously, it is noticed that vacuolar ATP synthase catalytic subunit A and inorganic pyrophosphorylase were higher in the fruiting body than those in sclerotium of *C. sinensis*. Especially, inorganic pyrophosphorylase, which can catalyse degradation of pyrophosphate and release energy, in the fruiting body of *C. sinensis* was about 6-fold higher than that in sclerotium (Table 1).

Proteins involved in protein synthesis and degradation

Elongation factors, eEF1A, eEF2 and eEF3, serve an essential function in translation cycle of protein synthesis in fungi. The transcript of eEF2 is also highly expressed in fruiting body cDNA library of medicinal fungus *Ganoderma lucidum* (Luo et al. 2010). In addition, the gene of elongation factor 1 is one of developmentally specific genes in primordium of *L. edodes* (Miyazaki et al. 2005), and eEF1A controls fruiting body formation of *P. anserina*, interacts with actin and tubulin to activate some proteins degradation and is probably involved in signal transduction and cell cycle regulation (Silar et al. 2001). Some factors of protein synthesis, including eukaryotic initiation factor 4A (eIF4A), elongation factors eEF2 and eEF3, and ribosomal L18ae protein family, were expressed at higher levels in the fruiting body of *C. sinensis* than that in sclerotium and mycelium. Similarly, the abundance of translation elongation factor EF-Tu in the fruiting body of *C. memorabilis* was higher than that in mycelium (Tables 1 and 2). Protein synthesis activity is very active during fruiting body formation of *C. sinensis*, which is consistent with a higher protein level (30.4%) in the fruiting body of natural *C. sinensis* than that (14.8%) in fermented mycelium (Hsu et al. 2002).

Besides proteases play an important role in turnover of nitrogenous compounds (e.g. protein and amino acids) during fruiting body formation (Terashita et al. 1998), proteolytic enzymes such as serine proteases, proteasome component PUP3, and probable proteasome subunit alpha were also highly expressed in the fruiting body rather than in mycelium and sclerotium of *C. sinensis* (Table 1). Similarly, although serine protease is active in all stages of fruiting body development in *Coprinopsis cinerea*, its expression is the most abundant during young tissue development (Heneghan et al. 2009). High abundance of serine protease in the fruiting body may decompose useless proteins for fruiting body development. But, serine protease in sclerotium may be beneficial for fungus to infect its host through digesting protein component of insect cuticles (Li et al. 2006b; Zhang et al. 2008). Two cuticle-degrading serine proteases from mycelium of fungus *C. sinensis* strain CS2 has been obtained (Zhang et al. 2008). These results show that the process of protein turnover is more active during fruiting body formation.
Amino acid status also has a strong impact on cleistothecium development in *A. nidulans* (Krappmann, and Braus, 2003). It is reported that the total level of amino acids in the fruiting body (16.4%) is higher than that in fermented mycelia (9.23%) (Hsu et al. 2002). Some enzymes catalysing amino acid synthesis, such as O-acetylhomoserine sulfhydrylase, cobalamin-independent methionine synthase and chorismate binding enzyme were highly expressed in the fruiting body of *C. sinensis* (Table 1), which indicated that amino acid biosynthesis is active in fruiting body formation. Indeed, homocysteine synthase (O-acetylhomoserine sulfhydrylase), which was over-expressed in mature ascoma of fungus *Tuber borchii*, might be involved in its fruiting body formation (Zeppa et al. 2010). Cobalamin-independent methionine synthase can be only observed in conidia rather than in mycelia of entomopathogenic fungus *Metarhizium acridum* (Barros et al. 2010). The enzyme is inducible by heat and estrogen in fungus *Candida albicans* (Burt et al. 1999). While homologous chorismate binding enzymes can catalyse the initial biosynthesis of tryptophan, menaquinone and siderophores (Zwahlen et al. 2007). Especially, chorismate mutase is the first enzyme of the branch of the shikimate pathway which catalyses a necessary step in biosynthesis of aromatic amino acids. Aromatic amino acids are not only the essential composition of proteins but also crucial precursors for many secondary metabolites (Pudelski et al. 2009). Fruit body formation and ascosporogenesis of *Aspergillus nidulans* significantly decreased with chorismate mutase gene aroC knocked out (Krappmann, and Braus, 2003). Unfortunately, chorismate mutase decreased in the fruiting body of *C. sinensis* rather than mycelia of *H. sinensis*. The mechanism need further study.

