Alternative Oxidase Gene Induced by Nitric Oxide is Involved in the Regulation of ROS and Enhance the Resistance of *Pleurotus Ostreatus* to Heat Stress

Ludan Hou

Chinese Academy of Agricultural Sciences

Mengran Zhao

Chinese Academy of Agricultural Sciences

Chenyang Huang

Chinese Academy of Agricultural Sciences

Qi He

University of the Chinese Academy of Sciences

Lijiao Zhang

Chinese Academy of Agricultural Sciences

Jinxia Zhang (✉ zhangjinxia@caas.cn)

Chinese Academy of Agricultural Sciences <https://orcid.org/0000-0002-5232-8222>

Research

Keywords: *Pleurotus ostreatus*, Nitric oxide, ROS, RNA-Seq, alternative oxidase, antioxidant enzymes.

Posted Date: August 31st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-63426/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Microbial Cell Factories on July 19th, 2021.
See the published version at <https://doi.org/10.1186/s12934-021-01626-y>.

Abstract

Background: *Pleurotus ostreatus* is easily affected by temperature during its cultivation. Nitric oxide (NO) plays an important regulatory role in the response to abiotic stress, and previous studies have found that NO can induce alternative oxidase (*aox*) in response to heat stress (HS) by regulating aconitase. However, the regulatory pathway of NO is complex, and the function and regulation of the *aox* gene in the response to HS remain unclear.

Results: In this study, we found that NO affected the NADH and ATP levels, reduced the H_2O_2 and O_2^- contents, and slowed the production of O_2^- . Further RNA-Seq results showed that NO regulated the oxidation-reduction process and oxidoreductase activity, affected the cellular respiration pathway and activated *aox* gene expression. The function of *aox* was determined by constructing overexpression (OE) and RNA interference (RNAi) strains. The results showed that the OE-*aox* strains showed obvious advantages in experiencing growth recovery after exposure to HS. During exposure to HS, the OE-*aox* strains exhibited reduced levels of NADH, the product of the TCA cycle, and decreased synthesis of ATP, which reduced the production and accumulation of ROS, whereas the RNAi-*aox* strains exhibited the opposite result. In addition, *aox* mediated the expression of antioxidant enzyme genes in the mycelia of *P. ostreatus* under HS through the retrograde signaling pathway.

Conclusions: This study shows that the *aox* gene in *P. ostreatus* mycelia can be induced by NO under HS, regulates the TCA cycle and cell respiration to reduce the production of ROS, and can mediate the retrograde signaling pathway involved in the mycelial response to HS.

Background

Pleurotus ostreatus is one of the most widely cultivated mushroom species globally and is mainly cultivated in horticultural facilities in China [1]. During the cultivation process of *P. ostreatus*, the high temperatures in summer limit the growth of mycelia, the formation of primordia and the development of fruiting bodies, and thereby seriously affect the yield. However, the response of mycelia to changes in temperature is very complex and includes different physiological and metabolic changes that affect the development of edible fungi as well as interactions between cells and molecules. Metabolomics analyses have shown that heat stress (HS) promotes the degradation of unsaturated fatty acids and nucleotides, increases the contents of amino acids and vitamins, and accelerates glycolysis and the tricarboxylic acid (TCA) cycle [2]. Furthermore, reactive oxygen species (ROS) accumulate in the mycelium during exposure to HS, which results in cell damage and even apoptosis [3]. The synthesis of trehalose and heat shock proteins is an important component of the response of edible fungi to high temperatures and to their resistance to heat [4]. Moreover, nitric oxide (NO) and calcium (Ca^{2+}) as signal molecules also play an active protective role during exposure to HS. In recent years, increasing attention has been paid to the mechanism through which fungi respond to HS.

NO, an essential endogenous signaling molecule that is involved in many biological processes in plants, animals, bacteria and fungi, is considered a broad-spectrum anti-stress molecule. The role of NO as a signal molecule in the growth, development and stress responses of plants has been widely studied and has become the focus of research. Previous studies have revealed that NO contributes to various stress tolerances by influencing ROS metabolism [5]. Ca^{2+} -mediated NO signal transduction is involved in the response of *Brassica* seedlings to metalloid stress [6], and in *Prunus persica* (L.) Batsch, treatment with NO solution can protect the fruit from pathogen infection by inducing activities of defense enzymes and the expression of pathogenesis-related genes [7]. In two ecotypes of reeds, NO significantly reduces the contents of hydrogen peroxide (H_2O_2) and Malondialdehyde in calli and significantly inhibits the increase in ion leakage, growth inhibition and decreases in cell viability induced by HS [8]. Previous studies have shown that NO can regulate the ROS content in two ways: by acting as a free radical that reacts with ROS to neutralize ROS or by acting as a signaling molecule to initiate gene expression via a molecular cascade [9]. In recent years, NO has also been found in edible fungi. Specifically, in *Ganoderma lucidum*, NO is involved in the regulation of ganoderma acid synthesis [10], and in *P. ostreatus*, NO can alleviate the oxidative damage induced in mycelia by HS [11]. However, the function and regulation of NO in fungi are less clear than those in plants.

