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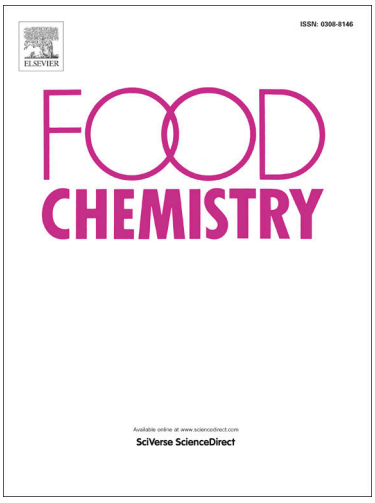

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Melatonin enhances the cadmium tolerance of mushrooms through antioxidant-related metabolites and enzymes

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Abstract:

Many wild mushroom species are known to immune heavy metals. However, the mechanism by which this occurs remains largely unsolved. Melatonin (MT) has been proven to play an important defensive role against various abiotic stresses in plants and animals. This study reports on the presence of MT in edible fungi and its role in the response to cadmium (Cd)-induced stress. We found that melatonin was widely distributed in all experimental species and could also relieve Cd-induced damage in the *Volvariella volvacea*. Comparative metabolic and proteome analyses reveal that tryptophan/proline/tyrosine metabolism, the citrate cycle, nitrogen and glutathione metabolism, and oxidation-reduction processes were enriched after Cd and/or MT addition, indicating an antioxidant mechanism was aroused. Finally, different MT and cadmium treatments were studied for their effects on the expression and activity of oxidation-related enzymes (superoxide dismutase, catalase, peroxidase, etc), which further verified the ameliorative influence of MT on Cd-induced oxidative stress.

Keywords: melatonin, mushroom, heavy metal, antioxidant metabolites, antioxidant enzymes

1. Introduction

Heavy metal pollution has become one of the most serious environmental problems faced today, both in China and worldwide (Yang, Li, Lu, Duan, Huang, & Bi, 2018). Of all heavy metal pollutants, Cd poses serious threats to both humans and crops because it is readily absorbed by plant roots and integrated into above-ground

tissues (Gill & Tuteja, 2011). Cd tends to induce toxic effects in all organisms at low doses (0.1-1 mg/kg) and resulting in inhibited growth and stunted reproduction in animals and plants. Furthermore, Cd-accumulation can cause a diverse range of adverse effects such as cell damage, promotion of lipid-peroxidation, and inhibition of photosynthesis, pollen germination, and tube growth (Hasan, Liu, Pan, Ahammed, Qi, & Zhou, 2018; Gill & Tuteja, 2011). Hence, the reduction of Cd-induced damage is imperative for the protection of humans, animals, and plants that have been exposed to high concentrations of Cd (Hasan, Ahammed, Sun, Li, Yin, & Zhou, 2019).

The abnormal accumulation of high levels of Cd and other heavy metals has been observed in the macro fungi (edible fungi) kingdom. Mushrooms appear to be immune to high concentrations of heavy metals, while equal concentrations in plants are decidedly toxic (Kalač, 2010). This indicates that mushrooms have evolved diverse mechanisms to protect themselves from toxicity caused by heavy metals. For instance, in *Pleurotus ostreatus*, Cd induces an increase in active oxygen and malonaldehyde and is accountable for the stimulation of the antioxidant system and glutathione metabolism, which behave as guards against Cd-induced damage and facilitate detoxification (Li, et al., 2017; Liu, et al., 2017). However, the exact effect of Cd tolerance is still a contentious issue, and it is difficult to clarify the regulatory pathways and modes solely by analyzing the enzymatic activity involved in the antioxidant defense system. Previously, it has been reported that excessive bioaccumulation of heavy metals in mushrooms may be attributed to compartmentation and chelation (Wu, et al., 2012). Several other agents, such as heat

shock proteins and ubiquitin, have been reported to play an important role in Cd tolerance (Shim, et al., 2009). In addition, main agronomic measures such as hybridization, grafting, and plant hormones are also reported to enhance tolerance to adversity (Lin, et al., 2020; Wang, et al., 2019; Li, et al., 2019; Xia, et al., 2020; Liu, et al., 2019). Nevertheless, the exact mechanism of fungal resistance to the bioaccumulation of heavy metals remains largely unsolved.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a pleiotropic molecule that is ubiquitous in nature. Not only does it play multifaceted roles in humans and animals, but also in plant growth and development. In animals, it acts as a neurohormone originating in the pineal gland, and its key functions include promoting sleep, regulating physiological events, and participating in cellular pathways as an antioxidizing agent (Arnao & Hernández-Ruiz, 2015). In plants and bacteria, the most well-known functions of MT are related to the amelioration of abiotic stresses arising from diverse factors, such as drought, radiation, extreme temperature, and chemical stresses, all of which have been reported to promote the generation of reactive oxygen species (ROS) (Arnao & Hernández-Ruiz, 2015). Many studies have shown that melatonin can mediate selenium-induced tolerance to Cd stress in tomatoes through Cd detoxification and can ameliorate water-deficit stress in grapes via antioxidant metabolites (Li, et al., 2016; Meng, Xu, Wang, Fang, Xi, & Zhang, 2014). Hence, it is possible that the same mechanisms of action occur in fungi in response to Cd-induced stress. Although the presence of melatonin in fungi within the phylum Ascomycota has been understood for a long time, little research has been conducted regarding the

presence of melatonin in members of the phylum Basidiomycota (Hardeland, 1999; Hardeland, & Poeggeler, 2003). Additionally, few studies have examined fungi as a source of MT, or the role of MT in the acquisition of Cd immunity to high concentrations of heavy metals. Hence, further research is needed to investigate whether melatonin has this supposed function in edible fungi, as well as the potential mechanism of action by which it occurs.

