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iTRAQ-based quantitative proteome revealed metabolic changes of *Flammulina velutipes* mycelia in response to cold stress

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ABSTRACT

Temperature is one of the pivotal factors influencing mycelium growth and fruit-body formation of *Flammulina velutipes*. To gain insights into hyphae growth and fruit-body formation events and facilitate the identification of potential stage-specific biomarker candidates, we investigated the proteome response of *F. velutipes* mycelia to cold stresses using iTRAQ-coupled two-dimensional liquid chromatography tandem mass spectrometry (2D LC-MS/MS) technique. Among 1198 proteins identified with high confidence, a total of 63 displayed altered expression level after cold stress treatments. In-depth data analysis reveals that differentially expressed proteins were involved in a variety of cellular processes, particularly metabolic processes. Among the 31 up-regulated proteins, 24 (77.42%) were associated with 22 specific KEGG pathways. These up-regulated proteins could possibly serve as potential biomarkers to study the molecular mechanisms of *F. velutipes* mycelia response to cold stresses. These data of the proteins might provide valuable evidences to better understand the molecular mechanisms of mycelium resistance to cold stress and fruit-body formation in fungi.

Biological significance: Low-temperature is one of the pivotal factors in some *Flammulina velutipes* industrial processes influencing mycelium growth, inducing primordia and controlling fruit-body development. Preliminary study has indicated that effectively regulating cultivation could augment the yield by controlling optimal cold stress level on mycelia. However, we are still far from understanding the molecular and physiological mechanisms of adaptation of these fungi at cold stress. In the present study, the experiments reported above were undertaken to investigate chronological changes of protein expression during *F. velutipes* mycelia in response to cold stress by using iTRAQ-coupled 2D LC-MS/MS technique. This result would provide new insights to the underlying mycelium growth and fruit-body formation mechanisms of basidiomycetes under cold stress.

1. Introduction

Flammulina velutipes (Curt. ex Fr.) Sing, a white-rot fungus, is known as golden-needle mushroom, winter mushroom or enokitake. Because of its high nutritional and medicinal value, *F. velutipes* is cultivated on a significant scale worldwide. Fruit-body quality of *F. velutipes* is determined by both genotype and environment. Both yield and quality of *F. velutipes* fruit-bodies differ largely depending on their primordium number, and the initiation of primordium is prone to environmental factors, especially cold stress during mycelium growth [1]. Cold stress is also a key factor in some *F. velutipes* industrial processes inducing primordia and controlling fruit-body development. Preliminary study has indicated that effectively regulating cultivation could augment the yield by controlling optimal cold stress level on mycelia [2–3]. Meanwhile, *F. velutipes* is a low-temperature fungus whose mycelia can grow vegetatively at 20–24 °C, and fruiting at an optimum temperature for 12–15 °C, and its favorable conditions can be conveniently built by using some refrigeration equipment. Therefore, *F. velutipes* has emerged as a potentially excellent model system for studies of fungal genetics, development, biochemistry and environmental stress [4–6]. Knowledge of chronological protein expression patterns in *F. velutipes* mycelia in response to cold stress is an important metabolic engineering strategy that considers the impact of novel genes and pathways on cold adaptation of fungi.

Proteomics techniques, defined as the global and quantitative measurement of cellular proteins in a particular biological state, are powerful tools for the identification of the quantitative changes in protein expression in response to stress exposure at cells, tissues or biological fluids in filamentous fungi [7]. The standard approaches to proteomics have been one-/two-dimensional gel electrophoresis (DE) or liquid chromatograph (LC) followed by mass spectrometry (MS) [8]. With the recent advent of chromatography, mass spectrometry, and bioinformatics, the labor intensive gel-based techniques have been challenged and complemented by LC-based methods, particularly in the area of high-throughput proteomic research [9]. Coupled two-dimensional

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liquid chromatography tandem mass spectrometry (2D LC-MS/MS), the isobaric tags for relative and absolute quantitation (iTRAQ) labeling technique has been proved that has the ability to analyze simultaneous changes and to classify the temporal patterns of protein accumulation in complex developmental processes of filamentous fungi [7,10]. Therefore, iTRAQ-coupled 2D LC-MS/MS technique is more suitable to gain insights into chronological changes of proteomic profiles during *F. velutipes* mycelia in response to cold stress.

In this study, we used the iTRAQ-coupled 2D LC-MS/MS technique to assess proteome changes of *F. velutipes* mycelia in response to cold stress. Chronological changes of protein expression patterns after cold stress treatment were investigated. Furthermore, proteins expressed characteristically and abundantly in different cold stress periods were compared and examined. This study will contribute to important metabolic processes for fungal mycelia response to cold stress. These data might also provide new insights to the underlying molecular mechanisms of mycelium growth and fruit-body formation under cold stress in basidiomycetes.

2. Materials and methods

2.1. Strains and culture conditions

The *F. velutipes* strain Fv13 mycelia were cultured on potato dextrose agar (PDA) (20% potato extract, 2% glucose, 2% agar, pH 7.0) plates at 23–25 °C in darkness. After being incubated for 25 days, control mycelia were collected. After cold stress treatment under 12–15 °C, whole plates of mycelia were collected for 3 days and 14 days, and sampled short term cold stress (STCS) and long term cold stress (LTCS), respectively. All samples were stored at −80 °C for protein extraction.