Mitochondria are the main site of ROS bursts and play a key role in cell energy metabolism. The electron transport chain (ETC) of the mitochondria consists of two pathways: the main cytochrome c pathway and the alternative pathway. Alternative oxidase (AOX), an integral protein (32–36 kDa) of the inner mitochondrial membrane [12], is a component of the mitochondrial respiratory pathway, which is widely found in higher plants and some fungi and algae, and is responsible for the activity of the alternative respiratory pathway [13]. In most fungi, *aox* transcripts are undetectable, or present at very low levels under classical growth conditions but become expressed when the cytochrome respiratory chain is restricted by inhibitors or by mutations [14]. Recently, the function of AOX has been widely studied. In plants, the AOX pathway plays an important role in maintaining metabolism and signal homeostasis during exposure to abiotic and biotic stress [15]. One of the possible functions of AOX is regulating the production of ROS [16, 17]. The electron transport flow generated by AOX bypasses proton pump complexes III and IV, which affects adenosine triphosphate (ATP) production and reduces electron leakage and ROS production [18]. In tobacco, AOX can maintain the balance of the plant environment and enhance the tolerance of cells to drought stress by controlling the respiration rate, photosynthesis and chlorophyll synthesis [19]. In *Medicago truncatula*, NO can induce the expression of *aox*, and *aox* can help regulate the accumulation of ROS, protect the photosystem, and enhance plant resistance to salt stress [20]. In addition, AOX enhances the tolerance of spring wheat to HS [21]. The function of AOX in fungi has also been reported; for instance, AOX is a determinant for growth and sporulation in the early diverging fungus *Blastocladiella emersonii* [22], and in *Aspergillus fumigatus*, the silencing of the mitochondrial *aox* gene makes the strain more sensitive to ROS and easier to kill by macrophages [23]. However, the function of AOX in edible fungi remains unclear.

The expression of the *aox* gene is also induced by a wide range of biotic and abiotic stresses [24], and previous studies have shown that the induced expression of the *aox* gene under stress conditions is associated with stress-dependent ROS production [25]. Our previous study revealed that NO, as a signal molecule under HS, can increase the content of citric acid by inhibiting aconitase. The accumulation of citric acid can induce the expression of the *aox* gene and enhance the resistance of mycelia to HS [11]. However, the regulatory pathway through which NO alleviates mycelial stress is complex, and the mechanism through which the *aox* gene of *P. ostreatus* enhances the thermostability of mycelia remains unclear; hence, further research is needed to obtain a more in-depth understanding of this phenomenon. In this study, RNA sequencing (RNA-Seq) technology was used to explore the possible regulatory pathways and key genes involved in the NO-mediated alleviation of HS-induced damage in *P. ostreatus*, and the regulatory effect of NO on the *aox* gene was studied in detail. Moreover, the relationship among *aox*, energy metabolism and ROS was explored using overexpression (OE) and RNA interference (RNAi) technology.

Results

Exogenous NO affected the energy metabolism of mycelia and reduced the production and accumulation of ROS

NO, an important signaling molecule, plays an important regulatory role in the growth and development of organisms and their responses to stress. Our previous study showed that NO plays an important role in the response of *P. ostreatus* to HS. In this study, the addition of exogenous NO promoted the growth recovery of *P. ostreatus* mycelia after HS, as shown in Fig. 1A (the red arrow indicates regenerated mycelia after HS). H_2O_2 and superoxide anion (O_2^-) are important components of ROS, and as shown in Fig. 1B, the content of H_2O_2 in mycelia increased significantly after HS. Exogenous sodium nitroprusside (100 μ M SNP) could significantly reduce the accumulation of H_2O_2 in mycelia after HS, but the level was still significantly higher than that in the control group. As shown in Figs. 1C and 1D, HS increased the O_2^- content in mycelia by 1.64-fold and the production rate of O_2^- by 60.74 %. In addition, compared with the levels found in the HS group, exogenous SNP decreased the O_2^- content and production rate by 12.85 % and 9.34 % respectively, whereas, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO (250 μ M) significantly increased the content and production rate of O_2^- . The results showed that exogenous NO could affect not only the content of H_2O_2 , but also the content and production rate of O_2^- .

Nicotinamide adenine dinucleotide (NAD) is a coenzyme that exists in all cells and is found in two forms: oxidized (NAD^+) and reduced (NADH). NADH is produced during the TCA cycle, participates in material and energy metabolism in cells and serves as a control marker in the energy production chain of mitochondria. As shown in Fig. 1E, HS significantly increased the content of NADH in mycelia compared with the level found in the control group, and this increase could be partially offset by treatment with SNP. HS also significantly affected the $NAD^+/NADH$ ratio, and the addition of exogenous SNP alleviated the imbalance in NAD caused by HS. In contrast, the addition of cPTIO significantly decreased the

NAD⁺/NADH ratio compared with that observed in the HS group (Fig. 1F). Because NAD⁺ is reduced to NADH by the TCA cycle in mitochondria and NADH serves as a substrate for the generation of ROS in the respiratory chain [26], it can be assumed that the TCA cycle is accelerated by HS and that NO can affect the accumulation of NADH by regulating the TCA cycle. ATP is commonly considered as an intracellular energy currency molecule, and the mitochondrial respiratory chain is the site of ATP production. As shown in Fig. 1G, the total respiration rate of mycelia decreased after HS, and this decrease might be caused by mitochondrial damage induced by HS. The addition of exogenous NO further inhibited the total respiratory rate. It has been suggested that NO can regulate the respiration rate of mycelium under stress and that NO might reduce ROS production by inhibiting respiration rate. Interestingly, the ATP content increased by 39.27 % after HS treatment compared with the level found in control group, and this increase is synchronous with the outbreak of ROS. The increase in the ATP content might be due to the observed increase in the NADH level (Fig. 1E). Exogenous NO significantly inhibited the increase in ATP induced by HS, whereas the addition of exogenous cPTIO significantly increased the ATP content under HS (Fig. 1H). The results further proved that NO can regulate the respiratory chain.

In conclusion, NO might alleviate ROS outbreaks by regulating the mitochondrial respiratory chain and thereby reducing mycelial damage induced by HS.

RNA-Seq analysis of the regulatory mechanism through which NO alleviates mycelial damage induced by HS

To fully understand the effect of NO on the transcriptome of *P. ostreatus* under HS, 12 RNA-Seq cDNA libraries were prepared. As shown in Additional file 1: Table S2, after removing adapters, low-quality regions and all possible contamination, each treatment group contained an average of 47.29 M clean reads with Q30 > 94.93 % and a GC percentage between 53.03 % and 53.64 %. The ratio of reads mapping to the *P. ostreatus* genome was high, ranging from 77.26 % to 82.93 %. This result indicated that the accuracy of the sequencing results was high and could be used for subsequent analysis.

