SIRT2-mediated deacetylation of Glutathione transferase alleviates oxidative damage and increases heat tolerance of Pleurotus ostreatus

Qianqian Chai
Henan Agricultural University
Huihui Li
Henan Agricultural University
Yuandong Liu
Zhoukou City Academy of Agricultural Sciences
Qing Wen
Henan Agricultural University
Qing Liu
Henan Agricultural University
Yuancheng Qi
Henan Agricultural University
Fengqin Wang
Henan Agricultural University
Jinwen Shen
Henan Agricultural University
Yanru Hu
huyanru12@163.com
Henan Agricultural University

Research Article

Keywords: High-temperature stress, acetylation, Pleurotus ostreatus, SIRT2

Posted Date: April 16th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4234214/v1

Additional Declarations: No competing interests reported.

Abstract

High-temperature stress (HS) is the most important environmental factor that affects crop growth. It seriously threatens agricultural production. *Pleurotus ostreatus* is cultivated in many parts of the world, and its growth is strongly affected by HS. In another study, we found that metabolic rearrangement occurred in HS, but the gene expression levels of some key enzymes remained unchanged. Therefore, in this study, we investigated the contribution of post-translational modification of proteins to HS resistance in *P. ostreatus*. Acetylation is a conserved and important post-translational modification of proteins in vivo, and its main function is to regulate metabolic processes. We found that the level of acetylation of *P. ostreatus* decreased under short-term HS treatment and increased as the duration of HS treatment increased. The acetylation omics showed that almost all metabolic enzymes were acetylated. Ten antioxidant-related proteins, four acetylated proteins, and one deacetylated protein (SIRT2) were screened and identified in the differentially modified group between heat stress and normal conditions. A low concentration of the SIRT family substrate NAM (1 mM) was added to decrease the intracellular acetylation level. We found that deacetylation under heat stress can improve the growth recovery ability of mycelia, matrix-degrading enzyme activity, and antioxidant content (NADPH and GSH). Deacetylation can also decrease the H_2O_2 level after heat stress. Further studies showed that SIRT2 increased the activity of GSTs by deacetylating GST1 66K, GST2 206K, and GST2 233K. We found that lysine acetylation occurred in *P. ostreatus*, and thus, we assessed the importance of the acetylation of non-histone proteins under HS.

1. Introduction

Harsh climatic conditions adversely affect crop growth. It is a serious problem that needs to be addressed urgently (Pfleiderer et al., 2019). A key factor that negatively affects crop yield is high temperature. Crops grow efficiently within a certain temperature range. Temperatures above the critical threshold may lead to high-temperature stress (HS). HS may occur across the crop growth period or developmental stages and affect physiological processes such as photosynthesis, primary metabolism, etc., thus reducing grain yield (Jagadish 2020). Considering the ever-increasing global population, the FAO (Food and Agriculture Organization) proposed that to cope with the hunger caused by population growth, world food production should be doubled by 2050 (Janni et al., 2020). However, HS is a major obstacle to achieving this goal. Hence, adaptive responses related to heat tolerance need to be understood, as it can improve our understanding of adaptability to HS and also help us breed new varieties of crops with greater heat resistance.

Edible mushrooms are popular as they are rich in nutrients, contain a unique flavor substance, and have pharmaceutical properties. Additionally, the cultivation of edible mushrooms is eco-friendly, as they can rapidly convert agricultural waste into high-quality protein at a low cost. Edible mushrooms are expected to become a fungal protein source and replace plant and animal proteins (Abigail et al., 2020; Lavelli et al., 2018). The edible mushroom industry plays an important role in addressing global food issues. *Pleurotus ostreatus* is the second most cultivated edible mushroom in the world (Roncero-Ramos and

Delgado-Andrade 2017). However, its cultivation is strongly affected by HS. HS strongly inhibits the growth of mycelia, increases cell death, and increases the risk of *P. ostreatus* infection caused by various bacteria, including *Trichoderma asperellum* (Song et al., 2014; Qiu et al., 2018). These adverse effects lead to huge economic losses. Reactive oxygen species (ROS) are signaling molecules that accumulate in large numbers under HS. When their numbers exceed a certain limit, they can destroy cell redox homeostasis and oxidize biological macromolecules. Thus, the removal of ROS is an effective way to improve the resistance of organisms to HS (Janni et al., 2020). Various substances, such as trehalose, salicylic acid (SA), and nitric oxide (NO), can enhance the ability of *P. ostreatus* to survive under HS by helping them maintain redox and metabolic homeostasis (Lei et al., 2019; Hou et al., 2020; Hu et al., 2022). Several metabolic enzymes, such as MnSOD1, PAL, aconitase, and alternative oxidase, can also help *P. ostreatus* cope with HS (Hou et al., 2019; Hou et al., 2020; Hou et al., 2021; Hou et al., 2022). In another study, we found that metabolism is rearranged under HS in *P. ostreatus*. However, the transcription level of several key stress-resistance genes did not differ significantly relative to the control (Hu et al., 2022). Therefore, we speculated that post-translational modifications (PTMs) of proteins may contribute to the HS resistance of *P. ostreatus*.

Post-translational modifications (PTMs) regulate the functions of proteins by altering protein stability, protein activity, interaction partners, and subcellular localization (Liao et al., 2021). PTMs modulate various physiological reactions, such as gene transcription and metabolic adaptability, in response to biotic and abiotic stress (Lämke and Bäurle 2017). Thus, PTMs contribute to the acclimation of organisms to environmental changes. Lysine acetylation (Kac) is evolutionarily highly conserved and occurs commonly in eukaryotes and prokaryotes (Narita et al., 2019); Kac was first discovered in histones (Allfrey and Faulkner 1964). It is a dynamic regulatory process regulated by lysine acetyltransferases (KATs) and lysine deacetylases (KDACs). KATs perform acetylation by transferring an acetyl group from acetyl-CoA to lysine, whereas KDACs remove the acetyl group from lysine (Verdin and Ott 2015). Kac plays an important role in enhancing tolerance to different types of stress, including salt (Li et al., 2022; Tilak et al., 2023), oxidative (Harris et al., 2015), glucose deprivation, drought, and heat stress (Sivananthan et al., 2023; Wang et al., 2022; Kim et al., 2020). However, the role of Kac in macrofungi is unclear.

In this study, we found that the acetylation level of *P. ostreatus* first decreased and then increased as the duration of heat stress increased. A reduction in the acetylation level of total protein enhanced the heat resistance of *P. ostreatus* through the removal of ROS. Ten antioxidant-related proteins, four acetylated proteins, and one deacetylated protein (SIRT2) were screened and identified in the differentially modified group under heat stress and normal conditions by acetylproteome sequencing. Further analysis showed that SIRT2 can enhance the activity of glutathione transferase, a key enzyme in glutathione metabolism, through deacetylation.

2. Materials and methods

2.1 Model strain and culture conditions

The model strain *Pleurotus ostreatus* New 831 was obtained from the College of Life Science, Henan Agricultural University. The mushroom complete medium (GYE: yeast extract 5 g/L, glucose 20 g/L, VB_1 0.01 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KH_2PO_4 1 g/L, and agar 2%) was used to culture the mycelium at 25°C.

The cultivation test was performed according to a previously described method (Hu et al., 2023). The production cultivation substrates contained cottonseed hull (97%), lime (2%), precipitated calcium carbonate (1%), and H_2O (65%). In each treatment, 30 bags and three replicates were maintained.

2.2 High-temperature stress and chemical treatments

First, a small piece of mycelium (5 mm) was attached to the center of the GYE medium and cultured at 25°C for five days. Then, the mycelium was cultured for 24 h at 40°C for heat-stress treatment (Hu et al., 2022).