In this study, ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS/MS) was used to determine the presence of MT in edible fungi (Szafrańska, Szewczyk, & Janas, 2014). In the experimental design (Fig. S1), varying doses of exogenous MT and Cd were administered to *V. volvacea* (a type of edible fungus) to investigate the role of MT in response to Cd stress, which was characterized by a shorter growth/cell cycle. The results demonstrate that MT can relieve Cd-induced damage in *V. volvacea.* Furthermore, a metabolic and proteomic analysis was conducted to investigate the potential mechanism of MT-mediated Cd tolerance in *V. volvacea*. Ultimately, the expression and activity of oxidation-related enzymes were investigated to confirm the role of MT in the reduction of Cd-induced oxidative stress. This study indicates that MT exists in all eight of the studied edible fungi and exert an ameliorative effect on Cd toxicity via antioxidant metabolites and enzymes.

Materials and methods

2.1. Mycelium culture and treatment

All eight edible fungi strains were obtained from the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences, China. All mycelium samples (*Hericium erinaceus*, *Hypsizygus marmoreus*, *Pleurotus geesteranus*, *Pleurotus eryngii*, *Ganoderma Lucidum*, *Flammulina velutiper*, and *Lentinus edodes*) other than *V. volvacea* were cultured in potato dextrose medium in an incubator shaker at shaking speed of 150 rpm and a temperature of 25 ^{o}C for 7 days. The *V. volvacea* strain was cultured in similar medium at 32 ^{o}C for 5 days. The samples were divided into four groups as follows: control group, Cd-treated group (2, 5, and 8 μM $CdCl_2$, designated as Cd2, Cd5, and Cd8, respectively), melatonin (MT)-treated group (50, 100, or 200 μM MT solutions, designated as MT50, MT100 and MT200, respectively), and Cd + MT-treated group (100 μM MT + 5 μM $CdCl_2$) (Sun, et al., 2015; Li, et al., 2016; Hasan, et al., 2015).

2.2. Melatonin qualitative and quantitative analysis

MT was extracted using protocols outlined in previous publications (Szafrańska, Szewczyk, & Janas, 2014). Then, the dried residue was re-dissolved in 100 μL of acetonitrile and subjected to MS/MS analysis (Acquity UPLC I-Class, MS/MS, Waters MA, USA). Mobile phase A, composed of methanol, and mobile phase B, composed of water containing 10 mM ammonium acetate and 0.1% formic acid, were applied at a flow rate of 0.4 mL/min. Aliquots of samples (5 μL) were injected on a column maintained at 45 ^{o}C using gradient conditions as follows: 0 min, 5:95; 1 min, 95:5; 3 min, 95:5; 3.6 min, 5:95; 4 min, 5:95. The mass spectrometer operated in positive electrospray ionization (ESI) mode during the analysis, and the optimized

settings were: drying gas (N_2) flow rate (1000 L/h), capillary voltage (2.5 kV), ion source temperature (150 ^oC), desolvation temperature (450 ^oC), conical pore gas (N_2) flow rate (150 L/h), and collision gas (argon) flow rate (0.11 L/h). The retention time of MT was 2.70 min. MS/MS detection was performed in positive ionization mode. The conditions for MS/MS detection of MT were optimized and MS/MS detection MRM pairs for MT were 233.0-174.0 m/z (CE = 12) - quantifier ion, 233.0-159.0 (CE = 24)- qualifier ion. A commercial ELISA kit (Meimian, Yancheng, Jiangsu, China) was used for MT quantitative analysis (Shi, Wang, Tan, Reiter, & Chan, 2015).

2.3. Determination of mycelial growth rate and dry weight

The *V. volvacea* mycelia were treated with Cd and MT, followed by incubation at 32 ^oC. After every 12 h, new lines were drawn on each plate. The distances between two adjacent lines were measured and used to calculate the mycelial growth rate. To determine the mycelial dry weight, mycelium was first cultured in a potato dextrose agar plate, and then transferred into potato dextrose liquid medium wherein different samples underwent Cd and MT treatment. After cultivation at 32 ^oC for 5 days, the mycelium was collected and dried at 80 ^oC until its measured weight was constant, which took 72 h.

2.4. Enzyme activities analysis

Samples for enzyme activity analysis were cultured using the method described in section 2.1. The samples were thoroughly ground in a pre-cooled mortar inside a liquid nitrogen environment. Powdered sample (0.1 g) was dissolved in 1 mL of potassium phosphate buffer (0.1 M, pH 7.8) and centrifuged at 32 ^oC for 10 min at

10000 g. The supernatant was tested for enzymatic activity according to the manufacturer's instructions. Enzyme activity kits for detecting superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and peroxidase (POD) were purchased from either Beyotime (Shanghai, China) or Jiancheng Bio-Engineering Institute (Nanjing, China). A BCA protein concentration determination kit from Beyotime (Shanghai, China) was used to measure the protein concentration of the collect supernatant of each sample. Enzyme-linked immunosorbent assay (ELISA) kit was purchased from Meimian (Yancheng, Jiangsu, China) and used for the quantification of endogenous MT, hydrogen peroxide (H_2O_2), and superoxide anion ($O_2^{\cdot-}$) (Shi, Wang, Tan, Reiter, & Chan, 2015; Shi, Ye, & Chan, 2013).

2.5. Real-time quantitative PCR (RT-qPCR) analysis

RT-qPCR analysis was performed to examine the gene expression level using previously reported protocols (Qian, et al., 2018; Zhao, et al., 2016). RT-qPCR was performed using LightCycler 480 (Roche Molecular Biochemicals, Mannheim, Germany) and AceQ qPCR SYBR Green Master Mix (Vazyme, Nangjing, China). The primer sequences used in this study are listed in Table S1. As outlined in a previous report, Alpha-tubulin (*TUBα*) gene served as a reference gene to normalize samples (Qian, et al., 2018). The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001). Primer specificity was confirmed by analyzing the melt curve of each gene (Fig. S2). The PCR efficiency (90-110% E) and the regression coefficient ($R^2 > 0.99$) also indicated that all primers amplified well and were suitable

for investigating the relative expression of genes (Fig. S2) (Qian, et al., 2018; Zhao, et al., 2016).

