Comparative phosphoproteome analysis to identify candidate phosphoproteins involved in blue light-induced brown film formation in Lentinula edodes

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Abstract

Background
Light plays an important role in the growth and differentiation of Lentinula edodes mycelia, and mycelial morphology is influenced by light wavelengths. The blue light-induced formation of brown film on the vegetative mycelial tissues of L. edodes is an important process. However, the mechanisms of L. edodes' brown film formation, as induced by blue light, are still unclear. Using a high-resolution liquid chromatography-tandem mass spectrometry integrated with a highly sensitive immune-affinity antibody method, phosphoproteomes of L. edodes mycelia under red- and blue-light conditions were analyzed.

Results
A total of 11,224 phosphorylation sites were identified on 2,786 proteins, of which 9,243 sites on 2,579 proteins contained quantitative information. In total, 475 sites were up-regulated and 349 sites were down-regulated in the blue vs red group. To characterize the differentially phosphorylated proteins, systematic bioinformatics analyses, including gene ontology annotations, domain annotations, subcellular localizations, and Kyoto Encyclopedia of Genes and Genomes pathway annotations, were performed. These differentially phosphorylated proteins were correlated with light signal transduction, cell wall degradation, and melanogenesis, suggesting that these processes are involved in the formation of the brown film.

Conclusions
Our study provides new insights into the molecular mechanisms of the blue light-induced brown film formation at the post-translational modification level.

Background
Lentinula edodes, also known as shiitake mushroom, belonging to Lentinus, is a valuable medicinal and edible fungus[1]. It is a popular edible mushroom and the third most cultivated mushroom in the world[2]. During cultivation, there are at least four growth stages: vegetative mycelial growth with growth substrate colonization, the light-induced brown film formation, primordial formation, and fruiting body development[3]. The brown film formation on the surface of mature mycelia usually
appears on the fruiting body primordia and may represent a speciation step[3-5]. In addition, the mycelial surface does not form a brown film, which is easily occupied by pathogenic organisms, such as bacteria, green molds and fungi[6]. Light signals are essential factors in the formation of brown films[7-9]. The basic genetic regulatory mechanisms of brown film formation and the influence of environmental factors, especially light, remain unclear. Comparative transcriptome studies revealed that the mechanisms of light-induced brown film formation are related to photosensitivity, signal transduction pathways, and melanin deposition[7]. Several gene ontology (GO) classifications related to brown film formation were revealed by two-dimensional electrophoresis combined with the matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry approach and included small molecule metabolic processes, response to oxidative stress, and organic substance catabolic processes[10]. Blue light is an important environmental factor in inducing primordial differentiation and the fruiting body development of mushrooms, such as Hypsizygus marmoreus, Pleurotus ostreatus, and Coprinus cinereus[11-13].

During the growth and development of fungi, the influence of light is very important, and it is also necessary for their growth and development[14]. As an external signal, light regulates mycelial growth, primordial differentiation, fruiting body formation, gene expression, and metabolite and enzyme activities through complex light-sensing systems[15-18]. At least 100 kinds of fungi have light-perception systems, including red, blue, green, and near-violet[19]. Photoreceptors are proteins that harvest light and produce signals that are then transported to the nucleus to activate the transcription of light-responsive genes[20]. The white collar-1/white collar-2 (WC-1/WC-2) complex is the main blue-light sensor in Neurospora crassa, a model organism for studying photoperiod[21, 22]. Other blue-light receptors have been successfully identified and cloned, such as the dst1 and dst2 genes in C. cinereus, phrA and phrB in L. edodes, Cmwc-1 in different strains of Cordyceps militaris, and Slwc-1 from Sparassis latifolia[12, 23-27]. However, the molecular mechanisms of blue light-induced brown film formation are still unknown.

With the determinations and in-depth analyses of genome and transcriptome sequences of model organisms, such as Arabidopsis thaliana, researchers have realized that it is impossible to understand
the functions of organisms from only a gene-based perspective[28]. Proteomics studies the compositions, expressions, structures, functions, interactions between proteins and their activities[29]. Isobaric tags for relative and absolute quantification/tandem mass tag (iTRAQ/TMT)-labeling combined with tandem mass spectrometry is a high-throughput quantitative proteomics application technology developed in recent years[30]. Compared with relatively stable genomes, proteins are diverse and changeable. In addition, the presence of post-translational modifications (PTMs) and protein processing, such as phosphorylation, glycosylation, and acetylation, are not comparable at the genome or RNA level[31]. Proteomics research is a cutting-edge technique in the edible fungi industry. With the effects of abiotic stresses on protein expression levels have been studied the most[32, 33].

In this study, an immunoaffinity analysis combined with high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to study the global phosphorylated proteome of brown films induced by blue light. This study provides new insights into the molecular mechanisms of blue light-induced brown film formation at the PTM level.