To evaluate the levels of H_2O_2, GSH, and NADPH, the samples were collected using tubes and immediately frozen using liquid nitrogen. Three replicates were maintained per group.

To determine the growth rate of mycelium, the contents of redox-related substances (H_2O_2, GSH, and NADPH) and acetylation level after treatment with NAM (Sigma–Aldrich, Madrid, Spain) were evaluated; NAM was added to the GYE medium directly. In the experiments in which substrate-degrading enzyme activity was analyzed after treatment with NAM (Sigma–Aldrich, Madrid, Spain), NAM was added to the MCM medium when the mycelium culture medium was changed from GYE to MCM. In the experiment, ddH_2O was used as the solvent control (Hu et al., 2023).

2.3 Detection of H_2O_2, GSH, and NADPH production

The contents of ROS and GSH were estimated using their corresponding assay kits (E004–1–1 and A006–2–1, Nanjing Jiancheng Bioengineering Institute).

The content of NADPH was evaluated using an assay kit (AAT Bioquest; 15274).

2.4 Enzyme activity assays

Substrate-degrading enzymes, including laccase, filterpaper cellulase, and carboxymethyl cellulase, were induced using lignin or cellulose as the sole carbon source (MCM: 0.05% $MgSO_4 \cdot 7H_2O$, 0.46% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, and 2 mL/L trace elements) (Hu et al., 2020). The *P. ostreatus* strains were first cultured in GYE medium under constant shaking for five days at 25°C. Then, they were collected and transferred to the MCM medium and cultured under constant shaking conditions at 25°C for two days. The samples were cultured at 40°C for 24 h if necessary.

The activities of carboxymethyl cellulase, filterpaper cellulase, laccase, and GST were measured as described in other studies (Hu et al., 2023; Zhang et al., 2022; Fafián-Labora et al., 2020). Three independent cultures were maintained per treatment.

2.5 Determination of the hyphal growth rate

The growth distance divided by the number of growing days was used to calculate the hyphal growth rate (Hu et al., 2022). Three independent cultures per treatment were maintained.

2.6 Western blotting analysis

The acetylation level was determined via Western blotting assays (Hu et al., 2020). The primary antibody (anti-acetylation antibody, Cat#PTM-101, PTM Bio, Hangzhou, China) was diluted at a ratio of 1:1000.

2.7 Acetylomic and proteomic analyses

The hyphae were incubated at 40°C for 24 h after cultivation at 25°C for five days. Then, they were sent to PTM Bio (Hangzhou, China) for acetylomic and proteomic analyses (Liao et al., 2021).

2.8 Prokaryotic expression and in vitro deacetylation reaction

The cDNA fragments of SIRT2, GST1, and GST2 were obtained by performing PCR using primers listed in Table S1. Then, the fragments were attached to PET28a and transformed into *E. coli* BL21. IPTG (1 mM) was used to induce protein expression. After the cells were cultured at 37°C for 4 h, they were collected, and the proteins were isolated using a standard protocol (Hu et al., 2020).

In vitro deacetylation: His-PoGST (10 μg) and His-PoSIRT2(10 μg) were mixed and incubated at 30°C for 4 h in HEPES buffer (HEPES 40 mM; $MgCl_2$ 1 mM; DTT 1 mM; NAD^+ 5 mM). Then, the level of acetylation was determined by Western blotting analysis (Chen et al., 2011).

2.9 Point mutation

The point mutant proteins of GST2 K66G, GST2 K66Q, GST3 K233R, GST3K233G, GST3 K206R, and GST3 K206G were obtained using a site-directed mutagenesis kit (B639281, Sangon Biotech, Shanghai, China). The mimic mutant primers are listed in Table S1.

2.10 Real-time qPCR

The level of expression of the genes was determined by real-time qPCR according to a protocol described in another study (Hu et al., 2020). The primers used are listed in Table S1.

2.11 Statistical analysis

GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA) was used for plotting graphs. The differences between groups were determined by Turkey's test (Version 26.0, IBM Corporation, NY). All differences between the two groups were considered to be statistically significant at $p < 0.05$. The data were represented by the average of three replicates, and the error bar represented the standard deviation. All statistical analyses were performed using the SPSS software.

3. Results

3.1 Changes in the total protein acetylation level of Pleurotus ostreatus under heat stress

Lysine acetylation (Kac) plays a key role in regulating almost all cellular processes and is reversibly catalyzed by KATs and KDACs (Narita et al., 2019). To evaluate the change in the acetylation level of *P. ostreatus*, we collected samples exposed to HS for different durations. Then, the total protein was extracted and determined by Western blotting assays. The results showed that there was considerable variation in the molecular weights of the acetylated proteins (Fig. S1). Kac decreased significantly after 0.5 h of HS and then increased significantly as the duration of heat stress increased (Fig. 1 and Fig. S1). These results suggested that protein Kac responded to HS in *P. ostreatus*.

3.2 Differences in the acetylated proteins between heat-stress and normal conditions

Label-free quantitative acetylome analysis was performed to quantify acetylated proteome (Liao et al., 2021). The proteins extracted from samples not exposed to HS (control) and from those exposed to HS for 24 h were analyzed via LC–MS/MS to determine the differences in the acetylated proteins between the two groups of samples. In total, 1,170 different modified proteins and 2,264 different modified sites were identified (Fig. 2A). The Kac levels of 105 proteins and 124 sites were lower, whereas the Kac levels of 1,065 proteins and 2,140 sites were higher in the HS treatment group compared to that in the control group. Subcellular localization showed that acetylated proteins were more abundant in the cytoplasm (39.87%), mitochondria (20.80%), and nucleus (20.61%), whereas few proteins were found in the extracellular matrix (4.70%), plasma membrane (4.43%), cytoskeleton (3.53%), or other parts (0.99%). Additionally, 5.06% of proteins were found in both the cytoplasm and the nucleus (Fig. 2B).

The function classification of clusters of orthologous groups of proteins (COG/KOG) was performed to understand the functions of differentially acetylated proteins. These functions included cellular processes and signaling (e.g., post-translational modification), information storage and processing (translation, ribosomal structure, and biogenesis, etc.), metabolism (energy production and conversion, amino acid transport and metabolism, etc.), and poorly characterized functions (Fig. 2C). These results indicated that acetylation was involved in many physiological processes.

3.3 Acetylation of proteins associated with different metabolic processes under HS in the hyphae of P. ostreatus

The most important function of acetylation is to regulate metabolism (Zhao et al., 2010). To better understand the acetylation of proteins associated with various metabolic processes under HS in the hyphae of *P. ostreatus*, KEGG pathway analysis was performed. The results showed that multiple important metabolic pathways, such as glycolysis/gluconeogenesis, citrate cycle (TCA cycle), and pyruvate metabolism, were enriched (Fig. 3A). Almost all metabolic enzymes were modified to different degrees. In glycolysis/gluconeogenesis, the Kac levels of PGM, PGK, GAPDH, PGI, PK, Gpml, FBP1, PGI,

and GALM increased, whereas the Kac levels of HK, PFK9, ALDO, TPI, and ENO decreased. In the TCA cycle, the Kac levels of aceE, IDH1, OGDH, DLD, SDHA, fumA, CS, PC, aceE, DLD, DLAT, and ACC increased, whereas the Kac levels of ACO, ACLY, IDH3, LSC1, sucD, and MDH1 decreased. The Kac levels of proteins associated with amino acid metabolism (GS, PPAT, GOGAT, and CAD) increased. Additionally, the Kac levels of ACC, which connects carbon metabolism with lipid metabolism, increased. Four sites were found in GDH, which connect carbon metabolism with amino acid metabolism; in three of those sites, Kac levels increased, whereas, in one site, Kac levels decreased (Fig. 3B).