2.6. Metabolite extraction and derivation

Thirty-two well-dried mycelium samples (60 mg each) were divided into 4 groups, with 8 biological repeats in each group. An individual sample was transferred to a 1.5 mL Eppendorf tube containing two small steel balls followed by the addition of 360 µL of cold methanol, and 40 µL of internal standard (2-chloro-l-phenylalanine) (0.3 mg/mL in methanol). The samples were cooled at -80 °C for 2 min. Subsequently, the samples were ground at 60 Hz for 2 min and then ultrasonicated at ambient temperature for 30 min. Each sample was combined with 200 µL of chloroform and 400 µL of ddH_2O and ultrasonicated for another 30 min. The samples were then centrifuged at 14000 rpm at 4 °C for 15 min. 200 µL of the supernatant was taken in a glass vial and freeze-dried for further analysis. To each freeze-dried sample, 80 µL of a methoxylamine hydrochloride solution (15 mg/mL in pyridine) was added and vortexed vigorously for 2 min, followed by incubation at 37 °C for 90 min for the oxidation reaction. Each sample was derivatized by adding 80 µL of BSTFA (with 1% TMCS) and 20 µL of *n*-hexane, vortexing vigorously for 2 min, and allowing it to dry further at 70 °C for 60 min. The samples were placed at ambient temperature for 30 min and filtered prior to gas chromatography mass spectrometry analysis (Hu, et al., 2016).

2.7. GC-MS and data analysis

The derivatized samples were analyzed using the GC-MS system (7890A-5975C,

Agilent Technologies Inc., CA, USA) equipped with an HP-5MS fused-silica capillary column (30 cm × 0.25 mm × 0.25 μm, Agilent Technologies Inc., CA, USA). Helium (>99.999%) was used as the carrier gas at a constant flow rate of 6.0 mL/min, and the temperature of the injection port was maintained at 280 °C. The injection volume for each sample was 1 μL under splitless mode. The column temperature was programmed as follows: 60 °C initial stage, ramped to 125 °C at a rate of 8 °C/min, increased to 190 °C at a rate of 10 °C/min, ramped to 210 °C at a rate of 4 °C/min, and ultimately maintained at 310 °C using a ramping rate of 20 °C/min for 8.5 min (Hu, et al., 2016). The temperatures of the MS quadrupole and the ion source were set to 150 and 230 °C, respectively. The collision energy was set at 70 eV. Mass data were acquired in full-scan mode (m/z 50-600) and the solvent delay time was fixed to 5 min. The acquired MS data from GC-MS was analyzed by ChromaTOF software (v 4.34, LECO, St Joseph, MI) and metabolites were identified using the Fiehn database linked to the ChromaTOF software. The resulting data were normalized to the total peak area of each sample in Excel 2007 (Microsoft, USA) and imported into SIMCA (version 14.0, Umetrics, Umeå, Sweden), wherein principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and orthogonal partial least-squares discriminant analysis (OPLS-DA) were performed.

2.8. Protein extraction and digestion

Two biological replicates from each of the four groups described above were subjected to iTRAQ analysis. Total protein extraction was performed according to a previously reported protocol (Shi, Wang, Tan, Reiter, & Chan, 2015; Shi, Ye, &

Chan, 2013). The proteins were then re-dissolved in 500 mM TEAB (triethylammonium bicarbonate) to determine their concentration using the BCA method (Shi, Wang, Tan, Reiter, & Chan, 2015; Shi, Ye, & Chan, 2013). From each sample, 100 μg of protein was harvested and transferred into a new tube, and the final volume was adjusted to 100 μL using 8 M urea. To reduce disulfide bonds, each sample was combined with 11 μL of a 1 M solution of dithiothreitol (DTT) and incubated at 37 °C for 1 h. The sample was transferred into a 10-kDa cutoff Microcon centrifugal filter (Millipore, Billerica, MA) to remove urea using three cycles of centrifugation at 4℃ and the addition of 100 mM TEAB. The filtrates from each sample were separately combined with 120 μL of a 55 mM solution of iodoacetamide (IAA) and incubated at ambient conditions for 20 min in the dark. An excessive quantity of IAA was quenched by the addition of an extra 100 mM of DTT. Finally, the proteins were digested with sequence-grade modified trypsin (1:50, Promega, Madison, WI) and lyophilized.

2.9. TMT labeling and nanoLC-MS/MS analysis

The resultant peptide mixture was labeled using a TMT-10plex isobaric mass tag labeling kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instructions. The labeled peptide samples were then pooled and lyophilized in a vacuum concentrator. The peptide mixture was re-dissolved in buffer A (20 mM ammonium formate in water, pH 10.0) and fractionated by high pH separation using the Ultimate 3000 system (Thermo Fisher Scientific, MA, USA) equipped with a reverse phase column (XBridge C18 column, 4.6 mm × 250 mm, 5 μm, Waters

Corporation Inc., MA, USA). High pH separation was performed using a linear gradient, from 5% B to 45% B, for 40 min (B: 20 mM ammonium formate in 80% ACN, pH 10.0). The column was re-equilibrated at initial conditions for 15 min by keeping a flow rate of 1mL/min and maintaining a temperature of 30 °C. A total of twelve fractions were collected, and each fraction was dried in a vacuum concentrator. The dried peptides were re-dissolved in 30 µL of solvent A (0.1% formic acid in water) and subjected to chromatographic separation. The labeled peptides were separated and identified through the UPLC system (NanoAcquity, Waters Corporation Inc., MA, USA) equipped with an analytical column (Acclaim PepMap C18, 75 µm × 15 cm) and on-line nanospray LC-MS/MS(Q Exactive™, Thermo Fisher Scientific, MA, USA). For peptide fractionation, 6 µL of peptide sample was injected into the analytical column and separated using the linear gradient: 3% B (B: 0.1% formic acid in ACN) to 38% B for 90 min. The column flow rate and temperature were maintained at 300 nL/min and 40 °C, respectively. The electrospray voltage versus the inlet of the mass spectrometer was maintained at 2 kV.