Results

**Characteristics of quantitative phosphoproteomic data in L. edodes mycelia**

Using affinity enrichment followed by LC-MS/MS, the phosphoproteomic changes in L. edodes mycelia grown in red or blue light were investigated. A flow chart of our experiment is exhibited in Fig.1a. Pearson’s correlation coefficient between the two groups showed sufficient reproducibility (Fig. 1b). In this study, 160,949 secondary spectra were obtained by MS analyses. After searching the theoretical protein data, the effective number of spectra was 22,857 and the utilization rate of the spectra was 14.2%. In total, 8,830 peptides and 7,777 phosphorylated peptides were identified. There were 11,224 phosphorylation modification sites on 2,786 proteins, of which 9,243 sites on 2,579 proteins provided quantitative information (Fig.1c). The first-order mass errors of most spectra are less than 10 ppm, which is in accordance with the high accuracy of the MS (Fig. 1d). Most of the peptides were distributed in 7–20 amino acids, which was in accordance with the general rules of trypsin-based enzymatic hydrolysis and high energy collision dissociation (HCD) fragmentation, indicating that the
sample preparation and the quality accuracy of the mass spectrometer reached the standard required (Fig.1e). The detailed information regarding the identified peptides are listed in Additional file 1: Table S1.

**Analysis of phosphorylation sites**

In *L. edodes* mycelia, 977 (35.07%) phosphoproteins were modified at a single site, 519 (18.63%) at two sites, and 1,290 (46.3%) at three or more phosphosites (Fig.2a). Interestingly, some proteins contained a large number of phosphosites. For example, there are 34 phosphosites in a non-specific serine/threonine protein kinase (A0A1Q3E061), 45 phosphosites in a regulatory transcript from a polymerase II promoter-related protein (A0A1Q3ERS8) and 53 phosphosites in a SRC Homology 3(Sh3) domain-containing protein (A0A1Q3ENM7) (Additional file 1: Table S1).

To analyze the density levels of the phosphorylation sites in each protein, the phosphorylated proteome of *L. edodes* was compared with those of other species. The average number of phosphorylation sites per protein in *L. edodes* is 3.22, which is similar to the numbers in Bombyx mori (3.07), Nicotiana tabacum (3.05), and Physcomitrella patens (3.44) (Fig.2b)[34-36].

**Characteristics of the identified phosphoproteins in *L. edodes***

To predict the possible functions of the identified phosphoproteins, a GO classification analysis was performed. Most of the proteins were classified into three GO categories (Fig. 3a). Specifically, 594 proteins were annotated as ‘metabolic process’, 519 proteins were annotated as ‘cellular process’, and 361 proteins were annotated as ‘single-organism process’. In the cellular component category, the largest terms were ‘cell’ (289 proteins), ‘organelle’ (186 proteins), and ‘macromolecular complex’ (154 proteins). In the molecular function category, ‘binding’ (846 proteins), ‘catalytic activity’ (627 proteins), and ‘transporter activity’ (51 proteins) were the three top dominant terms. The euKaryotic Ortholog Groups annotation clustered all the phosphoproteins into four major categories. The ‘cellular processes and signaling’ category contained the largest number of proteins (Fig.3b). Most identified phosphoproteins were grouped into 13 subcellular component categories predicted by WoLF PSORT software, including 783 nuclear, 380 cytoplasmic, and 275 mitochondrial proteins (Fig.3c). The detailed annotation information for all the identified phosphoproteins are listed in Additional file 2:
Table S2.

**Protein motifs associated with phosphorylation**

Among the identified phosphosites in *L. edodes*, 8,645 sites occurred at serine residues, 2239 sites at threonine residues, and 340 sites at tyrosine residues (Fig.4a). To understand the upstream pathway of the identified phosphorylated proteins, a motif analysis was carried out using MOMO and Motif-X software. A number of conserved phosphorylation motifs were enriched in the phosphorylated proteins of *L. edodes* (Additional file 3: Table S3). A total of 7,741 distinct sequences containing 13 residues were obtained, with 6 upstream and 6 downstream residues around each phosphosite (Additional file 4: Table S4). The five S-based motifs containing the largest numbers of sequences were ‘sP’, ‘RxxsP’, ‘PxsP’ ‘Gs’, and ‘RRxS’, and the five top T-based motifs were ‘tP’, ‘tPP’, ‘RxxtP’, ‘RxTP’, and ‘Rxxt’. A Y-based motif, ‘Rxxxxxy’, was identified. Two position-specific heat maps of upstream and downstream amino acids at all the identified phosphorylated serine or threonine sites. For the S-based motifs, strong preferences for glutamic acid, lysine, and arginine upstream, and aspartic acid, glutamic acid, and proline downstream, of the phosphorylation sites were observed. For the T-based motifs, preferences for lysine, proline, and arginine upstream, and aspartic acid and proline downstream, of the phosphorylation sites were observed (Fig. 4c).

**Differentially phosphorylated proteins (DPPs) in response to a blue-light treatment**

To compare the DPPs between red- and blue-light treated samples, expression profiles of the proteins generated by MeV software are shown in a heatmap (Fig. 5a). The screening of DPPs followed the following criteria: change threshold ≥1.5 times and t-test p-value < 0.05. Among these DPPs, 475 sites in 317 phosphorylated proteins were up-regulated and 349 sites in 243 phosphorylated proteins were down-regulated (Fig. 5b and Additional file 5: Table S5). Based on the subcellular localizations predicted by WoLF PSORT software, all the DPPs were classified into 10 subcellular components. There were 204 nuclear localized DPPs, 82 cytoplasmic localized DPPs, and 51 plasma membrane localized DPPs (Fig. 5C).