Gene ontology (GO) enrichment of the differentially acetylated proteins showed that they were involved mainly in ligase activity, forming carbon-oxygen bonds, aminoacyl-tRNA ligase activity, ribosome binding, antioxidant activity, protein oxidoreductase activity, and TBP-class protein binding (Fig. 4A). Among these functions, we were interested in the class of proteins with antioxidant activity, as removal of ROS can help organisms cope with HS (Janni et al., 2020). GO enrichment of the differentially acetylated motifs revealed that they were involved mainly in the proteasome subunit N-terminal signature, proteasome subunit, cytochrome b5-like heme/steroid binding domain, glutathione S-transferase, and ATP synthase (Fig. 4B). Among them, we investigated glutathione S-transferase. We identified nine antioxidant-related proteins (GPX, PRX1, PRX2, GST1, GST2, TRX, CCP, GR, and CAT) among the differentially acetylated proteins (Fig. 4C). These results suggested that protein Kac affects intracellular redox homeostasis in *P. ostreatus*.

3.4 Deacetylation of total protein enhanced the HS resistance of P. ostreatus

To study the effect of acetylation modification on the heat resistance of *P. ostreatus*, we intend to change the intracellular acetylation level. Lysine acetylation (Kac) is a conserved PTM and is reversibly catalyzed by KATs and KDACs (Narita et al., 2019). Two kinds of KATs (HAT and three NATs) and one kind of KDAC (SIRT2) were identified among the differentially acetylated proteins (Fig. 4C). The results of the qRT-PCR analysis showed that the level of expression of SIRT2 increased significantly ($P < 0.05$), but the expression of the four KATs did not change significantly ($P > 0.05$) (Fig. S2). SIRT2 belongs to the sirtuin deacetylase family, which is a highly evolutionarily conserved NAD^+-dependent protein deacetylase family (Narita et al., 2019). This family includes seven members (SIRT1–7), which are localized in different cellular compartments. SIRT2 is located in the cytoplasm (Narita et al., 2019). We further investigated the KDAC as its type was simple.

Niacinamide (NAM) is a precursor of NAD. A low concentration of NAM can promote the synthesis of NAD and deacetylation activity of sirtuin family, whereas a high concentration of NAM acts as an inhibitor of the sirtuin deacetylase family and inhibits deacetylation (Jiang et al., 2012; Hwang and Song 2017). In this study, the growth rate of the hyphae of *P. ostreatus* was significantly accelerated ($P < 0.05$) after adding 0.25, 0.5, 1, 2, and 4 mM NAM. However, the growth rate decreased significantly ($P < 0.05$) after adding 20, 30, 40, and 50 mM NAM under normal conditions (Fig. 5A and B). The recovery growth rate of the hyphae of *P. ostreatus* after HS was significantly accelerated ($P < 0.05$) after adding 0.25, 0.5,

1, and 2 mM NAM, but the growth rate decreased significantly ($P < 0.05$) after adding 20, 30, 40, and 50 mM NAM (Fig. 5C and D). The results of the Western blotting assays showed that the Kac level of total protein decreased after treatment with 1 mM NAM and increased after treatment with 20 mM NAM (Fig. S3). This result indicated that a low concentration of NAM (1 mM) promoted deacetylation, whereas a high concentration of NAM (20 mM) acted as an inhibitor of the sirtuin deacetylase family, thus inhibiting deacetylation. These findings were similar to those of previous studies (Jiang et al., 2012; Hwang and Song 2017). The culture experiment also showed that 1mM NAM promoted the growth rate of the hyphae of *P. ostreatus* under normal conditions and the recovery growth after HS (Fig. S4). To summarize, 1 mM NAM decreased deacetylation, promoted the growth of mycelia, and enhanced the recovery growth after HS, which helped *P. ostreatus* adapt to HS.

3.5 Deacetylation of total protein maintained the activities of substrate-degrading enzymes

P. ostreatus can secrete various substrate-degrading enzymes, such as laccase and cellulase, to degrade lignocellulose in the cultivation substrate for obtaining nutrients. Changes in environmental conditions can affect the growth rate of mycelia by affecting the activity of substrate-degrading enzymes. Thus, we determined the activities of the main substrate-degrading enzymes, including laccase, filter paper cellulase, and carboxymethyl cellulase. The activities of laccase, filterpaper cellulase, and carboxymethyl cellulase decreased significantly ($P < 0.05$) after HS (by 69.31%, 86.48%, and 95.15%, respectively) (Fig. 6). Under normal conditions, 1 mM NAM significantly increased ($P < 0.05$) the activities of laccase, filterpaper cellulase, and carboxymethyl cellulase, by 1.68-fold, 1.23-fold, and 1.15-fold, respectively, relative to their activities in the absence of NAM treatment. The promotion effect was stronger after HS treatment. The activities of laccase, filter paper cellulase, and carboxymethyl cellulase increased to 3.25-fold, 6.28-fold, and 4.86-fold, respectively, after treatment with HS and 1 mM NAM, compared to their activities recorded after treatment with HS only (Fig. 6). This result suggested that deacetylation of total protein maintained the normal function of substrate-degrading enzymes, which promoted the growth of *P. ostreatus* mycelia.

3.6 Deacetylation of total protein maintained redox homeostasis

We also investigated the antioxidant function of NAM, as previous results showed that acetylation might play a role in maintaining redox homeostasis of *P. ostreatus* (Fig. 4). H_2O_2 is a major and highly stable form of ROS (Lennicke and Cochemé 2021). The content of H_2O_2 significantly increased under HS treatment ($P < 0.05$). However, treatment with 1 mM NAM significantly decreased the content of H_2O_2 under normal and HS conditions ($P < 0.05$) (Fig. 7A). GSH and NADPH play key roles in maintaining the cellular redox environment. NADPH also supports the recycling of GSSG to GSH (Czarnocka and Karpiński 2018; Ferguson and Bridge 2019). We found that 1 mM NAM significantly increased the content of NADPH and GSH ($P < 0.05$) (Fig. 7B and C). This finding suggested that deacetylation of total protein

with 1 mM NAM maintained the redox homeostasis through an increase in the content of NADPH and GSH and the removal of ROS.

3.7 SIRT2 enhanced GST activity via deacetylation

As the only deacetylase in our differential acetylation group, Sirt2 influences various biological processes that maintain lipid and glucose homeostasis, decrease oxidative stress, and have other functions (Wang et al., 2020; Zhao et al., 2018; Zhang et al., 2021). The role of SIRT2 in HS was unclear. Arikhani et al. found that SIRT2 can promote oxidative stress-induced cell death by deacetylating c-Jun NH_2-terminal kinases (JNKs) (Arikhani et al., 2018). In our result, addition of 1 mM NAM to promote SIRT2 deacetylation activity was beneficial to the heat resistance of *P. ostreatus* and has antioxidant effect. This indicates that the role of SIRT2 in the regulation of oxidative stress might differ under different circumstances.

Glutathione transferase (GST) is the key enzyme controlling GSH-dependent redox processes (Ferguson and Bridge 2019). Our results showed that deacetylation under heat stress increased the contents of NADPH and GSH and decreased H_2O_2 levels. These changes favored the HS resistance of *P. ostreatus*. GSH is involved in the enzymatic reaction associated with the scavenging of oxygen by antioxidant enzymes, and it plays a central role in maintaining intracellular redox homeostasis by antioxidant enzymes (Ferguson and Bridge 2019). GST is the key enzyme in the GSH network of redox control and has multiple functions in endogenous metabolism, pathogen defense responses, and stress tolerance (Ferguson and Bridge 2019). We identified two GSTs among the differentially acetylated proteins (Fig. 4C). Two GSTs were identified among the differentially acetylated proteins (Fig. 4C).