2.10. iTRAQ data analysis

Tandem mass spectra were processed by PEAKS Studio version 8.5 (Bioinformatics Solutions Inc., Waterloo, Canada). Search criteria in the PEAKS DB included a fragment ion mass tolerance of 0.02 Da and a parent ion tolerance of 10 ppm. Carbamidomethylation (C) and TMT 10plex (K, N-term) were specified as the fixed modifications. Oxidation (M), Deamidation (NQ), and acetylation (Protein N-term) were specified as the variable modifications. Peptides were filtered by 1%

FDR and 1 unique. PEAKSQ was used to calculate peptide and protein abundance. Normalization was performed by averaging the abundance of all peptides, with median values used to determine the average. Distinct expressed proteins were filtered if their fold change exceeded 1.2 and they contained at least 2 unique peptides with significance values over 13 ($p < 0.05$). Hierarchical Cluster Analysis (HCA) was conducted using the pheatmap package (https://CRAN.R-project.org/package=pheatmap). A volcano plot was drawn using the ggplot2 package (http://ggplot2.org). Blast2GO version 4 was used for functional annotation. Pathway analysis was conducted via KOBAS (Xie, Mao, Huang, Ding, Wu, Dong, et al., 2011). Analysis and mapping of the whole protein sequence database were performed using BlastP and annotated using data from the gene ontology database.

2.11. Statistical analysis

All the physiological measurements reported in this study are the means of three replicates. Vertical bars represent ± S.E. Statistical analysis was performed by one-way ANOVA using Duncan's multiple range test ($p = 0.05$). Letter symbols, such as a, b, or c are defined as follows: different lower-case letters on a bar chart are distinct from each other and indicate a significant difference ($p < 0.05$) among their corresponding values. If the same lower-case letter appears in multiple places on a figure, then the corresponding values do not exhibit a significant difference ($p > 0.05$).

3. Results

3.1. Melatonin was widely distributed in all eight edible fungi species

To confirm the presence of MT in edible fungi UPLC-MS/MS analysis was conducted. MS/MS data analysis demonstrated that all eight of the common edible fungi (Fig. S3) had a detectable MT content. This was further confirmed using MT as a reference standard, along with its secondary fragment ions (Fig. 1A). MRM and MT quantification were carried out using 233.0/174.0 MRM transitions. The [M + H] peak, identified at 232.962, and the fragments, at 174.048 and 159.029, were consistent with the previous report (Zhang, et al., 2014). Additionally, quantification via the ELISA method revealed that the MT content of different edible fungi species varies from 5 - 25 $pg \cdot g^{-1}$ FW (Fig. 1B) (Rodriguez-Naranjo, Gil-Izquierdo, Troncoso, Cantos, & Garcia-Parrilla, 2011). Interestingly, *H. erinaceus* turned out to have the most abundant amount of MT (about 24 pg/g FW), which may contribute to its neuroprotective function (Shimbo, Kawagishi, & Yokogoshi, 2005). The presence of MT in edible fungi signifies that MT might function as an antioxidant, and may play an important role in the process of conferring physiological regulatory effects on edible fungi, which has been reported in plants and animals (Fan, Xie, Zhang, & Chen, 2018; Tan, Hardeland, Back, Manchester, Alatorre-Jimenez, & Reiter, 2016).

3.2. Physiological parameters changes of *V. volvacea* indicated melatonin could relieve $CdCl_2$-induced damage

To assess the role of MT in physiological regulation within edible fungi, heavy metal ($CdCl_2$)-induced damage was applied to simulate the abnormal accumulation of heavy metals that commonly occurs in edible fungi. Since *H. erinaceus* grows very

slowly, *V. volvacea* was selected as the potential candidate. Moreover, the regular circadian rhythms of MT concentration in *V. volvacea* also suggested that *V. volvacea* might be a good candidate for the assessment of the role of MT during physiological regulation in edible fungi (Fig. S4). Subsequent testing for whether the stimulation of endogenous MT biosynthesis can alter the response in varying exogenous MT and Cd concentrations revealed that treatment with MT alone does not alter mycelium growth, as confirmed by the relatively constant morphology, growth rate, and dry weight (Fig. 2). It is noteworthy that the addition of exogenous MT may induce the generation of endogenous MT. However, treatment of the 5-day-old *V. volvacea* with 2, 5, and 8 μM $CdCl_2$ solutions prompted significantly decreased mycelium growth, while MT levels were markedly increased. This significant increase in endogenous MT concentration indicates that the MT response is directly related to Cd stress. Compared to the samples treated with Cd alone, those treated with both MT and Cd possessed a similar mycelium diameter, but the intensive situation of mycelium had increased, indicating the mitigating role of MT in Cd-induced growth inhibition. This was consistent with the measured growth rates and the dry weights (Figs. 2B and 2C). To determine whether Cd and exogenous MT induce transcription in MT biosynthetic genes, we assayed the expression of *TDC*, *T5H*, *SNAT*, and *ASMT* genes, which are reported to be responsible for MT biosynthesis (Back, Tan, & Reiter, 2016). It can be inferred that the transcription levels of these genes were induced by the application of a 5 μM $CdCl_2$ solution (Fig. 2). The addition of both Cd and MT simultaneously may further increase the expression of genes involved in MT biosynthesis, especially *TDC,*

which initiates MT biosynthesis (Back, Tan, & Reiter, 2016). Thus, our results indicate that MT could relieve $CdCl_2$-induced damage in *V. volvacea,* although the exact mechanism is not fully understood yet.

3.3. Comparative metabolic analyses reveal that glutathione metabolism is involved in the ameliorative effects of MT on Cd stress

In order to investigate the possible mechanisms by which MT relieves $CdCl_2$-induced damage, a comparative metabolic analysis was conducted in *V. volvacea* samples with or without MT or $CdCl_2$ addition, as outlined in the methods section. A comparison of four groups using principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) revealed a significant difference among the different treatment groups (different colors in Fig. S5). More precisely, the results implied that Cd and MT treatments may alter the metabolite profiles of the *V. volvacea* samples. This was verified with a heatmap analysis of differential metabolites with VIP > 1 and p-value < 0.05 (Fig. S6, Table S3). As indicated by the overview of the heatmap, significant differences can be observed for most of the metabolites among the different treatment groups, especially tyrosine, alanine, methionine, and sorbose. For example, the content of tyrosine is low in the control group, and was burst in the Cd group. While, when MT was added, the content of tyrosine seems to flatten out. Generally, MT addition prompted significant changes in the metabolites involved in the citrate cycle, the pentose phosphate pathway, and the metabolism of some amino acids (Fig. S6 and S7). $CdCl_2$ addition, on the other hand, promoted significant changes to the metabolic pathways involved

in glutathione, starch sucrose metabolism. In addition, enhanced sulfur and proline metabolism indicated that a self-defense mechanism against Cd-induced damage was active. Considering that glutathione is a reductant, it can be inferred that the Cd-induced damage involved oxidation. Hence, MT might serve as an antioxidant or promote antioxidant levels in *V. volvacea* through the metabolism of amino acids (such as alanine, glutamate, and valine), the citrate cycle, or nitrogen and glutathione metabolism (Fig. S7).