**Functional enrichment analysis of the DPPs**

To understand the biological functions of these phosphorylated proteins, GO, KEGG and protein
domain enrichment analyses of DPPs were carried out. For biological process, cellular component, and molecular function categories, the DPPs were mostly enriched in ‘DNA conformation change’ (Fig. 6a); ‘nucleosome’ (Fig. 6b), and ‘transporter activity’ (Fig. 6c), respectively.

To reveal the metabolic pathways involved in the formation of brown films induced by blue light, the DPPs were further analyzed using the KEGG database. For the up-regulated DPPs, two KEGG pathways, ‘Ribosome biogenesis in eukaryotes’, and ‘ABC transporters’, were significantly enriched’ (Fig. 7a). For the down-regulated DPPs, four enriched KEGG pathways were identified, ‘Valine, leucine and isoleucine degradation’, ‘Phenylalanine metabolism’, ‘Galactose metabolism’, and ‘Fructose and mannose metabolism’ (Fig. 7b). We also found that the total polysaccharides of blue light treatment was significantly lower than that of red light treatment (Additional file 6: Fig. S1). A protein domain enrichment analysis revealed that the up-regulated DPPs were enriched in 19 protein domains, with ‘ABC transporter-like’, ‘P-type ATPase’, and ‘HAD-like domain’ being the most highly enriched. The down-regulated DPPs were most strongly associated with ‘Glutathione S-transferase, C-terminal-like’, ‘YTH domain’ ‘VPS9 domain’, ‘Domain of unknown function DUF1708’, and ‘High mobility group box domain’.

**Identification of DPPs related to signal transduction mechanisms and carbohydrate-active enzymes (CAZymes)**

To better understand the DPPs related to blue light-induced mycelial brown film formation, a functional classification of DPPs was conducted using euKaryotic Ortholog Groups. A total of 319 DDPs were grouped into 23 subcategories (Additional file 7: Fig. S2). For the ‘signal transduction mechanisms’ subcategory, 50 phosphosites in 29 phosphorylated proteins were identified (Table 1). Among these, 30 phosphosites were up-regulated and 20 were down-regulated.

CAZymes, including auxiliary activity (AA), carbohydrate-binding modules (CBM), carbohydrate esterase (CE), glycoside hydrolase (GH), glycosyl transferase (GT), and polysaccharide lyase (PL), were involved in the hydrolysis of plant cell wall polysaccharides and play an important role in the degradation of substrates[37]. In the present study, 13 DPPs were identified as CAZymes, including 11 phosphosites in three CBMs, two phosphosites in two CEs, four phosphosites in three GHs, and six
phosphosites in five GTs (Table 2). Interestingly, the GHs were up-regulated, while the CBMs were down-regulated.

Discussion

With the completion of various biological genome sequences, proteomics has become an increasingly important analysis of important proteins based on the differential recognition of their expression levels. Protein phosphorylation is an important PTM, which can rapidly control enzyme activity, subcellular localization, and protein stability, and involves the regulation of metabolism, transcription, and translation, as well as protein degradation, homeostasis, cell signaling, and communication[38-40]. Recently, large-scale quantitative phosphoproteomics analyses were performed in many plants to elucidate the growth, development, and diverse response mechanisms, but the technology has rarely been applied to L. edodes[39]. Here, we report a comprehensive analysis of phosphoproteomic responses to blue light-induced mycelial brown film formation of L. edodes through a combination of affinity enrichment and LC-MS/MS.

Protein phosphorylation is a common PTM, but the level of phosphorylation varies with species. In L. edodes, the number of phosphoproteins (2,786 phosphorylated proteins) was more than in most published species, such as Abelmoschus esculentus (2,550 phosphorylated protein), Ammopiptanthus mongolicus (2,019 phosphoproteins), Aspergillus nidulans (647 phosphoproteins) B. mori (2,112 phosphoproteins), Catalpa fargesii (1,646 phosphoproteins), Lotus japonicus (1,154 phosphoproteins), N. tabacum (1,311 phosphoproteins), and Sus domesticus (966 phosphoproteins). [34-36] The number of phosphorylation sites in each protein is 3.22, which is higher than most published phosphorylation proteomes, indicating that the degree of phosphorylation in the L. edodes proteome is very high. The large number of identified phosphoproteins provide an opportunity to comprehensively analyze the mechanism of blue light-induced mycelial brown film formation. The ‘sP’ motif most frequently occurred in many species, including L. edodes[41-43]. ‘sP’ is a target of the following kinases: cyclin-dependent kinase, mitogen-activated protein kinase (MAPK), and sucrose non-fermenting1-related protein kinase 2[41, 43]. The ‘tP’ motif also provides a target for MAPKs[44].