To assess how Kac affected the activity of the enzymes, we first expressed and purified two GSTs and Sir2 (Fig. S5). The results of the in vitro acetylation experiment showed that SIRT2 deacetylated the GSTs and increased their activity (Fig. 8). To evaluate the effect of the site of acetylation on the acetylation level and protein activity, we mutated the modification sites identified by MS (Fig. 8). We individually mutated GST1 K66R, GST2 K233R, and GST2 K206R to mimic deacetylation (Wang et al., 2020). We found that GST1 K66R significantly decreased the acetylation level and increased the activity of GST2. K233R and K206R significantly decreased the acetylation level and increased the activity of GST3. Although SIRT2 could not further reduce the acetylation levels of GST1 K66R and GST2 K206R proteins, it could further reduce the acetylation levels of GST2 K233R and increased its activity (Fig. 8B). This finding indicated that SIRT2 could act on acetylation sites other than K233. These results suggested that K66 is the major acetylation site of SIRT2 present on GST1. K206 is the major acetylation site of SIRT2 present on GST2. To summarize, SIRT2-mediated deacetylation of GST can alleviate oxidative damage and increase the heat tolerance of *P. ostreatus* (Fig. 9).

4. Discussion

In this study, we found that the acetylation level of *P. ostreatus* first decreased and then increased as the duration of heat stress increased. The acetylation omics showed that almost all metabolic enzymes were

acetylated. Ten antioxidant-related proteins, four acetylated proteins, and one deacetylated protein (SIRT2) were screened and identified in the differentially modified group between heat stress and normal conditions (Fig. 4). A low concentration of the SIRT family substrate NAM (1 mM) was added to reduce the intracellular acetylation level. We found that deacetylation under heat stress enhanced the growth recovery ability of mycelia, the activity of the matrix-degrading enzyme, and the antioxidant content (NADPH and GSH) and decreased H_2O_2 levels after heat stress. Further assessment showed that SIRT2 increased its activity by deacetylating GST. Overall, SIRT2 was found to play an important role in the regulation of redox homeostasis and resistance to HS in *P. ostreatus*.

Lysine acetylation (Kac) plays a key role in regulating almost all cellular processes and is reversibly catalyzed by KATs and KDACs (Narita et al., 2019). Many studies have found that HS affects histone acetylation. In yeast, the deacetylase Rpd3 is essential for activating transcription to cope with HS (Ruiz-Roig et al., 2010). In *Arabidopsis*, the histone acetyltransferase GCN5 can activate HSFA3 and UVH6 by acetylating H3K9 and H3K14 to improve temperature adaptability (Hu et al., 2015). In *C. elegans*, HS can activate the transcription of resistance genes by increasing the level of histone acetylation (Zhou et al., 2019). Some researchers have found that non-histone acetylation is also common and involved in almost all important physiological processes (Narita et al., 2019). For example, the mitochondrial deacetylase sirtuin-3 (Sirt3) deacetylates MnSOD, thus increasing MnSOD activity, following oxidative stress (Tao et al., 2010). However, the role of non-histone acetylation in HS remains unclear, and related study about the role of Kac in macrofungi has only just begun. We found that the differentially acetylated proteins were most abundant in the cytoplasm, followed by the mitochondria and the nucleus. Thus, non-histone acetylation significantly changed under HS. Further investigation showed that deacetylation increased the HS resistance of *P. ostreatus*, and SIRT2-mediated deacetylation of Glutathione transferase (GST) played an important role in this process.

SIRT2 belongs to the NAD^+-dependent sirtuin deacetylase family. This family includes seven members (SIRT1–7), which are localized in different cellular compartments. SIRT2 is located in the cytoplasm (Narita et al., 2019). It influences various biological processes that maintain lipid and glucose homeostasis, decrease oxidative stress, and have other functions (Wang et al., 2020; Zhao et al., 2018; Zhang et al., 2021). Arikhani et al. found that SIRT2 can promote oxidative stress-induced cell death by deacetylating c-Jun NH_2-terminal kinases (JNKs)(Arikhani et al., 2018). This indicates that the role of SIRT2 in the regulation of oxidative stress might differ under different circumstances. The role of SIRT2 in HS was unclear. We found that SIRT2 deacetylated GST2 66K and GST3 206 K and enhanced their activities under HS in *P. ostreatus*.

GSH and NADPH play key roles in maintaining the cellular redox environment. NADPH also supports the recycling of GSSG to GSH (Ferguson et al., 2019). Our results showed that deacetylation under heat stress increased the contents of NADPH and GSH and decreased H_2O_2 levels. These changes favored the HS resistance of *P. ostreatus*. GSH is involved in the enzymatic reaction associated with the scavenging of oxygen by antioxidant enzymes, and it plays a central role in maintaining intracellular redox homeostasis by antioxidant enzymes (Ferguson et al., 2019). GST is the key enzyme in the GSH network

of redox control and has multiple functions in endogenous metabolism, pathogen defense responses, and stress tolerance (Ferguson et al., 2019). We identified two GSTs among the differentially acetylated proteins (Fig. 4C). Further examinations showed that the deacetylation of GSTs enhanced their activities (Fig. 8), which promoted the function of GSH in removing ROS. The point mutation results showed that K66 was the major acetylation site of SIRT2 on GST1. As K66R significantly decreased the Kac level of GST1, SIRT2 could not further decrease the Kac level of the GST1 K66R protein (Fig. 8A). The acetylation of K206 contributed more to the acetylation of GST2 than the acetylation of K233. K206R significantly decreased the Kac level of GST2 and could not be deacetylated further by SIRT2. However, K233R had a weaker effect on decreasing the acetylation level of GST2 than K206R. Thus, the GST2 K206R protein could be deacetylated by SIRT2. This finding indicated that SIRT2 could act on acetylation sites other than K233 (Fig. 8B).

However, the overall acetylation level of total protein increased as the duration of heat stress increased (Fig. S1). The activity of deacetylases decreases after they are acetylated (Yang et al., 2015). Therefore, we speculated that as the duration of heat stress increased, SIRT2 was acetylated, and its activity decreased, which led to an overall increase in the acetylation level of total protein. It might have occurred because the long-term heat stress caused serious damage to *P. ostreatus*, or other regulatory mechanisms were involved in the long-term heat stress resistance. This also indicated that the acetylation and deacetylation of proteins are dynamically regulated (Narita et al., 2019).

5. Conclusion

To summarize, our study showed that SIRT2-mediated deacetylation of GST alleviated oxidative damage and increased the heat tolerance of *P. ostreatus*. However, the overall acetylation level of total protein increased as the duration of heat stress increased (Fig. S1). The activity of deacetylases decreases after they are acetylated (Yang et al., 2015). Therefore, we speculated that as the duration of heat stress increased, SIRT2 was acetylated, and its activity decreased, which led to an overall increase in the acetylation level of total protein. It might have occurred because the long-term heat stress caused serious damage to *P. ostreatus*, or other regulatory mechanisms were involved in the long-term heat stress resistance. This also indicated that the acetylation and deacetylation of proteins are dynamically regulated (Narita et al., 2019). In this study, we identified the non-histone substrate proteins of SIRT2 and the new lysine acetylation sites under HS. Our findings suggested a way to increase heat tolerance through the activation of SIRT2.

Declarations

Funding statement

Thanks to the National Natural Science Foundation of China (32202571), the China Agriculture Research System (Project No. CARS20), Henan Provincial Joint Project of Tackling Key Problems in Agricultural Varieties (2022030101) for their support to the study.

Author contributions

Y.R.H., Q.F.W., and J.W.S. designed the research, Q.Q.C., Y.D.L., H.H.L. performed research, Q.Q.C., Q.W., Q.L., and Q.F.W. performed MS analysis. Y.R.H. and Y.C.Q. supervised research and wrote the paper.

Ethics approval and consent to participate

This article has no studies with human participants or animals.