3.4. Proteomic analyses reveal that the action of MT on Cd tolerance is reduction of oxidative stress

To gain further insight into how MT relieves Cd-induced damage, iTRAQ-based proteome data were obtained for the *V. volvacea* samples corresponding to the groups used for the metabolomics analysis. When cut-off values were set at a significance of ≥13 (p <0.05, PEAKSQ), unique peptides ≥2, and fold change ≥1.2, it was found that compared to the control, there were a total of 370 differential proteins in MT, 19 in Cd , 27 in MT + Cd, and 74 in MT + Cd vs. Cd groups (Fig. S8). The differential proteins were then enriched by gene ontology (GO) via level 4 molecular function. This demonstrated that oxidoreductase activity, coenzyme binding, and peptidase regulator activity comprise the main GO functional cluster in all four sample groups (Fig. 3). These results imply that redox processes participated in both Cd-induced damage and the damage mitigation mechanism of MT. To further confirm these results, the differential proteins in the MT + Cd vs. Cd group were analyzed by the GO biological process cluster (Fig. 4 and Table S4). As shown in Fig. 4A, the

differential proteins were found to be distributed at almost all 10 levels of biological processes, which represent responses to chemical exposure (level 3, 5 proteins), oxidation-reduction processes (level 4, 17 proteins), detoxification (level 5, 4 proteins), and cellular oxidant detoxification (level 7, 4 proteins). In addition, 4 proteins were found to participate in the metabolic process of tryptophan, the precursor to MT. This suggests that tryptophan metabolism suffers destabilization upon the addition of $CdCl_2$ or MT. Compared with the Cd group, the MT + Cd group exhibited up-regulation of the DNA damage-responsive protein VVO_08879, GMC oxidoreductase VVO_09541, DyP-type peroxidase VVO_10281, and oxidoreductase VVO_10307. On the contrary, aldehyde dehydrogenase VVO_06008, FAD-binding protein VVO_01252, and GMC oxidoreductase VVO_10808 were all down-regulated. The KEGG cluster indicates that the differential proteins are distributed in fatty acid degradation, glutathione and amino acid metabolism (tryptophan, proline, tyrosine, etc.), and oxidative phosphorylation (Fig. S9). Generally, our proteome data suggest that oxidation, anti-oxidation, and the metabolism of glutathione and amino acids comprise the primary mechanism involved in the amelioration of Cd-induced damage by MT. A combined proteomic and metabolomic analysis further corroborated this conclusion and suggested that the metabolic processes impacted by MT and/or Cd exposure are cysteine and methionine metabolism, glutathione metabolism, and oxidative phosphorylation (Fig. S10).

3.5. Melatonin could reduce Cd-induced oxidation and improve the activity and gene expression levels of antioxidant enzymes

In order to confirm the results of the metabolomic and proteomic analyses, we measured the proline and total sugar content of the *V. volvacea* samples treated with MT and/or Cd. As depicted (Figs. 5A and 5B), MT was able to reduce the levels of both proline and total sugar content after treatment with highly concentrated Cd (Cd5). However, the extent of reduction was not significant. The level of endogenous ROS (H_2O_2 and O_2^-) appears to experience significant reduction after MT and Cd treatment compared with treatment with Cd alone (Figs. 5C and 5D). These results indicate that the action of MT on Cd tolerance resembles the scavenging of free radicals, which is consistent with the results of the proteomic analyses in this study (Fig. 4). Comparison of the activities of antioxidant enzymes involved in scavenging H_2O_2 and O_2^- reveals that treatment with either MT or Cd alone can improve the activity of SOD, CAT, POD, GR, and APX (Fig. 6). Furthermore, the activities of all antioxidant enzymes were further elevated by supplementation with MT, indicating that MT may support the activity of antioxidant enzymes through a free radical scavenging pathway. The gene expression levels of antioxidant enzymes were also investigated and found to be in close agreement with the enzyme activity results. Interestingly, the addition of MT alone was found to exert a light impact on the expression of *CAT, SOD, POD, GR,* and *APX* despite its ability to improve the corresponding enzyme activities. However, combined treatment with both MT and Cd significantly elevated all gene expression levels, which provides further evidence to support that MT may enhance antioxidant capacity in coordination with Cd. In summary, Cd in a concentration of 5 μM may induce oxidative stress in *V. volvacea,*

in which the levels of proline, total sugar, H_2O_2 and O_2^- are all improved (Fig. 5). However, when 100 μM of MT was added, the level of endogenous ROS reduced, and accordingly, the enzyme activities and the gene expression of *CAT*, *SOD, POD, GR,* and *APX* are all improved (Fig. 6).