In Basidiomycetes, light is a crucial environmental factor that affects fruiting body induction and
development[45, 46]. In recent years, in fungi, the effects of different light wavelengths on mycelial morphology, metabolites, and enzymatic activities have been studied. In Monascus, red and blue light can affect the formation of mycelia and spores, as well as the production of secondary metabolites[17]. In this study, we found that blue light can promote the formation of a brown film associated with L. edodes mycelia, but no correlation was found with a red-light treatment. The effects of blue light on the expression levels of phosphorylated proteins during brown film formation were studied. Phosphorylation proteomics revealed that 560 phosphorylated proteins were differentially expressed during a blue-light treatment.

Brown film formation at the transcriptional level is correlated with photoreceptor activity, light signaling pathways, and pigment formation[7]. Most fungi perceive blue light through homologues of the white collar complex, which is a complex of photoreceptors and transcription factors that was first found in Neurospora crassa[47]. The N-terminus of WC-1 is a lov domain, which is a special Per-Arnt-Sim (PAS) domain that can bind to flavin adenine dinucleotide[14]. In the present study, three flavin adenine dinucleotide-binding domains and an FMN-binding domain differentially accumulated, indicating that the L. edodes mycelia could have perceived blue light when the brown film was formed. The MAPK cascade is an important signal transduction pathway connecting light responses and the biological clock[48]. MAPK also regulates various secondary metabolic activities in Aspergillus nidulans and Colletotrihum lagenarium, and it controls light-influenced melanin biosynthesis in B. cinerea[49-52]. The MAPK signal transduction pathways may be directly involved in brown film formation[7]. Several MAPK signal transduction pathways related to DPPs were identified in this study, suggesting that these signal pathways are involved in the formation of brown films.

The differential expression of CAZymes were observed in L. edodes mycelia under two light conditions. GHs mainly hydrolyze glycosidic bonds between carbohydrates or between carbohydrates and non-carbohydrates[53]. The GH61 family contains copper-dependent lytic polysaccharide monooxygenase[54]. CEs catalyze the deacylation of esters or amides, in which sugar plays the role of alcohol and amine[55, 56]. They are currently divided into 16 different families, which have a great diversity in substrate specificity and structure[56]. CE10 (two DPPs) were down-regulated by blue
light. CBMs are noncatalytic, individually folded domains that are attached to the catalytic enzyme modules by linkers\[57\]. Some CE1 enzymes may contain a CBM48 family protein, which is associated with starch binding\[58, 59\]. Our research showed that these CAZymes play important roles in the degradation of lignocellulose and provide sufficient nutrition for the formation of the brown film of mushroom mycelia.

To survive, fungi have evolved the ability to adapt to different environmental conditions, and various metabolic pathways secrete different metabolites\[60\]. The regulation of these metabolites is not only related to fungal growth and development, but also to light stimulation and responses. The shorter the light wavelength, the more polysaccharides accumulated in the cells of *Pleurotus eryngii*\[61\].

Blue-light treatments significantly improved the synthesis of ergosterol and polyphenols in the fruiting body of *Pleurotus eryngii*, and the scavenging ability of the free radicals was the greatest compared with other light treatments\[61\]. In our study, the KEGG-enrichment analysis showed that four DPPs belonged to ‘Galactose metabolism’ and ‘Fructose and mannose metabolism’, suggesting that the blue light affected the sugar metabolism of *L. edodes*. Phenolic compounds were correlated with pigment formation\[62\]. Phenylalanine ammonia-lyase and tyrosinase-encoding genes were significantly up-regulated in *P. eryngii* under blue-light conditions\[63\]. Two ‘Phenylalanine metabolism’ pathway phosphoproteins, amidase (A0A1Q3E9W2) and aspartate aminotransferase (A0A1Q3EG41), were down-regulated in mycelia under blue-light conditions. These results suggested that blue light may promote the formation of melanin and inhibit the formation of other phenolic compounds. Polyketide synthase (PKS) is an essential enzyme in the biosynthesis of fungal secondary metabolites\[64, 65\]. PKSs modify the polyketide backbone with other enzymes, such as Cytochrome P450 monooxygenases, oxidoreductase, and omethyltransferase\[64\]. P450-linked monooxygenases mediate oxidation-reduction steps in aflatoxin biosynthesis, and omethyltransferase was involved in yellow pigment biosynthesis through an aflatoxigenic Aspergillus strain\[66\]. In our study, the phosphorylation levels of PKS, O-methyltransferase, P450 monooxygenase, and oxidoreductase changed in brown film formation, indicating that they may play roles in pigment production.

**Conclusions**
Using a high-resolution LC-MS/MS integrated with a highly sensitive immune-affinity antibody method, phosphoproteomes of *L. edodes* mycelia under red- and blue-light conditions were analyzed. In this study, 11,224 phosphorylation sites were identified on 2,786 proteins, of which 9,243 sites on 2,579 proteins contained quantitative information. In total, 475 sites were up-regulated and 349 sites were down-regulated in the blue vs red group. Then, we carried out a systematic bioinformatics analyses of proteins containing quantitative information sites, including protein annotations, functional classifications, and functional enrichments. Our study provides new insights into the molecular mechanisms of the blue light-induced brown film formation at the PTM level.