### Proteins involved in cell division control

It is a very complicated transformation that from wire-like hyphal filaments into complex and sometimes container-like fruit bodies, which is necessary for the transformation to reconstruct major cells (Busch and Braus 2007).

Actins are highly conserved proteins involved in various types of cell motility, and tubulins are involved in complex structures like the mitotic spindle, centrioles, cilia, flagella and cytoskeleton (Poetsch et al. 1989). During fruiting body formation of fungus *Physarum polycephalum*, the most prominent syntheses of an actin decreased while two tubulins T1 and T2 were strongly increased (Putzer et al. 1984; Poetsch et al. 1989). Increased tubulins suggest the cellular reconstruction is active during fruiting body formation. Actually, predicted similar to Actin-5C isoform 1, actin 6, beta actins were down-regulated, but beta tubulins were consonantly up-regulated in the fruiting body of *C. sinensis* (Table 1). Alpha 2 tubulin gene was highly expressed in the immature fruiting body of *L. edodes* (Chum et al. 2011), while α-tubulins are constituents of microtubules responsible for cytoskeleton and further cell shape regulation (Juuti et al. 2005). It was noticed that two different tubulins simultaneously decreased in the mature fruiting body of *C. militaris* rather than in early stage, which suggested that tubulins possibly involved in fruiting body initiation rather than maturation.

UDP-N-acetylglucosamine pyrophosphorylase is a major enzyme in amino sugar synthesis during cyst wall formation (encystment) of *Giardia* and that its allosteric anabolic activation may shift the equilibrium of this pathway towards UDP-Gal-NAc synthesis (Bulik et al. 2000). UDP-N-acetylglucosamine pyrophosphorylase was increased in the fruiting body of *C. sinensis* (Table 1), which was in accordance with the previous report (Jin 2005).

### Proteins involved in cell reconstruction

Septin, as a cell division control protein, is involved in septa formation during cell division, and the highest expression of septin is found in the transitional zone between cap and stipe of mature mushroom *A. bisporus* (De Groot et al. 1998; Zeppa et al. 2002). The expression of this enzyme is strongly induced during fruiting body development and maturation of mushroom *A. bisporus* (De Groot et al. 1997).

Mago nashi proteins, highly conserved among eukaryotes, not only participate in oogenesis, embryogenesis and germ-line sex determination during animal development, but also play important roles in pollen tube growth, root development and spermatogenesis during plant development (Chen and Chu 2010; Lewandowski et al. 2010). They also participate in fungi development and are abundantly expressed in natural basidiomes (fruiting bodies) of
medicinal fungus *Antrodia cinnamomea* (Chu et al. 2009).

In this study, septin, mago nashi protein and cell division control protein 3 distinctly increased in the fruiting body of *C. sinensis*, besides higher septin was in the mature fruiting body of *C. militaris* (Table 1). These indicate that cell division contributes to the fruiting body formation.

In summary, this study identified 198 differential expression proteins that may relate to fruiting body development of *Cordyceps*, and 24 proteins have been proven their roles in fruiting body development in other fungi (Table 2). Among the identified proteins, acetaldehyde dehydrogenase, beta-tubulin, elongation factor 2, enolase, malate dehydrogenase, heat shock 70 kDa protein are the key proteins for fruiting body formation and development of *C. sinensis*. Especially, enolase and malate dehydrogenase were first proposed in fruiting body development of mushroom. Besides, the cAMP signal pathway as well as glycometabolism, protein metabolism, energy metabolism, cell division and cell reconstruction are presumed to be related to fruiting body development of *C. sinensis* (Figure 4). A map of metabolic pathways involved in fruiting body development of *C. sinensis* was also hypothesised (Figure 5). It has become evident that fruiting body formation of *C. sinensis* is a highly complex differentiation process and requires precise integration of a number of fundamental biological processes. Although the fruiting body formation mechanisms for all these activities remain to be further elucidated, the study presented here provides a framework for understanding them.

![Figure 4. Hypothesised cAMP signal pathways involved in fruiting body formation of *C. sinensis.*](image-url)
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Disclosure statement

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