The RNA-Seq analysis showed that HS caused significant changes in gene expression in *P. ostreatus*. To further explore the relationships among differentially expressed genes (DEGs) identified from the four different comparisons, Venn diagrams were constructed using all the identified DEGs (a total of 2400). Among the HS-responsive genes, 742 DEGs were significantly upregulated, and 1079 DEGs were significantly downregulated. A total of 817 DEGs were regulated by the addition of SNP or cPTIO. As shown in Fig. 2, 63 upregulated and 38 downregulated DEGs were found in CK_HS and SNP_cPTIO. Moreover, 626 specific upregulated DEGs and 957 specific downregulated DEGs were found in the CK_HS group, whereas 429 and 150 DEGs were significantly upregulated and significantly downregulated, respectively, in the SNP_cPTIO group. These results indicated that the molecular responses to HS between the group treated with SNP and cPTIO were strikingly different. The specific upregulated and downregulated DEGs identified from the SNP_cPTIO comparison were selected for further functional characterization to explore the possible molecular mechanism through which NO alleviates the oxidative damage to mycelia caused by HS.

Based on the above-described analysis, the functions of 579 DEGs identified from the SNP_cPTIO comparison were examined to elucidate the possible mechanism through which NO alleviates the oxidative damage to mycelia induced by HS. In the gene ontology (GO) analysis, the DEGs identified from the SNP_cPTIO comparison were classified into three categories: 'biological process', 'cellular component' and 'molecular function' (Fig. 3). Among the biological process category, genes corresponding to metabolic process, cellular process and single organism process were the most abundant. Membrane and membrane parts were the most abundant of the cellular components, and in the molecular function category, catalytic activity and binding were the most abundant. These results indicated that NO might participate in the response of mycelia to HS by regulating cell metabolism, affecting cell membrane components and structure, and affecting the catalytic activity of proteins.

To understand the functions of the DEGs identified from the SNP_cPTIO comparison, a pathway enrichment analysis was performed. The results showed that the DEGs were mainly concentrated in the following pathways: oxidoreductase activity, oxidation-reduction process, cofactor binding, protein kinase activity, phosphotransferase activity, alcohol group as acceptor, and protein phosphorylation (Fig. 4A). Previous studies have shown that HS can lead to the production and accumulation of ROS in mycelia, and ROS can further cause oxidative damage. As shown in Fig. 4A, exogenous NO can affect the oxidation-reduction process and oxidoreductase activity. In addition, considering the close relationship between the antioxidant system and ROS clearance, the expression pattern of the genes after the addition of a NO donor or scavenger was further analyzed with a heatmap. As shown in Fig. 4B, 69 DEGs identified after the addition of SNP or cPTIO were enriched in the oxidation-reduction process pathway and oxidoreductase activity, and these included 62 significantly upregulated DEGs and seven downregulated DEGs. It can thus be hypothesized that NO can activate the activity of oxidoreductase and accelerate redox reactions under HS.

NO affected the expression of key genes in the respiratory chain under HS

The respiratory chain is closely related to ROS. Based on previous studies, to explore whether NO can regulate the respiratory chain to alleviate mycelial damage under HS, six DEGs related to the respiratory chain were identified from 579 DEGs via functional enrichment (Table 1). Two of these DEGs are not annotated, and the remaining four DEGs are g3097, g11376, g12148 and g12952, which encode the succinate dehydrogenase iron-sulfur subunit, AOX, hypothetical protein and mitochondrial chaperone bcs1, respectively. As illustrated in the heatmap shown in Fig. 5, an exogenous NO donor (SNP) inhibited the expression of five DEGs and upregulated the expression of g11376 (AOX). Mitochondrial chaperone bcs1 is a transmembrane chaperone found in the mitochondrial inner membrane and is required for the assembly of mitochondrial respiratory chain complex III (<http://www.ebi.ac.uk/interpro/entry/InterPro/IPR027243/>) [27]. The succinate dehydrogenase iron-sulfur subunit is involved in the synthesis and assembly of mitochondrial respiratory chain complex II (<https://www.uniprot.org/uniprot/A1AZJ0>). These results indicate that exogenous NO can inhibit the cytochrome pathway and activate the alternative oxidation pathway.

NO induced *aox* gene expression under HS

ROS are mainly produced by a high-oxygen environment and the respiratory chain at a high reduction state during the transition of mitochondria from complex III to complex IV, which results in the leakage of a large number of electrons and the reduction of oxygen molecules. AOX prevents excessive reduction of downstream complexes (cytochrome pathway) by introducing a branch into the ETC at the ubiquinone pool. When AOX bypasses complexes III and IV of the cytochrome pathway, it significantly reduces ATP production and single electron leakage, which results in the reduction of ROS production [18]. Moreover, as determined through RNA-Seq analysis, *aox* can be regulated by NO, participates in the oxidation-reduction process pathway and has oxidoreductase activity.

To further verify the regulatory effect of NO on *aox* under HS, the effects of exogenous NO donors and scavengers on *aox* gene expression were detected. As shown in Fig. 6A, the relative expression of the *aox* gene in *P. ostreatus* mycelia changed steadily with increases in the time of exposure to HS. During the first 24 h of exposure to HS, *aox* gene expression first increased and then decreased within a small range. An increase in the exposure time to 48 h significantly increased the relative expression of the *aox* gene, and the level detected after 48 h was approximately 8-fold higher than that at 0 h. As shown in Fig. 6B, the relative expression of the *aox* gene was significantly increased after HS, whereas exogenous SNP treatment almost completely enhanced this effect, and cPTIO blocked the effect of SNP on *aox* gene expression. The results showed that NO can promote the expression of the *aox* gene in mycelia after HS. In conclusion, NO might participate in the response of *P. ostreatus* to HS by regulating the expression of the *aox* gene.