Declaration of competing interest

There is no conflict of interest between the authors.

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Figures

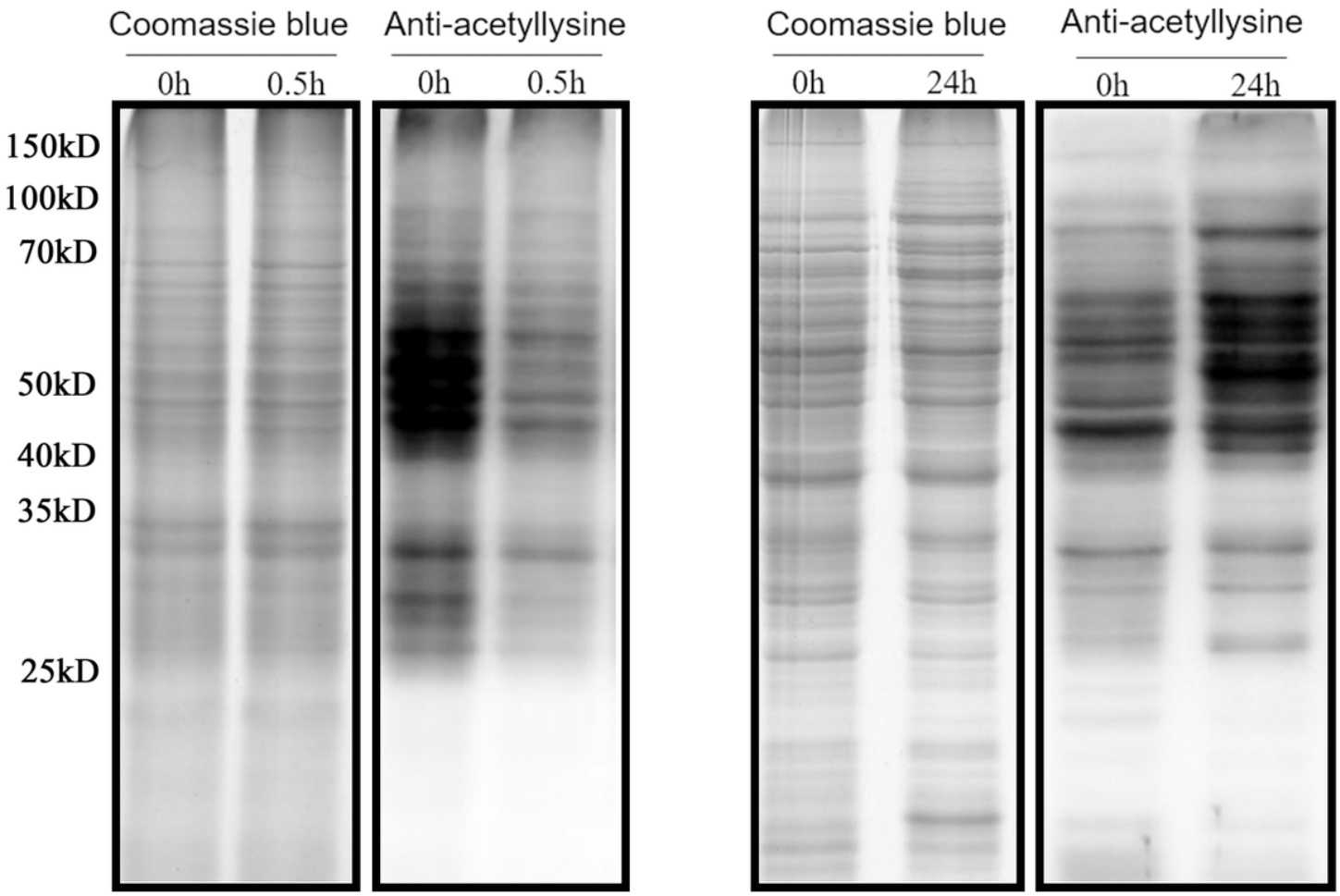

Figure 1

Changes in total protein acetylation in *Pleurotus ostreatus* after different durations of heat stress.

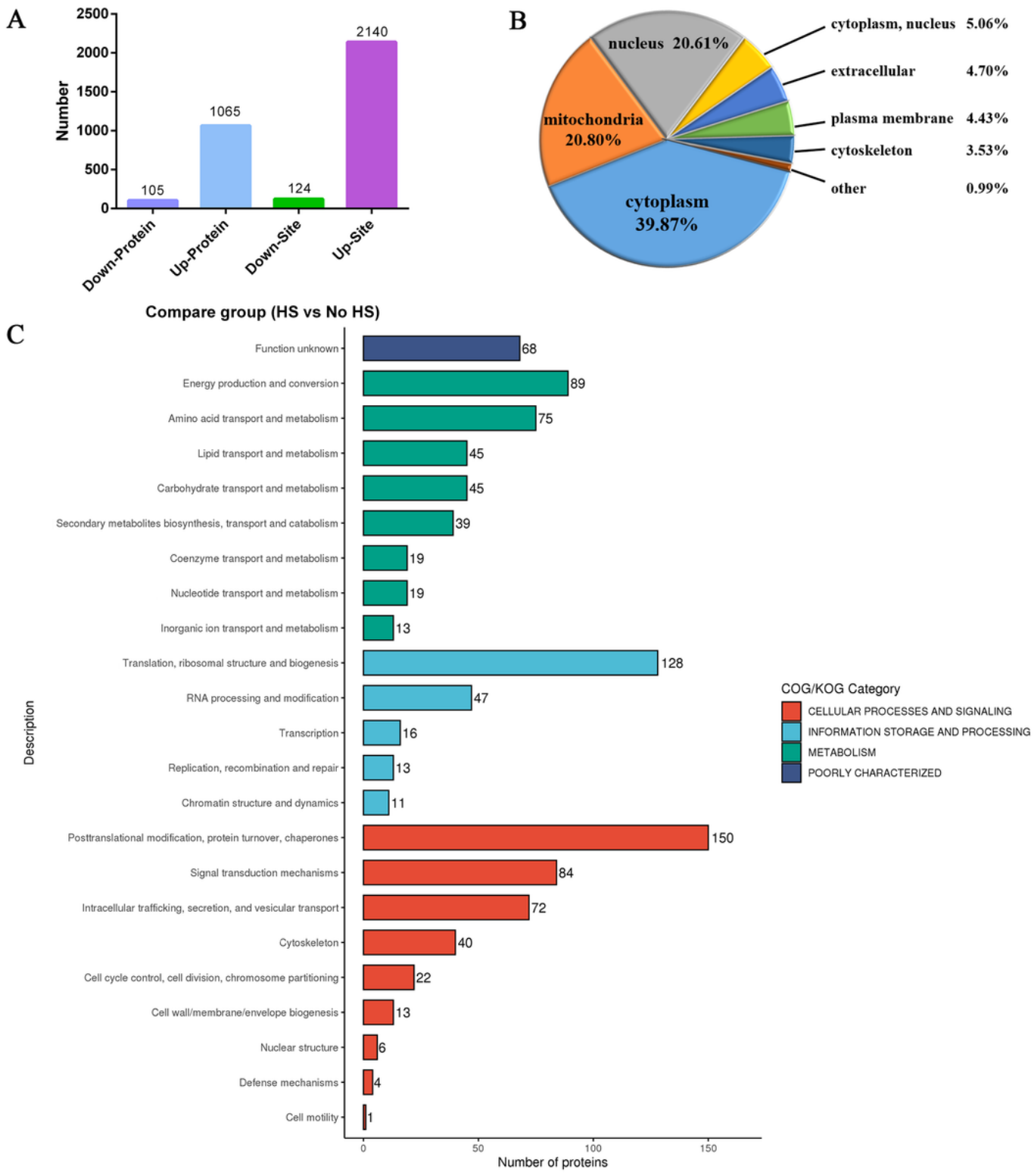

Figure 2

Differences in the acetylated proteins between heat stress and normal conditions. A. Analysis of the number of proteins and sites. B. Subcellular localization analysis. C. COG/KOG function classification.