4. Discussion

Numerous species of wild mushrooms are well-known to accumulate high levels of heavy metals; however, the mechanism of resistance is not yet fully understood. MT has been reported to play an important defensive role against various abiotic stresses in plants and animals. In this study, we report the existence of MT in eight common edible fungi and elaborate on the role of MT in response to Cd stress (Figs 1, 2 and S3). Since MT acts as an antioxidant in almost all organisms in which it occurs, it was inferred that MT might act similarly in edible fungi and play an antioxidizing role in their response to heavy metals (Fan, Xie, Zhang, & Chen, 2018; Tan, Hardeland, Back, Manchester, Alatorre-Jimenez, & Reiter, 2016). We found that MT could improve the growth of *V. volvacea* mycelium and increase the content of endogenous MT in the Cd and Cd + MT treatment groups (Fig. 2). In addition, all genes involved in MT biosynthesis were up-regulated, and the rate-limiting step (TDC) gene being particularly notable (Fig. 2). This insinuated that Cd stress could prompt the activation of endogenous MT biosynthesis. Furthermore, the addition of exogenous MT may reinforce this process, suggesting that the Cd-related MT response may be related to an oxidative stress response. Despite the similarity of this phenomenon to the gene expression mode observed during the postharvest

physiological deterioration of cassava storage roots, the exact mechanism remains unclear (Ma, Zhang, Zhang, & Wang, 2016). Interestingly, the regular circadian rhythms of MT levels in *V. volvacea* mirror those in mammals and plants and provide further support for our assumption (Fig. S4) (Kolář & Macháčková, 2005).

To gain insight into the potential mechanisms of MT, metabolic analyses were conducted in a similar manner to those reported in previous studies on plants (Hu, et al., 2016; Shi, Wang, Tan, Reiter, & Chan, 2015). As shown in Fig. S7, glutathione, sulfur, and proline metabolic pathways are enriched upon MT addition, which is consistent with the Cd tolerance observed in other mushrooms (Albert, et al., 2018; X. Li, et al., 2017; Melgar, Alonso, & García, 2016). Due to its unique redox and nucleophilic properties, glutathione serves an important defensive role against ROS, xenobiotics, and heavy metals in bio-reductive reactions. Thus, oxidation pressure may be the most important element to be managed. This experiment revealed that the endogenous ROS (H_2O_2 and O_2^-) level is significantly enhanced upon Cd addition; however, the ROS level is down-regulated in the absence of MT (Fig. 5). Proteome analysis further corroborated these results; 370 differential proteins were identified in the MT vs. control group, and 74 differential proteins were identified in the MT + Cd vs. Cd group (Figs. S7 and S8). These differential proteins participate in multiple pathways and are characterized by the molecular functions of either oxidoreductase activity, coenzyme binding, or peptidase regulator activity or by responses to oxidation-reduction processes, detoxification, and cellular oxidant detoxification in biological processes (Figs. 4, 5, and S9). In addition, amino acid and nitrogen

metabolism, the citrate cycle, and the pentose phosphate pathway are also enhanced by Cd or MT addition, which indicates the initiation of a systematic regulatory process. These findings were consistent with the results obtained in previous investigations of MT in plants and animals (R. Zhang, Sun, Liu, Jin, & Sun, 2017). Hence, the Cd-induced oxidation pressure and the reduction of ROS via the antioxidant function of are two main factors that contribute to the Cd tolerance mechanism of *V. volvacea*. This was confirmed by the thorough analysis of the activity changes of antioxidant enzymes involved in free radical scavenging after MT vs. Cd addition (Fig. 6). These results are in close alignment with the typically reported mechanism by which MT relieves abiotic stresses in plants and/or bacteria, which indicates the potential for MT to act as an endogenous antioxidant and reinforce the ability of edible fungi to adapt to harsh environments.

Generally, Cd can induce oxidative stress in *V. volvacea*, reflected by both suppressed growth of the mycelium, and boosted ROS levels. SOD induces the catalysis of O_2^- into H_2O_2, which is subsequently decomposed by CAT and POD (Fig. S11). The corresponding GO function cluster and transcriptome indicate the involvement of a redox process. The relatively enhanced enzyme activity and gene expression, which occur after MT addition, reveal the potential of MT to improve the capacities of SOD, CAT, and POD in scavenging ROS. Furthermore, the ascorbate-glutathione cycle is also stimulated to eliminate excessive ROS, which is confirmed by enhanced APX and GR activity and expression (Dusart, Gérard, Le Thiec, Collignon, Jolivet, & Vaultier, 2019). The comparative metabolism and KEGG

pathway analyses verified that amino acid metabolism, the citrate cycle, and nitrogen and glutathione metabolism contribute to the antioxidant process, especially in response to MT treatment. MT addition can also stimulate the expression of genes involved in MT biosynthesis and endow *V. volvacea* with Cd tolerance (Fig. S11).

5. Conclusions

In this study, the ubiquitous existence of MT in edible fungi was confirmed. The existence and the regular circadian rhythms of MT in edible fungi suggest that MT might also be present as an endogenous substance in edible fungi and can play an important role in physiological regulation. It was also found that MT could promote the antioxidant activity of *V. volvacea* through amino acid metabolism, glutathione metabolism, oxidation-reduction processes, detoxification, and cellular oxidant detoxification, which evidences the protective effects of exogenous MT on Cd-induced oxidative stress in edible fungi. The results were finally confirmed through analysis of the expression and activity of oxidation-related enzymes after different MT and Cd treatments. The work provides sight on the tolerance of mushrooms to the abnormal accumulation of high levels of Cd and other heavy metals.

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Declaration of competing interest

The authors declare no conflict of interest.

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Legend to Figures

Figure 1 Validation of MT existence and determination of MT content in different edible fungi. (A) The primary scan and secondary fragments of MT and proposed fragmentation pathway. (B) MT content of eight edible fungi. Results depicted the MT content in 1 g of fresh mycelium and the bars represent the mean ± SD (n = 3).

Figure 2 Cd stress resistance conferred by the application of exogenous MT in *V. volvacea*. (A) Representative images of mycelia with and without MT pretreatments under Cd stress conditions. (B) Growth rate of mycelia with different MT and $CdCl_2$ treatments for 1 day. (C) Dry weight of mycelia with different treatments. (D) MT

content of mycelia with different treatments. (E) The effect of different concentrations of MT and Cd on the expression of MT biosynthetic genes (*TDC*, *T5H*, *SNAT*, *COMT,* and *ASMT*) in *V. volvacea*. The heavy metal treatment involved the addition of 2, 5, or 8 μM CdCl2 solutions, with the corresponding samples designated Cd2, Cd5, and Cd8, respectively. The samples were harvested and analyzed after 5 days of treatment. Each result shown represents the mean ± SD (n = 3).