The OE of *aox* promoted the recovery of mycelial growth after HS

Using previously reported methods, we successfully constructed an RNAi-*aox* plasmid (Additional file 1: Fig. S1) and established *aox*-transformed strains via *Agrobacterium* mediated transformation. The *hyg* gene fragment was amplified for preliminary selection, and the relative expression of this target gene was amplified by (quantitative PCR) qPCR to screen the RNAi-*aox* strains. The results are shown in Additional file 1: Fig. S2. To further explore the function of the *aox* gene in the response of *P. ostreatus* to HS, the plates containing the various strains were cultured at 28 °C for 5 d, transferred to 40 °C for 48 h, and then incubated at 28 °C to allow growth recovery. After 3 d of growth recovery after HS, mycelial germination was observed in the wild type (WT), OE-*aox* and RNAi-*aox* strains, as shown in Fig. 7A. After 5 d of growth recovery, compared with the WT strain, the OE-*aox* strains exhibited a faster recovery rate and a complete colony edge, whereas RNAi-*aox* strains presented a slower mycelial recovery rate and showed defects on the edge of the colony. In conclusion, the *aox* gene plays an active role in the recovery of *P. ostreatus* mycelia after HS. Figs. 7B, 7C and 7D show the changes in the H₂O₂ content and the O₂⁻ content and production rate of the *aox*-transformed strains under HS. Under HS, the accumulation of H₂O₂ in the OE-*aox* 47 and OE-*aox* 71 strains was significantly lower (by 9.05 % and 12.28 % respectively) than that in the WT strain. In addition, the H₂O₂ content in the OE-*aox* 34 strain was 4.29 % lower than that in the WT strain. In contrast, the H₂O₂ contents in the RNAi-*aox* 12, RNAi-*aox* 29 and RNAi-*aox* 7 strains were 4.59 %, 4.59 %, and 4.59 %, respectively.

17.71 % and 21.11 % higher, respectively, than that in the WT strain. As shown in Fig. 7C-D, the O_2^- production rate and content of the OE-*aox* strains under HS were significantly lower than those of the WT strain, whereas that of the RNAi-*aox* strains increased significantly. These results indicate that the *aox* gene can regulate the production and accumulation of ROS. ROS are mainly caused by electron leakage in respiratory chain. As shown in Figs. 7E, 7F and 7G, compared with the WT strain, the average NADH and ATP contents in the OE-*aox* strains were decreased by 26.47 % and 9.82 %, respectively, and the average $NAD^+/NADH$ ratio in these strains was 1.55-fold higher. In contrast, the average NADH and ATP contents of the RNAi-*aox* strains were 66.86 % and 29.79 %, respectively, higher than those in the WT strain, and the $NAD^+/NADH$ ratio was decreased by 26.52 %. Therefore, it can be speculated that the *aox* gene plays an important role in regulating the mitochondrial ETC, energy metabolism and maintaining mitochondrial homeostasis.

In conclusion, the *aox* gene can affect the production and accumulation of ROS by regulating mitochondrial respiration, and participates in the response of mycelia to HS.

***aox* gene regulates the expression of key antioxidant enzyme genes in mycelia after HS**

The signaling from organelles controlling nuclear gene expression is called retrograde signaling, and previous studies have shown that AOX serves as a marker gene for mitochondrial retrograde regulation [28]. AOX also acts as a facilitator for signaling molecules conveying the metabolic status of mitochondria to the nucleus and is thus able to influence nuclear gene expression [29]. Moreover, studies have shown that AOX can affect the production and accumulation of ROS. Antioxidant systems (such as antioxidant enzymes and nonenzymatic oxidants) play critical roles in the defense against oxidative stress [30]. We detected the changes in the expression of genes that encode four key antioxidant enzymes, namely catalase (CAT), superoxide dismutase (SOD), thioredoxin reductase (TrxR) and glutathione peroxidase (GSH-PX). In the genome of *P. ostreatus*, two genes (*cat1* and *cat2*) encode CAT, four genes (*SOD1*, *SOD2*, *SOD3* and *SOD4*) encode SOD, one gene encodes TrxR, and one gene encodes GSH-PX [31]. To study whether *aox* in *P. ostreatus* can alleviate ROS outbreaks by regulating the antioxidant enzyme system, we measured the expression of these key antioxidant enzyme genes in *aox*-transformed strains exposed to HS at 40 °C for 48 h. As shown in Fig. 8A, after 48 h of exposure to HS, the relative expression of *cat1* in the OE-*aox* 47-, OE-*aox* 71- and OE-*aox* 34- transformed strains was significantly downregulated to 26.5 %, 25.96 % and 35.30 % of the level found in the WT strain, respectively. In RNAi-*aox* 12 and RNAi-*aox* 29 strains, the relative expression of *cat1* was significantly increased by 1.57-fold and 7.28-fold compared with that found in WT strain, but no significant change in the expression of this gene was detected in the RNAi-*aox* 7 strain. As shown in Fig. 8B, compared with that in the WT strain, the relative expression of *cat2* in the OE-*aox* and RNAi-*aox* strains was significantly downregulated and significantly increased, respectively. In conclusion, *aox* can negatively regulate *cat* gene expression under HS. As shown in Figs. 8C and 8D, the expression of the *TrxR* and *GSH-PX* genes was significantly downregulated in the OE-*aox*-transformed strains. In addition, the *TrxR* gene was significantly upregulated in all RNAi-*aox* strains, and the relative expression of the *GSH-PX* gene was significantly upregulated in RNAi-*aox*12 and RNAi-*aox* 29, and slightly upregulated in RNAi-*aox* 7. SOD is