**Materials And Methods**

**Materials treatment and protein extraction**

The *L. edodes* strain L901 which is a new hybrid strain was obtained from the Zhejiang Academy of Agricultural Sciences. Fungal mycelia were grown at 22°C under red- and blue-light conditions for 22 d. Samplings were taken after mycelial changed colour under blue light conditions. The determination of total polysaccharides was performed according to Zhang's description [67]. For protein extraction, a proper amount of sample was ground in liquid nitrogen into a cellular powder and then transferred to a 5-mL centrifuge tube. The samples were treated with four volumes of lysis buffer (10 mM dithiothreotol, 1% protease inhibitor, and 1% phosphatase inhibitor) and then sonicated three times. The supernatant was centrifuged for 10 min at 4°C and 5,500 g with an equal volume of Tris equilibrium phenol. The supernatant was taken and precipitated overnight with a fivefold volume of 0.1 M ammonium acetate/methanol. The protein precipitation was washed sequentially with methanol and acetone. The protein was redissolved in 8 M urea, and the protein concentration was determined using a bicinchoninic acid assay kit (P0012, Beyotime, Shanghai, China) according to the manufacturer’s instructions.

**Trypsin digestion, TMT labeling, and HPLC fractionation**

For digestion, the final concentration of dithiothreotol in the protein solution was 5 mM and was reduced at 56°C for 30 min. The 11-m final concentration of iodoacetamide was incubated at room temperature for 15 min. Finally, the urea concentration of the sample was diluted to less than 2 M.
Trypsin was added at a mass ratio of 1:50 (trypsin:protein), and enzymatic hydrolysis was carried out overnight at 37°C. The trypsin was added at a mass ratio of 1:100, and the enzymatic hydrolysis continued for 4 h.

The trypsinase-hydrolyzed peptide segments were desalted using a Strata X C18 (Phenomenex) and then freeze-dried in a vacuum. The peptide segment was dissolved in 0.5 M Triethylammonium bicarbonate and labeled according to the instructions of the TMT kit. The simple operation was as follows: the labeled reagent was dissolved in acetonitrile after thawing, incubated at room temperature for 2 h after mixing with the peptide segment, desalinated after mixing with the labeled peptide segment, and freeze-dried in a vacuum.

The tryptic peptides were fractionated using high pH reverse-phase HPLC on an Agilent 300Extend C18 column (5-μm particles, 4.6-mm ID, 250-mm length). Briefly, peptides were first separated using a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into six fractions and dried by vacuum centrifugation.

**Affinity enrichment**

Peptide mixtures were first incubated with an immobilized metal ion affinity chromatography (IMAC) microsphere suspension and vibrated in loading buffer (50% acetonitrile and 6% trifluoroacetic acid). The IMAC microspheres enriched with phosphopeptides were collected by centrifugation, and the supernatant was removed. To remove nonspecifically adsorbed peptides, the IMAC microspheres were washed with loading buffer and 30% acetonitrile plus 0.1% trifluoroacetic acid, sequentially. To elute the enriched phosphopeptides from the IMAC microspheres, elution buffer containing 10% NH4OH was added, and the enriched phosphopeptides were eluted with vibration. The resulting peptides were desalted with C18 ZipTips (Millipore) and lyophilized for the LC-MS/MS analysis.

**LC-MS/MS analysis**

The tryptic peptides were dissolved in 0.1% formic acid and directly loaded onto a home-made reversed-phase analytical column (15-cm length and 75-μm i.d.). The gradient increased from 6% to 23% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23% to 35% in 8 min and to 80% in 3 min. It was then held at 80% for the last 3 min, at a constant flow rate of 400 nL/min on an EASY-
nLC 1000 UPLC system.

The peptides were subjected to an NSI source followed by MS/MS in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1,800 for a full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using normalized collision energy (NCE) set as 28, and the fragments were detected in the Orbitrap at a resolution of 17,500. The data-dependent procedure alternated between one MS scan and 20 MS/MS scans with a 15.0-s dynamic exclusion. The automatic gain control was set at 5E4. The fixed first mass was set as 100 m/z.

**Database search**

The resulting MS/MS data were processed using the Maxquant search engine (v.1.5.2.8). The MS/MS spectra were used as query against a human uniprot database concatenated with a reverse decoy database. Trypsin/P was specified as the cleavage enzyme, allowing up to four missing cleavage events. The mass tolerance for precursor ions was set as 20 ppm in the first search and 5 ppm in the main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as a fixed modification, and acetylation and oxidation of Met were specified as variable modifications. The FDR was adjusted to < 1%, and the minimum score for modified peptides was set > 40.

**Annotation methods and functional enrichment**

The GO annotation on the proteomics level was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). First, the system converted the protein ID to UniProt ID, matched the GO ID with the UniProt ID, and then extracted the corresponding information from the UniProt-GOA database based on the GO ID. If there was no protein information queried in the UniProt-GOA database, then algorithm software based on the protein sequence, InterProScan, was used to predict the GO function of the protein.