2.2. Protein preparation

Approximately 2 g frozen samples from three samples above mentioned were ground into fine powders in liquid nitrogen and extracted with 30 mL acetone containing 10% w/v trichloroacetic acid (TCA). The samples were kept at −20 °C overnight and then centrifuged at 25,000 × *g* for 15 min at 4 °C. The pellets were washed twice with 90% methanol buffer (90% methanol, 50 mM Tris-HCl pH 7.8) and air dried. The frozen sample powders were re-suspended and homogenized in 10 ml extraction buffer (4% SDS, 1 mM DTT, 150 mM Tris-HCl, pH 8.0). After 5 min incubation in boiling water, the homogenate was sonicated on ice for 4 min. The crude extract was then incubated in boiling water again for 5 min and clarified by centrifugation at 16,000*g* at 25 °C for 10 min. Total protein concentration was determined by the Bradford assay using bovine serum albumin (BSA) from Applied Biosystems as the standard.

2.3. Protein digestion and iTRAQ labeling

200 μg of proteins for each samples were incorporated into 30 μl STD buffer (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0), incubated at boiling water for 5 min, cooling to room temperature. 200 μl UA buffer (8 M Urea, 150 mM Tris-HCl pH 8.0) was added and centrifuged at 14,000*g* for 15 min. This step was repeated twice. Then, 100 μl 0.05 M iodoacetamide in UA buffer was added and the samples were incubated for 20 min in darkness and centrifuged at 14,000*g* for 10 min. After the filters were washed three times with 100 μl UA buffer, 100 μl DS buffer (50 mM tri ethyl ammonium bicarbonate at pH 8.5) were added to the filters and the samples were centrifuged at 14,000*g* for 10 min. This step was repeated twice. Finally, 2 μg trypsin solution (2 μg trypsin from Promega in 40 μl DS buffer) was added to the filters. The samples were incubated overnight at 37 °C. The resulting peptides were collected by centrifugation and the peptide content was tested by BCA method. The filters were rinsed with 40 μl 10× DS buffer again. iTRAQ labeling performed according to the manufacturer's instructions. The tryptic peptides were incubated with 8-plex iTRAQ labeling kit (AB Sciex, Foster City, CA, USA). Control samples (Fv13) were labeled with reagent 119; short cold stress (STCS) and long cold stress (LTCS) samples were labeled with reagent 115 and 116, respectively.

2.4. LC-MS/MS analysis and database search

The peptides were fractionated on a waters UPLC using a C18 column (waters bed c18 2.1 × 50 mm, 1.7 μm). Peptides were eluted at a flow rate of 600 μl/min with a linear gradient of 5–35% solvent B (acetonitrile) over 10 min, the solvent A is 20 mM ammonium formation with pH adjusted to 10. The absorbance at 214 nm was monitored, and a total of 20 fractions were collected. The fraction was separated by nano-HPLC (Eksigent Technologies) on the secondary RP analytical column (Eksigent, C18, 3 "μm", 150 mm × 75 "μm"). Peptides were subsequently eluted using the following gradient conditions with phase B (98% ACN with 0.1% formic acid) from 5 to 45% B (5–100 min) and total flow rate was maintained at 300 nl/min. Electrospray voltage of 2.5 kV versus the inlet of the mass spectrometer was used. Triple TOF 5600 mass spectrometer was operated in information-dependent data acquisition mode to switch automatically between MS and MS/MS acquisition. MS spectra were acquired across the mass range of 350–1250 m/z using 250 ms accumulation time per spectrum. Tandem mass spectral scanned from 100 to 1250 m/z in high sensitivity mode with rolling collision energy. The 25 most intense precursors were selected for fragmentation per cycle with dynamic exclusion time of 25 s.

All LC-MS/MS data were processed using MASCOT software. Tandem mass spectra were extracted by [unknown] version [unknown]. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.0). Mascot was set up to search the Agaricales database (unknown version, 138,749 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.20 Da and a parent ion tolerance of 25 PPM. Carbamidomethyl of cysteine and iTRAQ 8 plex of lysine and the n-terminus were specified in Mascot as fixed modifications. Oxidation of methionine and iTRAQ 8 plex of tyrosine were specified in Mascot as variable modifications.

2.5. Quantitative data analysis

Scaffold Q+ (version Scaffold_4.4.6, Proteome Software Inc., Portland, OR) was used to quantitate Label Based Quantitation (iTRAQ, TMT, SILAC, etc.) peptide and protein identifications. Peptide identifications were accepted if they could be established at >5.0% probability to achieve an FDR <1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at >95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [11]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Channels were corrected by the matrix [0.000,0.000,0.929,0.0689,0.00220]; [0.000,0.00940,0.930, 0.0590,0.00160]; [0.000,0.0188,0.931,0.0490,0.001000]; [0.000,0.0282, 0.932,0.0390,0.000700]; [0.000600,0.0377,0.933,0.0288,0.000]; [0.0009 00,0.0471,0.933,0.0188,0.000]; [0.00140,0.0566,0.933,0.00870,0.000]; [0.000,0.000,0.000,0.000,0.000]; [0.00270,0.0744,0.921,0.00180,0.000] in all samples according to the algorithm described in i-Tracker [12]. Acquired intensities in the experiment were globally normalized across all acquisition runs. Individual quantitative samples were normalized within each acquisition run. Intensities for each peptide identification were normalized within the assigned protein. The reference channels were normalized to produce a 1:1 fold change. All normalization calculations were performed using medians to multiplicatively normalize data.

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