one of the major defense systems used to remove O_2^- . The relative expression level of the four SOD-encoding genes in the *aox*-transformed strains under HS are shown in Figs. 8E-8H. As shown in Fig. 8E, compared with the level in the WT strain, *SOD1* gene expression was significantly increased in the OE-*aox* strains and decreased to 72.66 %, 67.71 % and 79.93 % in the RNAi-*aox* 12, RNAi-*aox* 29 and RNAi-*aox* 7, respectively. In addition, as shown in Figs. 8F-8H, the expression level of *SOD2*, *SOD3* and *SOD4* in the OE-*aox* strains was significantly downregulated, to 34.93 %, 60.33 % and 32.16 % of the level found in the WT strain, respectively. However, *SOD2* and *SOD3* gene expression was not significantly changed in the RNAi-*aox* strains, and the expression of *SOD4* was slightly downregulated in these strains compared with the WT strain. Because the *SOD1* gene encodes Gu-SOD, which mainly exists in the cytoplasm, it can be concluded that the OE of *aox* during HS can regulate the expression of SOD-encoding gene.

In conclusion, in addition to *SOD1*, the OE-*aox* strains exhibited downregulated expression of key antioxidant enzyme coding genes under HS (40 °C for 48 h), and the RNAi-*aox* strains exhibited upregulated CAT-, GSH-PX- and TrxR-encoding genes. It can thus be hypothesized that the significant upregulation of the *aox* gene after HS (40 °C for 48 h) can further affect the expression of key antioxidant enzyme-encoding genes.

Exogenous benzohydroxamate (BHAM) regulated the expression of antioxidant enzyme-encoding genes in mycelia under HS

AOX is sensitive to inhibitors of the cytochrome pathway such as cyanide, antimycin A, or miyxoethiazol, but is inhibited by primary hydroxamic acids, such as BHAM [32, 33]. To further prove whether *aox* interference can regulate the expression of key antioxidant enzyme-encoding genes, an experiment in which different concentrations of an AOX inhibitor (BHAM) were added was then performed. The results showed that the growth rate of *P. ostreatus* mycelia at 28 °C was affected by exogenous BHAM. As shown in Additional file 1: Figs. S3A and S3B, a low concentration of BHAM (50-100 μ M) had no significant effect on the colony and mycelial growth rate of *P. ostreatus*, but the addition of exogenous BHAM at a concentration higher than 200 μ M affected mycelial growth of mycelia and significantly reduced the mycelial growth rate. Furthermore, whether exogenous BHAM could regulate the expression of the *aox* gene was assessed in the experiment. As shown in Additional file 1: Fig. S3C, the addition of BHAM at a concentration of 50-200 μ M significantly downregulated the expression of the *aox* gene compared with the CK level, which indicated that low concentrations of BHAM can inhibit the expression of the *aox* gene. However, a BHAM concentration of 400 μ M upregulated the expression of the *aox* gene, and because this concentration also significantly inhibited the mycelial growth rate, it can be speculated that 400 μ M BHAM might affect the growth environment of mycelia and induce abiotic stress, which eventually leads to the induction of *aox* gene expression. In addition, because the mycelial growth of the RNAi-*aox* strains did not significantly differ from the normal growth of *P. ostreatus* mycelia, 50-200 μ M was used as the BHAM concentration in the subsequent experiments.

As shown in Fig. 9A, the addition of exogenous BHAM at different concentrations could slow down the recovery of mycelial growth after HS compared with that obtained with the WT strain, and this result was

consistent with the results obtained with the RNAi-*aox* strains. The effects of exogenous BHAM on the H_2O_2 content and the O_2^- content and production rate were then further detected. The results showed that compared with the CK-HS group, the H_2O_2 content and O_2^- content and production rate of mycelia under HS were significantly increased by different concentrations of BHAM (Figs. 9B-9D). These results indicated that exogenous BHAM could promote the production and accumulation of ROS in mycelia under HS. The effects of exogenous BHAM on the expression of key antioxidant enzyme-encoding genes in mycelia under HS are shown in Figs. 9E-9L. As illustrated in the figures, the addition of exogenous BHAM significantly increased the expression of antioxidant enzyme-encoding genes under HS. Specifically, the expression levels of *cat1* (Fig. 9E), *cat2* (Fig. 9F), *TrxR* (Fig. 9G), *GSH-PX* (Fig. 9H), *SOD2* (Fig. 9J) and *SOD4* (Fig. 9L) increased with increases in the BHAM concentration, and the highest expression levels of these genes, which were 47.25-fold, 17.76-fold, 1.99-fold, 6.86-fold, 4.76-fold and 3.98-fold higher than those found in the CK-HS group, respectively, were detected with a BHAM concentration of 200 μM . In addition, the highest expression levels of *SOD1* and *SOD3*, which were 1.90-fold and 2.42-fold higher than the control levels, respectively, were obtained with the exogenous addition of 100 μM BHAM (Figs. 9I and 9K).

In conclusion, exogenous BHAM can promote the production and accumulation of ROS under HS and then regulate the expression of antioxidant enzyme-encoding genes.

Discussion

NO is a type of free radical involved in many types of stress and physiological processes and serves as a key regulator of physiological processes [34]. NO has been widely studied as a signal molecule in animals and plants [35, 36], but its role in fungi is less understood, although much progress has been made. For example, during fungal development, NO might not only induce sexual development [37] but also be involved in conidiation, spore germination, and formation of the parasitic structure of appressoria, among other processes [38, 39]. In addition, studies have shown that NO can participate in the regulation of secondary metabolism of fungi. For example, NO can stimulate the activities of phenylalanine ammonia lyase and chalcone synthase in fruiting bodies to induce the accumulation of phenols and quinolines [40]. In addition, NO participates in the response pathway of fungi to various environmental stresses [41, 42]. Although our previous studies showed that NO can enhance the resistance of *P. ostreatus* to HS, the possible regulatory pathway remains unclear. The current study revealed that NO reduces the production of ATP and ROS by activating the alternative oxidation pathway, maintaining mitochondrial function, and enhancing the resistance of *P. ostreatus* to HS. In addition, AOX can act as a retrograde signaling pathway to regulate the expression of nuclear genes (antioxidant enzyme-encoding genes) in response to HS. This finding provides a new perspective of the fungal regulatory mechanism of NO in response to abiotic stress.