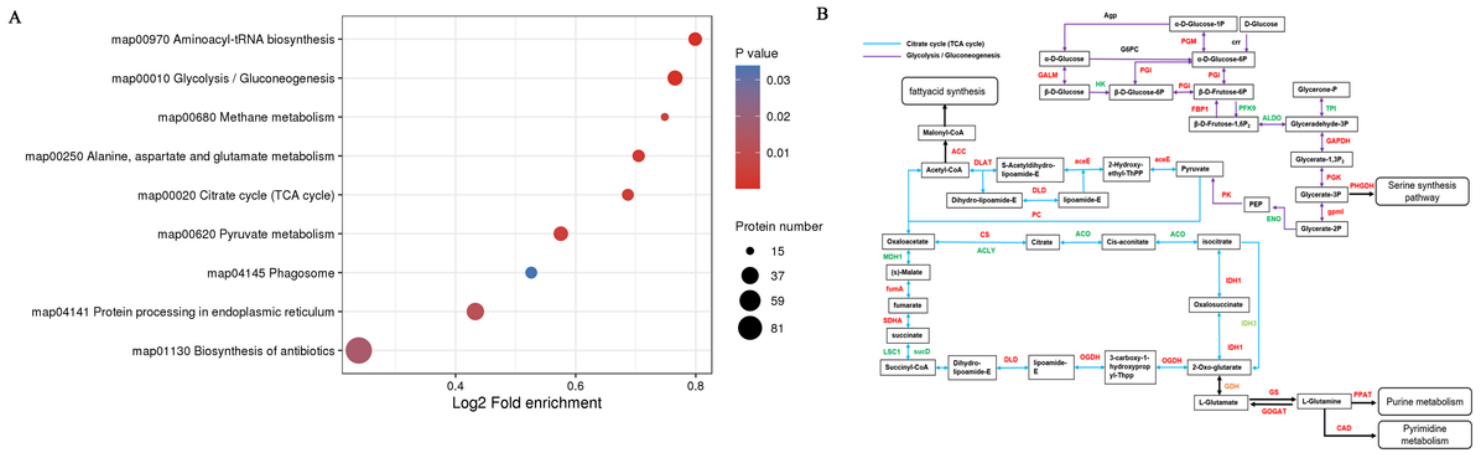

Figure 3

Acetylation of proteins associated with various metabolic processes under HS in the hyphae of *Pleurotus ostreatus*. A. KEGG pathway analysis. B. Enrichment of differentially acetylated proteins in central carbon metabolism (glycolysis and the TCA cycle).

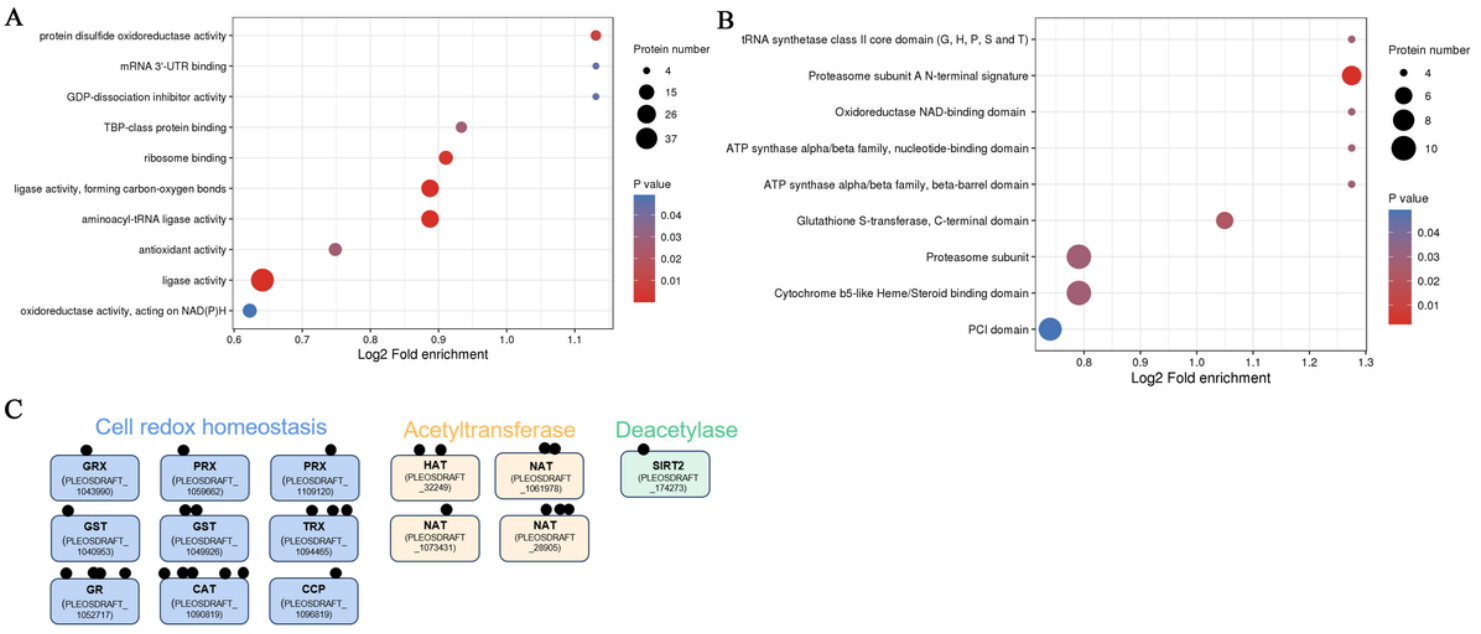

Figure 4

Functional analysis of differentially acetylated proteins. A. GO enrichment of proteins. B. GO enrichment of motifs. C. Antioxidant enzymes and acetylation-related proteins in differentially acetylated proteins. The black dots indicate the distribution of sites where the protein underwent acetylation.