The KEGG database was used to annotate protein pathways. First, the KEGG online service tool KAAS was used to annotate the submitted proteins, and then KEGG mapper was used to place the annotated proteins into the corresponding pathways in the database. WoLF PSORT, a software for
predicting subcellular localization, was used to annotate the submitted proteins for subcellular localization. Fisher’s exact test was used to detect differentially modified proteins against the background of identified proteins. A P-value of less than 0.05 was considered significant. The softwares motif-x and MoMo were used to analyze the models of sequences that contained the amino acids in specific positions of modified 13-mers (six amino acids upstream and downstream of the site) in all the protein sequences.

Supplementary Information

Additional files

Additional file 1: Table S1. The detail information of identified peptides pertinent to detected proteins.

Additional file 2: Table S2. The detail information of all identified peptides.

Additional file 3: Table S3. Statistics of phosphorylation site sequence.

Additional file 4: Table S4. The detail information of protein motifs associated with phosphorylation.

Additional file 5: Table S5. The detail information of differential phosphosites.

Additional file 6: Fig. S1. Determination of total polysaccharide contents in two lights treatments group of L. edodes.

Additional file 7: Fig. S2. KOG analysis of the DPPs between two different illumination treatments

Abbreviations

AA: Auxiliary activity; CBM: Carbohydrate-binding modules; CE: Carbohydrate esterase; DPP: Differentially phosphorylated protein; GH: Glycoside hydrolase; GO: Gene Ontology; GT: Glycosyl transferase; iTRAQ: Isobaric tags for relative and absolute quantification; KEGG: Kyoto Encyclopedia of Genes and Genomes; LC-MS: Liquid chromatography electrospray ionization tandem mass spectrometry; MAPK: Mitogen-activated protein kinase; PKS: Polyketide synthase; PTM: post-translational modification; QC: Quality control; TMT: Tandem Mass Tag; WC: White collar

Declarations

Ethics approval and consent to participate

The L. edodes strain L901 was used in our study. This project uses plant materials and does not utilize transgenic technology. It does not require ethical approval.
Consent to publish
Not applicable

Availability of data and materials
The datasets generated and analysed during the current study are available in the Proteome EXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016536 (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD016536).

Competing interests
The authors declare that they have no competing interests.

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Author Contributions
TTS, YYS and WMC conceived and designed the research. TTS and YYS performed the experiments. TTS wrote the manuscript. QLJ and WLF analyzed the data, and LJF formatted and revised the manuscript. WMC supervised the project. All authors have read and approved the final manuscript.

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Table 1. List of differentially expressed signal transduction mechanisms related phosphosites