Figure 3 GO molecular function (MF) categorization of deferentially accumulated proteins identified in different treatment groups. The number of differentially accumulated proteins in different molecular functions is shown in percentage in different colors. The concentrations used for MT and $CdCl_2$ treatment were 100 and 5 μM (5 days for treatment), respectively.

Figure 4 GO biological process (BP) categorization and hierarchical cluster analysis of differentially accumulated proteins identified in the MT + Cd vs. Cd group. (A) All the GO BP categorization of differentially accumulated proteins identified in the MT + Cd vs. Cd group and GO bar plot in level 4. The number of differentially accumulated proteins and their corresponding percentages are provided in the figure. (B) Hierarchical cluster analysis of differentially expressed proteins in the MT + Cd vs. Cd group. Green indicates significantly down-regulated proteins and red indicates significantly up-regulated proteins. The concentrations used for MT and $CdCl_2$ treatment were 100 and 5 μM (5 days for treatment), respectively.

Figure 5 Effects of exogenous MT on the response of *V. volvacea* to Cd stress. (A) Proline concentration. (B) Total sugars concentration. (C) Quantification of H_2O_2 content in different treatment groups. (D) Quantification of O_2^- content in different treatment groups. Each result shown is the mean ± SD (n = 3). The concentrations used for MT and $CdCl_2$ treatment were 100 and 5 μM (5 days for treatment), respectively.

Figure 6 Effects of exogenous MT on the enzyme activity and gene expression of ROS-related antioxidants in *V. volvacea*. Quantifications of (A) CAT activity, (B) SOD activity, (C) POD activity, (D) GR activity, and (E) APX activity of mycelia with or without MT and Cd treatment. (F) Corresponding gene expression of these ROS-related antioxidants. Each result shown is the mean ± SD (n = 3). The concentrations used for MT and $CdCl_2$ treatment were 100 and 5 μM (5 days for treatment), respectively.

Declaration of interests

✓ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT authorship contribution statement

Yingnyu Gao and Ying Wang: Writing - original draft, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing, Visualization, Project administration. **Jiang Qian and Wenshuai Si**: Methodology, Supervision, Writing - review & editing. **Qi Tan and Jiyang Xu**: Methodology, Validation, Conceptualization, Supervision, Funding acquisition, Writing - review & editing. **Yucheng Zhao**: Methodology, Validation, Conceptualization, Data curation, Formal Analysis, Writing - review & editing, Visualization, Supervision.

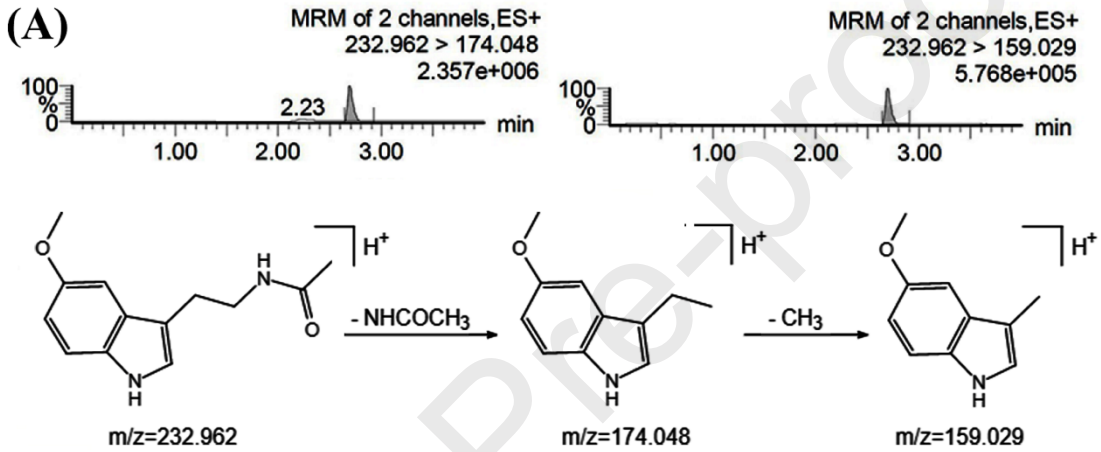
Highlights:

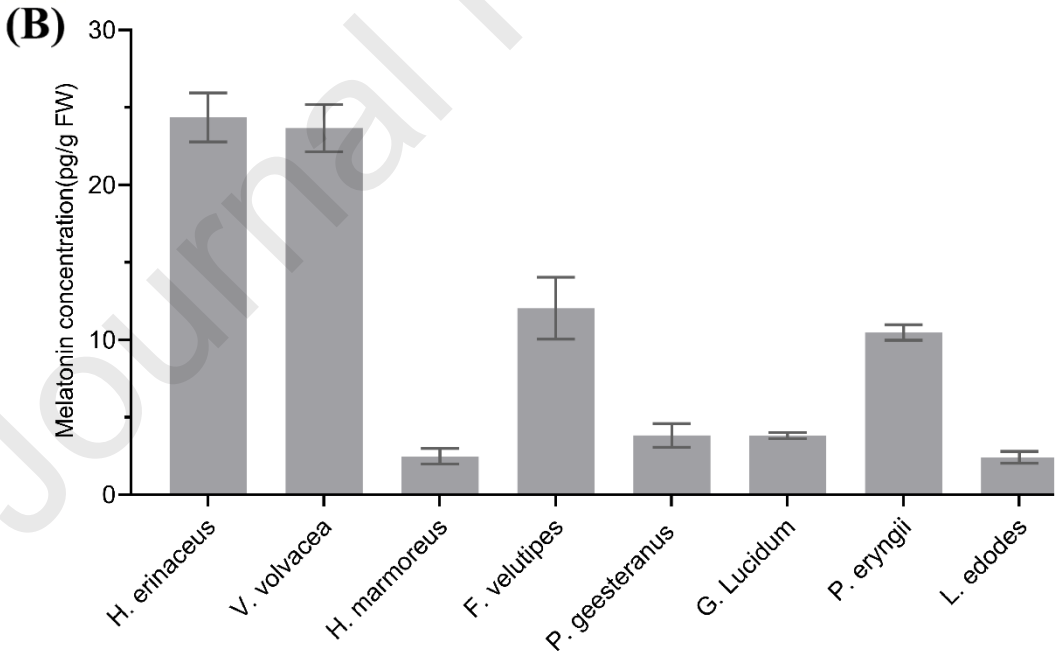
1. Melatonin exists in in edible fungi and it could response to Cd-induced stress.