In recent years, increasing studies have shown that the application of exogenous NO is useful for allaying oxidative stresses caused by drought, high temperature and salinity [43–45]. HS refers to an increase in temperature to a level that exceeds the optimal growth temperature of the organism of interest, which

causes irreversible damage to its growth [46]. HS induces an increased generation of ROS. The production rate of ROS is strongly dependent on the membrane potential, and a change in the membrane potential increases the probability of electron leakage and thus the production of O_2^- , which can be used as a substrate to produce H_2O_2 and hydroxyl radicals [47]. In addition, excessive production and accumulation of ROS can cause lipid peroxidation, membrane leakage, enzyme inactivation, and DNA fragmentation or mutation, which can result in serious cell damage [48, 49]. During its cultivation, *P. ostreatus* is often exposed to high temperature. The results obtained in this study show that exogenous NO can reduce the content of NADH and the production of ATP under HS. Previous studies have shown that NO can regulate the $NAD^+/NADH$ ratio in *Ganoderma lucidum* under HS [50], which is consistent with our results. Second, the $NAD^+/NADH$ ratio is mainly regulated by the TCA cycle, and NADH serves as a substrate for the generation of ATP in the respiratory chain [26]. Therefore, it can be speculated that exogenous NO can affect ATP production by regulating the NADH content in the TCA cycle. In addition, the production and accumulation of ROS in mycelia were decreased by exogenous NO addition. Previous studies have shown that NO can reduce ROS accumulation under HS by regulating different pathways. For instance, in wheat, the addition of exogenous NO donors can activate antioxidant enzymes, which results in the reduction of ROS and improvements in the heat resistance of wheat coleoptiles [51]. In rice, NO might protect photosynthesis from HS and alleviate oxidative stress by scavenging ROS [52]. The results of this study are consistent with the findings in plants. Because ROS are mainly caused by electron leakage in the respiratory chain, it can be speculated that exogenous NO can reduce the production and outbreak of ROS under HS by regulating the respiratory chain.

In plants, stress-induced gene expression changes are key components of the molecular mechanism underlying plant adaptation to environmental challenges [53]. In recent years, RNA-Seq has become an effective means to study changes in gene expression under stress. For example, in *Camellia sinensis*, a total of 89 putative AP2/ERF transcription factors related to the temperature response were identified by RNA-Seq [54]. Under drought stress, 5689 DEGs in *Populus trichocarpa* leaves were obtained by RNA-Seq analysis, and genes related to the drought stress response and involved in photosynthesis, cell wall organization, and osmoprotectant metabolism were also mined [55]. Therefore, RNA-Seq is an effective method for exploring the response mechanism of organisms to stress based on changes in gene expression. Our previous study found that NO can induce *aox* gene expression in *P. ostreatus* mycelia by inhibiting the expression of aconitase gene and protein, which results in citric acid accumulation [11]. In this study, RNA-Seq was used to further explore the response mechanism through which NO alleviates mycelial damage under HS. The results identified 1821 DEGs in the mycelia of *P. ostreatus* under HS (40 °C for 48 h), and after the exogenous addition of SNP and cPTIO, 579 DEGs specifically regulated by NO were obtained. Thus, these 579 genes were classified based on their GO functions. Based on the findings, NO might participate in the response of *P. ostreatus* mycelia to HS by regulating cell metabolism, affecting the composition and structure of the cell membrane, and modifying the catalytic activity of proteins. The pathway enrichment analysis revealed that 69 DEGs were significantly enriched in the oxidation-reduction process pathway and oxidoreductase activity, and 62 DEGs were upregulated after exogenous SNP addition. These results indicate that NO can regulate the oxidation-reduction process

pathway and oxidoreductase activity in response to HS. Interestingly, the functional enrichment analysis identified six among the 579 DEGs that are not only regulated by NO but also closely related to the respiratory chain. Among these six DEGs, mitochondrial chaperone bcs1 (g12952) and succinate dehydrogenase iron-sulfur subunit (g3097) are necessary for complexes II and III, respectively, which are closely related to the cytochrome pathway, and AOX (g11376) is involved in the alternative oxidation pathway. Further analysis showed that only AOX was upregulated after SNP addition, whereas the other DEGs were downregulated. This finding is consistent with our previous results [11]. In plants, it has also been reported that NO can regulate the expression of the *aox* gene. For instance, Fu *et al.* showed that NO is an inducer of *aox* in tobacco plants infected with *tobacco mosaic virus* [56], and another study found that *aox* is induced in *Arabidopsis* cell suspensions treated with NO [57]. Our results are consistent with the findings obtained with plants, which further indicated that exogenous NO can inhibit the cytochrome pathway and activate the alternative oxidation pathway and that AOX plays an important role in the response of mycelia to HS.