| Protein accession | Position | Ratio | Regulated | Type | P value | Amino acid | Protein description |
|-------------------|----------|-------|-----------|------|---------|------------|---------------------|
| A0A1Q3DXT2        | 147      | 2.041 | Up        |      | 0.000701| S          | Actin cytoskeleton-regulatory complex protein pan1 |
| A0A1Q3DXT2        | 940      | 0.661 | Down      |      | 0.0212  | S          | Actin cytoskeleton-regulatory complex protein pan1 |
| A0A1Q3DXT2        | 340      | 1.566 | Up        |      | 0.00104 | S          | Actin cytoskeleton-regulatory complex protein pan1 |
| A0A1Q3DXT2        | 538      | 1.503 | Up        |      | 0.000622| S          | Actin cytoskeleton-regulatory complex protein pan1 |
| A0A1Q3DXT2        | 145      | 2.041 | Up        |      | 0.000701| S          | Actin cytoskeleton-regulatory complex protein pan1 |
| A0A1Q3DXT2        | 626      | 1.573 | Up        |      | 0.00602 | S          | Arf gtpase activator |
| A0A1Q3EQA0        | 342      | 0.666 | Down      |      | 0.0269  | S          | Carbohydrate-binding module family 21 protein |
| A0A1Q3EH65        | 144      | 0.457 | Down      |      | 0.00398 | S          | Carbohydrate-binding module family 21 protein |
| A0A1Q3EH65        | 184      | 0.46  | Down      |      | 0.000337| S          | Carbohydrate-binding module family 21 protein |
| A0A1Q3EH65        | 1114     | 0.634 | Down      |      | 0.00106 | S          | Carbohydrate-binding module family 21 protein |
| A0A1Q3EH65        | 391      | 0.512 | Down      |      | 0.0000778 | S          | Carbohydrate-binding module family 21 protein |
| ID       | Value | Fold | Change | p-value  | Description                                         |
|----------|-------|------|--------|----------|-----------------------------------------------------|
| A0A1Q3EIZ3 | 287   | 0.542| Down   | 0.0000823 | Casein kinase II subunit beta                       |
| A0A1Q3EIZ3 | 363   | 0.472| Down   | 0.000779  | Casein kinase II subunit beta                       |
| A0A1Q3EHC7 | 350   | 1.787| Up     | 0.0024    | Ck1 ckl ck1-d protein kinase                        |
| A0A1Q3EMY6 | 60    | 0.596| Down   | 0.00757   | Gtpase-activating protein gyp7                       |
| A0A1Q3DYY9 | 5     | 1.542| Up     | 0.00764   | Guanine nucleotide-binding protein                   |
| A0A1Q3EYY9 | 220   | 1.778| Up     | 0.00163   | Guanine nucleotide-binding protein                   |
| A0A1Q3EBC7 | 120   | 0.663| Down   | 0.00414   | HCP-like protein                                     |
| A0A1Q3EQ51 | 108   | 2.293| Up     | 0.0123    | Kinase-like protein                                  |
| A0A1Q3EEF5 | 191   | 0.653| Down   | 8.16E-07  | Map kinase                                           |
| A0A1Q3EEF5 | 189   | 0.641| Down   | 1.16E-06  | Map kinase                                           |
| A0A1Q3EEF5 | 194   | 0.572| Down   | 0.000373  | Map kinase                                           |
| A0A1Q3E4D7 | 4     | 0.566| Down   | 0.00739   | Mitogen activated protein kinase-like protein        |
| A0A1Q3EI7  | 44    | 0.59 | Down   | 0.00196   | mRNA stability protein OS=Lentinula edodes           |
| A0A1Q3E289 | 28    | 0.651| Down   | 0.00319   | Neutral alkaline nonlysosomal ceramidase             |
| A0A1Q3EKW8 | 21    | 1.828| Up     | 0.00182   | Non-specific serine/threonine protein kinase         |
| A0A1Q3EKW8 | 19    | 1.717| Up     | 0.000797  | Non-specific serine/threonine protein kinase         |
| A0A1Q3E982 | 790   | 0.53 | Down   | 0.023     | Non-specific serine/threonine protein kinase         |
| A0A1Q3E1S4 | 101   | 0.642| Down   | 0.00332   | Otu-like cysteine                                    |
| A0A1Q3E102 | 696   | 1.667| Up     | 0.0108    | Phosphatidylinositol 3-kinase VPS34                  |
| A0A1Q3E326 | 400   | 0.496| Down   | 0.000677  | Protein phosphatase 2c                               |
| A0A1Q3E326 | 588   | 0.554| Down   | 0.000166  | Protein phosphatase 2c                               |
| A0A1Q3E326 | 402   | 0.5  | Down   | 0.000243  | Protein phosphatase 2c                               |
| A0A1Q3E326 | 586   | 0.51 | Down   | 0.000438  | Protein phosphatase 2c                               |
| A0A1Q3E326 | 393   | 0.655| Down   | 0.00102   | Protein phosphatase 2c                               |
| A0A1Q3EFRO | 271   | 0.543| Down   | 0.000024  | Protein serine threonine phosphatase 2C              |
| A0A1Q3EY4  | 138   | 1.593| Up     | 0.000198  | Ras guanyl-nucleotide exchange factor                |
| A0A1Q3EH9  | 765   | 0.636| Down   | 0.000277  | Rho gtpase activator                                |
| A0A1Q3DW25 | 452   | 0.335| Down   | 0.000459  | Serine threonine-protein kinase                      |
| A0A1Q3EBM7 | 137   | 0.648| Down   | 0.00158   | SGS-domain-containing protein                        |
| A0A1Q3EKV3 | 265   | 1.703| Up     | 0.039     | Signal transducer                                    |
| A0A1Q3EKV3 | 267   | 2.602| Up     | 0.000018  | Signal transducer                                    |
| A0A1Q3EKV3 | 263   | 2.391| Up     | 9.39E-07  | Signal transducer OS=Lentinula edodes              |
| A0A1Q3EB1  | 1413  | 1.776| Up     | 0.000573  | Sin component scaffold protein cdc11               |
| A0A1Q3DX25 | 818   | 0.602| Down   | 0.0273    | TKL TKL-ccin protein kinase                         |
| A0A1Q3EHP8 | 698   | 2.269| Up     | 0.000503  | Uncharacterized protein                             |
| A0A1Q3EY7  | 1099  | 1.628| Up     | 0.000258  | Uncharacterized protein                             |
| A0A1Q3EHP8 | 695   | 2.269| Up     | 0.000503  | Uncharacterized protein                             |
| A0A1Q3E1M6 | 515   | 0.586| Down   | 0.00232   | YTH domain-containing protein 1                     |
| A0A1Q3E1M6 | 531   | 0.639| Down   | 0.0227    | YTH domain-containing protein 1                     |