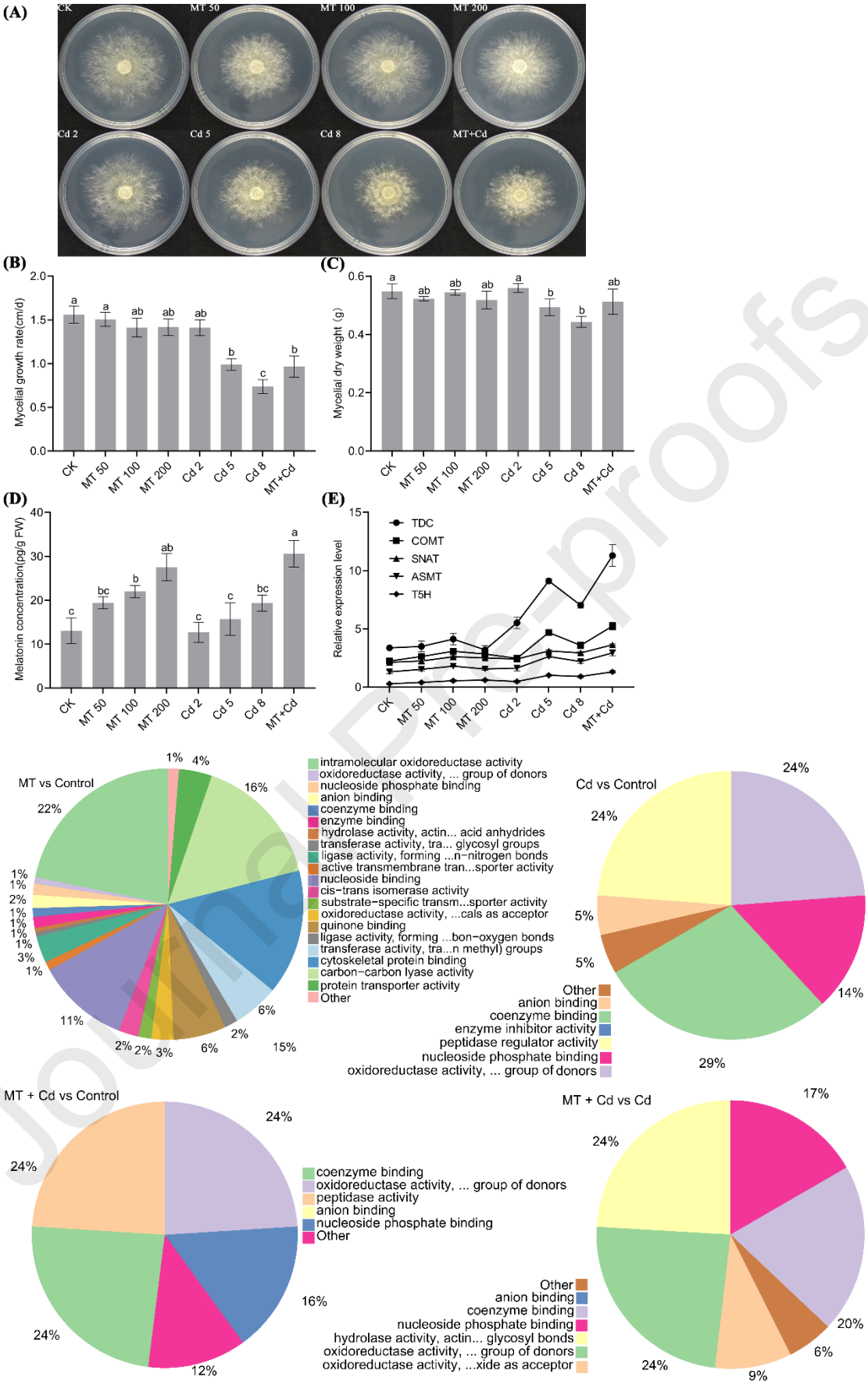
2. Melatonin can promote the antioxidant activity of *Volvariella volvacea.*

3. Antioxidant mechanism of melatonin was analyzed by metabolism and proteome.

4. Oxidation-related enzymes are responsible for Melatonin's ameliorative effects.






(A)
CK
MT 50
MT 100
MT 200
Cd 2
Cd 5
Cd 8
MT+Cd
(B)
Mycelial growth rate(cm/d)
(C)
Mycelial dry weight (g)
(D)
Melatonin concentration(pg/g FW)
(E)
Relative expression level
TDC
COMT
SNAT
ASMT
T5H
MT vs Control
intramolecular oxidoreductase activity
oxidoreductase activity, ... group of donors
nucleoside phosphate binding
anion binding
coenzyme binding
enzyme binding
hydrolase activity, actin... acid anhydrides
transferase activity, tra... glycosyl groups
ligase activity, forming ...n−nitrogen bonds
active transmembrane tran...sporter activity
nucleoside binding
cis−trans isomerase activity
substrate−specific transm...sporter activity
oxidoreductase activity, ...cals as acceptor
quinone binding
ligase activity, forming ...bon−oxygen bonds
transferase activity, tra...n methyl) groups
cytoskeletal protein binding
carbon−carbon lyase activity
protein transporter activity
Other
Cd vs Control
Other
anion binding
coenzyme binding
enzyme inhibitor activity
peptidase regulator activity
nucleoside phosphate binding
oxidoreductase activity, ... group of donors
MT + Cd vs Control
coenzyme binding
oxidoreductase activity, ... group of donors
peptidase activity
anion binding
nucleoside phosphate binding
Other
MT + Cd vs Cd
Other
anion binding
coenzyme binding
nucleoside phosphate binding
hydrolase activity, actin... glycosyl bonds
oxidoreductase activity, ... group of donors
oxidoreductase activity, ...xide as acceptor