AOX is a nonenergy-conserving terminal oxidase in the plant mitochondrial ETC, and previous studies have shown that AOX functions to balance energy stability and keep the ETC flowing through mitochondria by limiting the formation of mitochondrial ROS [24, 25]. The function of *aox* in plants has been studied in more depth than that in fungi, and these studies have shown that *aox* plays important roles in countering abiotic stresses, including drought, light, temperature and salinity [21, 58, 59]. In *Medicago truncatula*, high *aox* expression can regulate ROS, protect the light system and enhance the resistance to salt stress [20], and under drought stress, the recoverability of tobacco to *aox* knockdown is strongly compromised [60]. In this study, the OE-*aox* strains rapidly resumed their growth after HS, and *aox* OE could reduce the contents of NADH and ATP and the content and production rate of O_2^- . In contrast, the opposite results were obtained with the RNAi-*aox* strains. Previous studies with *Arabidopsis thaliana* have shown that under hypoxic stress, *aox* can regulate energy metabolism, affect ROS production and enhance resistance [61], and we obtained similar results. The formation of ROS is mainly due to the “single electron leakage” of respiratory chain components, and complexes I and III are considered the main sites of this electron leakage [62]. Therefore, it can be speculated that the *aox* gene might reduce the production of O_2^- by regulating the respiratory pathway and maintaining mitochondrial homeostasis and function in *P. ostreatus*.

A large number of studies have shown that AOX mediates a retrograde signaling pathway that in turn regulates gene expression both transcriptionally and post transcriptionally in response to stress. AOX serves as a link between metabolic activities (mitochondria) and signaling (nucleus) [29, 63]. In this study, the expression of antioxidant enzyme-encoding genes in the OE-*aox* strains was downregulated after HS, whereas the expression of these genes in the RNAi-*aox* strains was upregulated, and these finding might be related to the regulation of ROS by *aox*. BHAM is an inhibitor of AOX, and exogenous BHAM also promoted the expression of antioxidant enzyme-encoding genes under HS, which was very similar to the results obtained with the RNAi-*aox* strains. Therefore, it can be speculated that in response to HS, the *aox*

gene can mediate the retrograde signaling pathway in *P. ostreatus* mycelia to regulate the expression of antioxidant enzyme-encoding genes.

Conclusions

In summary, the results of this study indicated that NO responds to HS by reducing the production and accumulation of ROS in *P. ostreatus* mycelia, and can induce *aox* gene expression. The construction of OE-*aox* and RNAi-*aox* strains showed that high *aox* gene expression can affect cell respiration by reducing the content of NADH, which is a product of the TCA cycle, and thereby reducing the production and accumulation of ROS. Further studies have shown that under HS, *aox* can mediate the reverse signaling pathway to regulate the expression of antioxidant enzyme genes in *P. ostreatus* and thereby regulates the response of mycelia to ROS. Based on these findings, a potential cascade of the cellular events comprising the NO-mediated alleviation of ROS production via *aox* gene expression was proposed (Fig. 10). Our study improves the understanding of the biological functions and regulatory pathways of NO in *P. ostreatus* under HS and provides new ideas for further studies on the functions of AOX in fungi.

Methods

Strains and growth conditions

The *P. ostreatus* CCMSSC00389 strain was provided by the China Center for Mushroom Spawn Standards and Control. The WT, OE-*aox*, and RNAi-*aox* strains were incubated on PDA plates. *Agrobacterium tumefaciens* GV3101 (IMCAS, Beijing, China) was grown in Luria-Bertani medium (Oxoid, England) containing 100 µg/mL kanamycin (VWR Life Science, USA) and 50 µg/mL rifampin (MP Biomedicals, France).

HS and growth recovery of mycelia

According to research methods used in our previous study [4], the different strains were incubated at 28 °C for 5 d, and then exposed to 40 °C for 48 h. Some of the plates were used to collect mycelia, and the collected mycelia were rapidly placed in liquid nitrogen and stored in a refrigerator at -80 °C for subsequent experiments. After HS, the rest of the strains were placed at 28 °C to allow them to resume growth, and the mycelial growth after 3 and 5 d was observed and recorded.

Determination of the H₂O₂ content

The intracellular H₂O₂ content was determined using a hydrogen peroxide assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. H₂O₂ reacts with molybdcic acid to form a complex. The amount of complex formation was determined by measuring the absorbance at 405 nm, and the content of H₂O₂ was then calculated.

Determination of the O₂⁻ content and production rate

Excessive accumulation of O_2^- will destroy the cell membrane. The content and production rate of O_2^- in the mycelia of the different strains subjected to the different treatments were detected using an O_2^- content detection kit (Solarbio, Beijing, China) according to the manufacturer's instructions. O_2^- reacts with hydroxylamine hydrochloride to form nitrite ions, which form a red compound under the action of p-aminobenzenesulfonic acid and naphthalene ethylenediamine hydrochloride, and this compound has a characteristic absorption peak at 530 nm. The content and production rate of O_2^- in mycelia were then calculated based on the absorbance value at 530 nm.

Determination of the NADH and NAD^+ content

In this study, the changes in the NADH content and the $NAD^+/NADH$ ratio in the WT and *aox*-transformed strains under HS were determined using an $NAD^+/NADH$ assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Ethanol is oxidized to acetaldehyde in the presence of alcohol dehydrogenase, and during this process, NAD^+ is reduced to NADH. Subsequently, NADH reduces 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfobenzene)-2H-tetrazole to orange formazan in the presence of 1-methoxy-5-methylpenazinium methyl sulfate, and the product has a characteristic absorption peak at 450 nm. The samples from the different treatment groups were ground with liquid nitrogen, and 30 mg of the samples was added to 400 μ L of extraction solution, homogenized, incubated on ice for 10 min, and centrifuged at 12,000 g and 4 °C for 10 min. The supernatant was then collected. The total amounts of NAD^+ and NADH were determined according to the instructions provided with the kit, and the $NAD^+/NADH$ ratio was calculated.

Determination of the ATP content and total respiratory rate

Respiration is the core process of mitochondrial metabolism, and a large amount of free energy is released by oxidative phosphorylation for the production of ATP [64]. In this study, the total respiratory rate was measured according to previous studies [65]. Specifically, the changes in the ATP content during different treatment were measured using an enhanced ATP assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. This assay is based on the fact that firefly luciferase needs ATP to provide energy for the production of fluorescence. The samples were lysed with the ATP extract in the kit, homogenized for 10 min and centrifuged at 12,000 g and 4 °C for 10 min. The supernatant was then used to determine the ATP content and protein concentration, and the ATP content in the different samples was calculated.