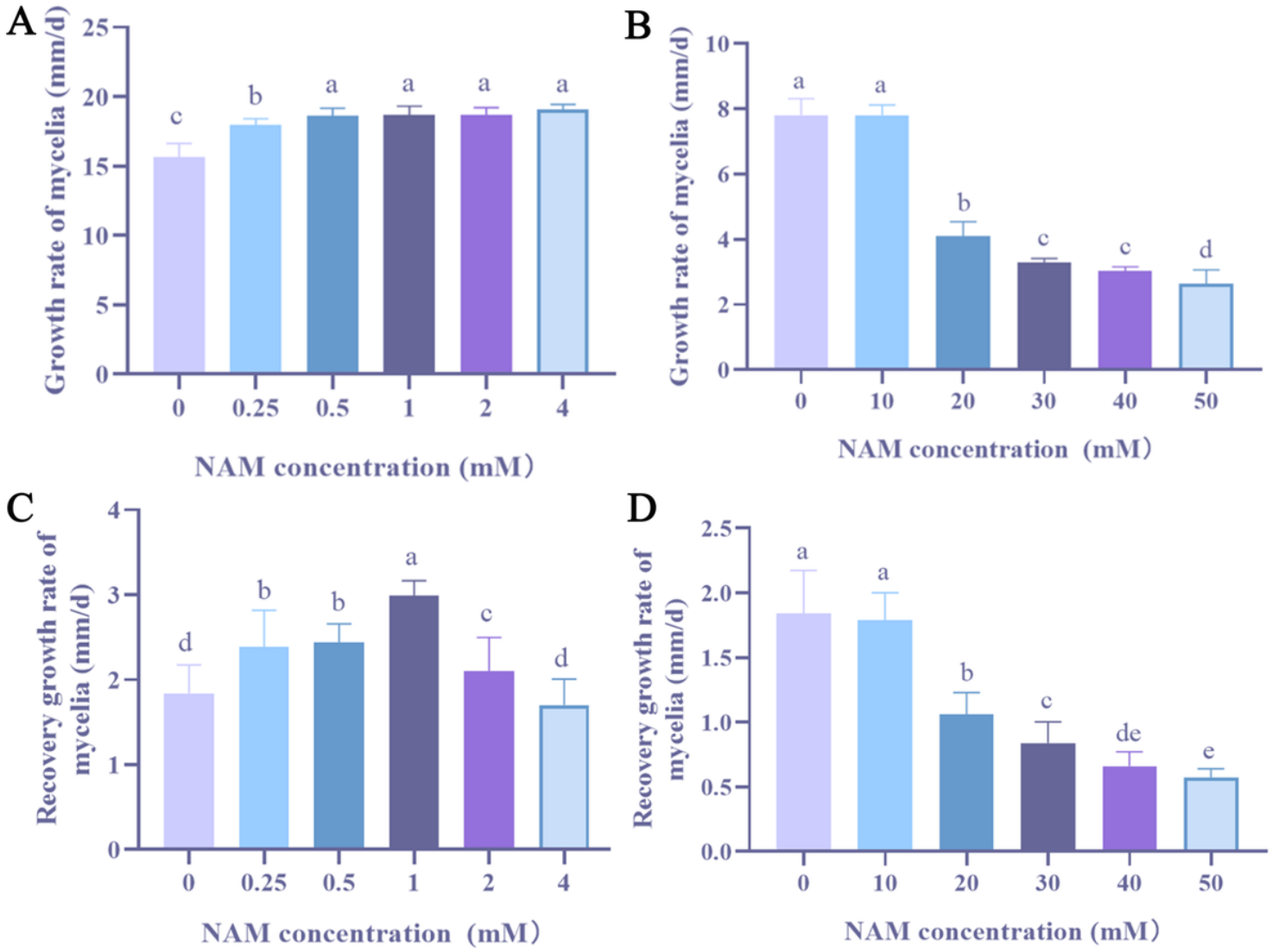

Figure 5

Effects of the addition of nicotinamide on the growth rate of mycelia. A. Effect of low concentrations of NAM (0, 0.25, 0.5, 1, 2, 4 mM) on the growth rate of mycelia under normal conditions. B. Effect of high concentrations of NAM (0, 10, 20, 30, 40, 50 mM) on the growth rate of mycelia under normal conditions. C. Effect of low concentrations of NAM on the recovery growth rate of mycelia after heat stress treatment. D. Effect of high concentrations of NAM on the recovery growth rate of mycelia after heat stress treatment. Different letters indicate a significant difference between groups at $P < 0.05$. The data are represented by the average of three replicates, and the error bar represents the standard deviation.

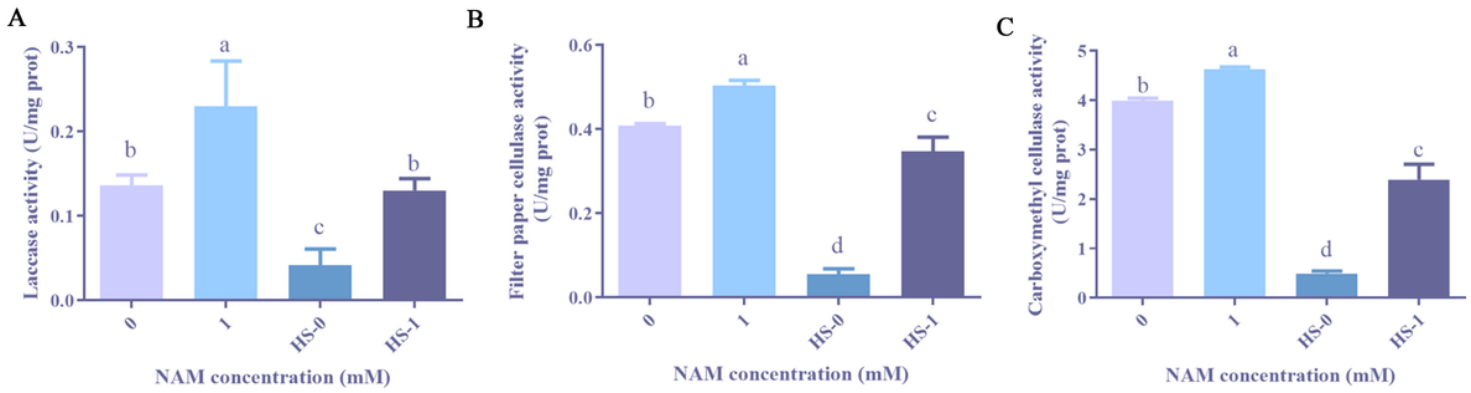

Figure 6

Effects of nicotinamide on substrate-degrading enzyme activities. A-C. The activities of laccase, filter paper cellulase, and carboxymethyl cellulase. Different letters indicate a significant difference between the groups at $P < 0.05$. The data are represented by the average of three replicates, and the error bar represents the standard deviation.

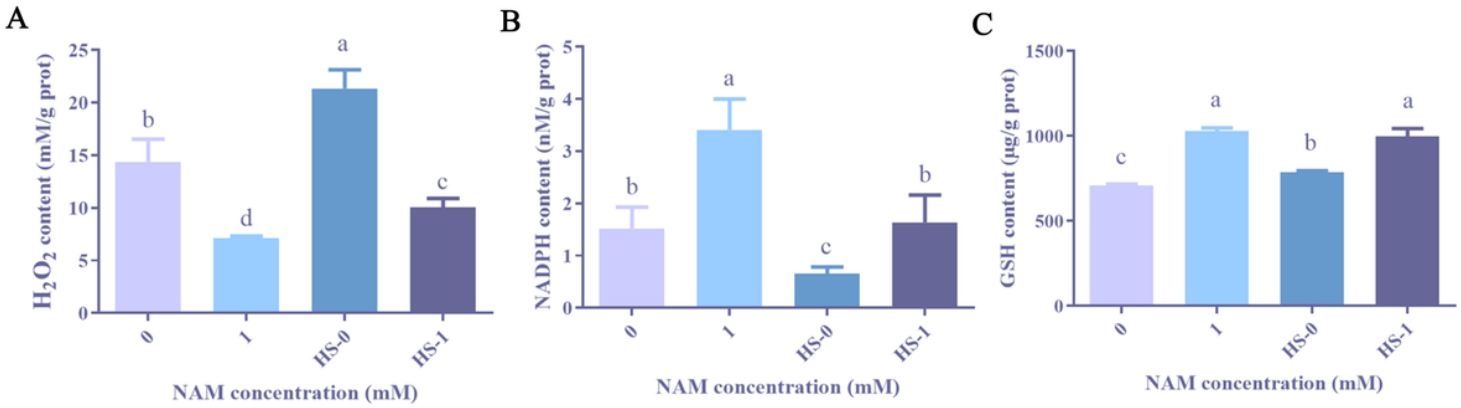

Figure 7

Effect of nicotinamide on intracellular redox homeostasis. A. H_2O_2 content under different treatment conditions. B. NADPH content under different treatment conditions. C. GSH content under different treatment conditions. Different letters indicate a significant difference between the groups at $P < 0.05$. The data are represented by the average of three replicates, and the error bar represents the standard deviation.