**Table2.** List of differentially expressed carbohydrateactive enzymes family phosphosites

Figures
| Protein accession | Position | Ratio | Regulated Type | P value | Amino acid | Protein description |
|------------------|----------|-------|----------------|---------|------------|---------------------|
| glycoside hydrolase |
| A0A1Q3DVW4       | 888      | 1.522 | Up             | 0.00618 | S          | Glycoside hydrolase family 105 |
| A0A1Q3DVY0       | 489      | 1.774 | Up             | 0.000241| S          | Glycoside hydrolase family 1 pr |
| A0A1Q3DVY0       | 481      | 1.508 | Up             | 0.0398  | S          | Glycoside hydrolase family 1 pr |
| A0A1Q3EE19       | 429      | 1.931 | Up             | 0.0000401| S         | Glycoside hydrolase family 61 p |
| carbohydrate-binding module |
| A0A1Q3DXJ6      | 394      | 0.555 | Down           | 0.0000212| T          | Carbohydrate-binding module family 68 |
| A0A1Q3DXJ6      | 396      | 0.556 | Down           | 0.000962 | T          | Carbohydrate-binding module family 68 |
| A0A1Q3DXJ6      | 377      | 0.483 | Down           | 0.0000158| S          | Carbohydrate-binding module family 68 |
| A0A1Q3DXJ6      | 409      | 0.503 | Down           | 0.000161 | S          | Carbohydrate-binding module family 68 |
| A0A1Q3DXJ6      | 380      | 0.508 | Down           | 0.0000025| S          | Carbohydrate-binding module family 68 |
| A0A1Q3DXJ6      | 388      | 0.451 | Down           | 0.0000568| S          | Carbohydrate-binding module family 68 |
| A0A1Q3E7W8       | 134      | 0.561 | Down           | 0.000969 | S          | Carbohydrate-binding module family 68 |
| A0A1Q3EH65      | 1114     | 0.634 | Down           | 0.00106  | S          | Carbohydrate-binding module family 68 |
| A0A1Q3EH65      | 391      | 0.512 | Down           | 0.000778 | S          | Carbohydrate-binding module family 68 |
| A0A1Q3EH65      | 184      | 0.46  | Down           | 0.000337 | S          | Carbohydrate-binding module family 68 |
| A0A1Q3EH65      | 144      | 0.457 | Down           | 0.00398  | S          | Carbohydrate-binding module family 68 |
| carbohydrate esterase |
| A0A1Q3E195      | 171      | 0.312 | Down           | 0.000639 | S          | Lipase from carbohydrate esterase family 16 |
| A0A1Q3EGY1      | 39       | 0.605 | Down           | 0.000116 | T          | Lipase from carbohydrate esterase family 16 |
| glycosyl transferase |
| A0A1Q3DXW9      | 1240     | 1.663 | Up             | 0.000342 | S          | Glycosyltransferase family 20 p |
| A0A1Q3E591      | 146      | 0.655 | Down           | 0.00272  | T          | Glycosyltransferase family 2 pr |
| A0A1Q3E591      | 24       | 2.532 | Up             | 0.0291   | S          | Glycosyltransferase family 2 pr |
| A0A1Q3EH60      | 1581     | 1.585 | Up             | 0.000836 | S          | Glycosyltransferase family 2 pr |
| A0A1Q3E3I6      | 235      | 0.64  | Down           | 0.009    | S          | Glycosyltransferase Family 22 p |
| A0A1Q3ERC2      | 75       | 0.622 | Down           | 0.00812  | T          | Glycosyltransferase family 2 pr |
Figure 1

Overview of the phosphorylation proteomes. a) The pictures showed the fungal mycelia under different illumination for 22 days. Experimental strategy for the quantitative analysis of phosphorylation proteomes from red and blue light treatment groups. b) Pearson’s correlation of the phosphorylation proteomes from two sample groups (three biological replicates for each group). c) Basic statistical data of MS results. d) Mass error distribution of all identified phosphorylated peptides. X-axis: Peptide Score; Y-axis: Peptides mass delta. e) Length distribution of all identified phosphorylated peptides. X-axis: No. of Peptide; Y-axis: Peptide length.
Figure 2

Analysis of the density of phosphorylation sites. a) Modification phosphorylated sites distribution of all identified peptides. b) Comparison of the average densities of phosphorylation sites per protein among various species.
Figure 3

Annotation and classification of all identified phosphorylated proteins and differentially phosphorylated proteins (DPPs). a) GO analysis of all phosphorylated proteins and DPPs. All proteins were classified by GO terms based on their biological process, cellular component and molecular function. b) Subcellular locations of all phosphorylated proteins. c) Subcellular locations of DPPs.
Phosphosite types and peptide motifs associated with phosphorylation. a) The distribution of phosphosites between serine, threonine and tyrosine residues. b) Motif analysis of the amino acids surrounding the phosphosites. Sequence logo representation of 5 S-based and 5 T-based conserved phosphorylation motifs. c) A plot showing the relative abundance of amino acids flanking a phosphorylated serine (S) and threonine (T) using the intensity map.
Figure 5

Impacts of illumination treatment on phosphorylation proteome levels in fungal mycelia. a) Heat map for the accumulation levels of all the identified phosphorylated proteins. Red indicates up-regulation and green indicates down-regulation. The heatmap scale ranges from 0 to +2. b) All DPPs were analyzed and clustered into four major Clusters by K-means method. c) The numbers of up- and down-regulated sites and proteins in red and blue light treatment comparison.
Figure 6

GO enrichment analysis of DPPs based on biological process (a), cellular component (b) and molecular function (c).
Figure 7

KEGG and domain enrichment analysis of the DPPs in fungal mycelium between two different illumination treatments. a) KEGG enrichment analysis of up- and down-regulated phosphorylated proteins. b) protein domains enrichment analysis of up- and down-regulated phosphorylated proteins.

Supplementary Files

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