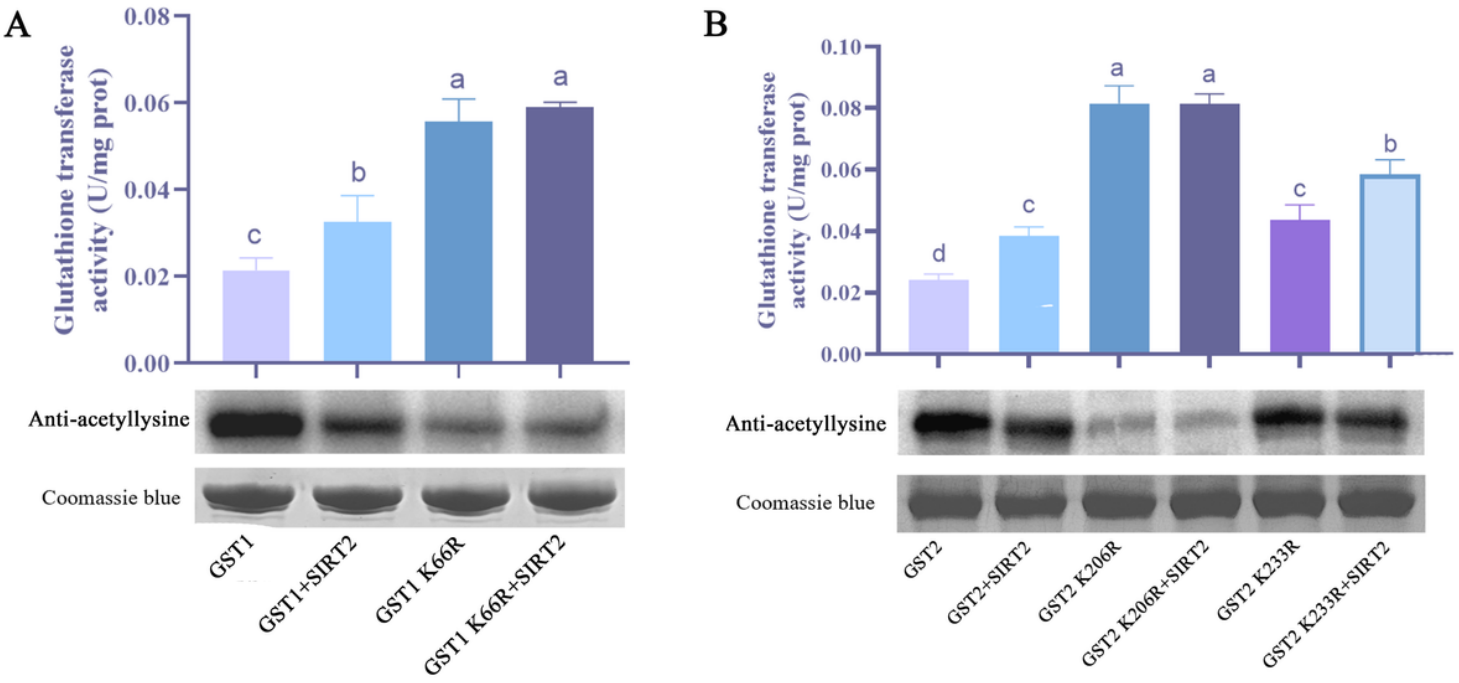

Figure 8

Effect of SIRT2 on deacetylation modification and the activity of GST. Effect of SIRT2 on the deacetylation modification and activity of A. GST1 and B. GST2.Different letters indicate a significant difference between the groups at $P < 0.05$. The data are represented by the average of three replicates, and the error bar represents the standard deviation.

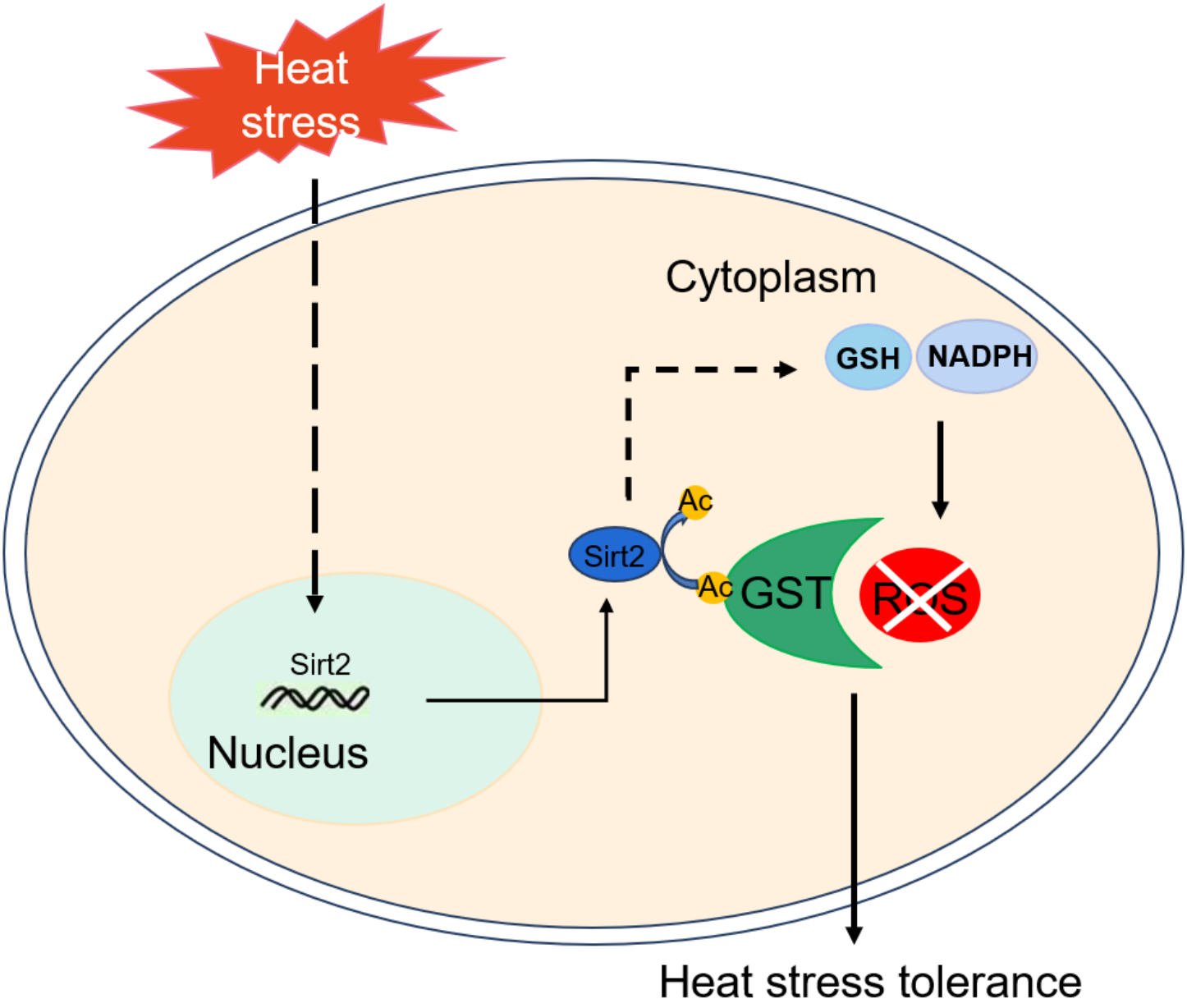

Figure 9

Schematic diagram of the mechanism by which SIRT2 enhances the tolerance of *Pleurotus ostreatus* to heat stress. SIRT2 was synthesized under heat stress, and it deacetylated glutathione transferase, thus enhancing its activity. SIRT2 also increased the accumulation of the antioxidants NADPH and GSH. Several antioxidant pathways were activated to increase the ROS clearance rate, which in turn increased the tolerance of *Pleurotus ostreatus* to heat stress.

Supplementary Files

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