RNA extraction, cDNA library construction, and RNA-Seq

SNP is a donor of NO, and cPTIO is a scavenger of NO. To understand the regulatory mechanism through which NO alleviates the mycelial damage in *P. ostreatus* induced by HS, four different treatments (CK, HS, SNP_HS and cPTIO_HS) were established for RNA-Seq analysis. Total RNA was extracted from the mycelia of *P. ostreatus* using the TRIzol® reagent (Invitrogen, USA) according the manufacturer's instructions, and genomic DNA was removed using DNase I (TaKaRa, Japan). The RNA quality was

determined using a 2100 Bioanalyzer (Agilent, CA, USA). The RNA-Seq transcriptome library was prepared using the TruSeq™ RNA sample preparation Kit from Illumina (San Diego, CA, USA) and 1 µg of total RNA. Briefly, messenger RNA was isolated according to the polyA selection method using oligo (dT) beads and then fragmented using fragmentation buffer. Double-stranded cDNA was then synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA, USA) with random hexamer primers (Illumina). The synthesized cDNA was then subjected to end repair, phosphorylation and 'A' base addition according to library construction protocol established by Illumina. The libraries were subjected to size selection to obtain cDNA target fragments of 200-300 bp using 2 % Low Range Ultra Agarose followed by 15 cycles of PCR amplification with Phusion DNA polymerase (NEB). After quantification by TBS380, the paired-end RNA-Seq sequencing library was sequenced with Illumina NovaSeq 6000 sequencers (2×150-bp read length), and the sequencing data were deposited into the Sequence Read Archive of the National Centre for Biotechnology Information (NCBI) with the accession number SRP277542.

Analysis of DEGs

To identify the DEGs between the CK and HS, SNP_HS and cPTIO_HS groups, the expression level of each transcript was calculated using the fragments per kilobase of exon per million mapped reads (FRKM) method. RSEM (<http://deweylab.github.io/RSEM/>) was used to quantify the gene abundances [66]. Differential expression analysis was performed using edgeR software in the R statistical package [67]. In addition, the GO functional enrichment of the DEGs was analyzed.

Construction of RNAi-*aox* plasmids and strains

Previous studies have shown that gene knockout and gene transformation using vectors constitute an effective strategy for exploring the function of fungal genes. In addition, OE-*aox* strains were successfully obtained in a previous study [11]. In this study, the RNAi-*aox* vector of *P. ostreatus* was constructed, and the *aox*-sense and *aox*-anti fragments were amplified using primers with homologous arms, as shown in Additional file 1: Table S1. Homologous recombination was then performed to connect the target fragments to the plasmid one by one. The constructed plasmid map is shown in Additional file 1: Fig. S1. The *aox*-JC-F/R primers were used to amplify the target fragment and thus detect whether the interference plasmid was successfully constructed. The RNAi-*aox* plasmid was then transformed into *P. ostreatus* mycelia by *A. tumefaciens* GV3101. The *hyg* fragment was amplified to screen the transformed strains, and qPCR was performed to identify the strains with high interference for further experiments.

RNA extraction, reverse transcription and qPCR

According to our previous study [68], qPCR was performed to analyze the mRNA expression levels of the *aox* gene and key antioxidant enzyme genes in the CCMSSC00389 strain and *aox*-transformed strains subjected to the different treatments. The *β-actin* and *β-tubulin* genes were used as internal reference genes, and the relative gene expression was determined according to the $2^{-\Delta\Delta CT}$ method. The qPCR amplification program was as follows: 95 °C for 3 min, 40 cycles of 95 °C for 3 s and 60 °C for 32 s, and a final extension at 72 °C for 30 s.

Statistical analysis

GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) was used for the statistical analyses. The values are reported as the means $\pm$ SEs and were analyzed by one-way ANOVA, with a *P* value <0.05 was considered significant.

Declarations

Acknowledgments

We thank all the members of the research group for their cooperation in the laboratory.

Authors' contributions

LDH conceived, designed and performed the experiments, analyzed the data, wrote and revised the manuscript. MRZ collaborated in bioinformatics analysis and in the manuscript writing. QH carried out part of experiments. CYH and LJZ designed and revised the manuscript. JXZ conceived and designed the experiments. All authors have read and approved the final manuscript.

Funding

This work was supported by the earmarked fund for China Agriculture Research System (CARS20), National Basic Research Program of China (No. 2014CB138303) and the Fundamental Research Funds for Central Nonprofit Scientific Institution (No.1610132020004).

Availability of data and materials

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Jaworska G, Bernas E, Mickowska B. Effect of production process on the amino acid content of frozen and canned *Pleurotus ostreatus* mushrooms. Food Chemistry. 2011, 125:936-943.
2. Yan ZY, Zhao MR, Wu XL, Zhang JX. Metabolic response of *Pleurotus ostreatus* to continuous heat stress. Frontiers in Microbiology. 2020, 10:3148.
3. Song C, Chen Q, Wu XL, Zhang JX, Huang CY. Heat stress induces apoptotic-like cell death in two *Pleurotus* species. Current Microbiology. 2014, 69:611-616.
4. Lei M, Wu XL, Huang CY, Qiu ZH, Zhang JX. Trehalose induced by reactive oxygen species relieved the radial growth defects of *Pleurotus ostreatus* under heat stress. Applied Microbiology Biotechnology. 2019, 103:5379-5390.
5. Peto A, Lehotai N, Feigl G, Tugyi N, Ordog A, Gemes K, Tari I, Erdei L, Kolbert Z. Nitric oxide contributes to copper tolerance by influencing ROS metabolism in *Arabidopsis*. Plant Cell Reports. 2013, 32:1913-1923.
6. Singh R, Parihar P, Prasad SM. Interplay of calcium and nitric oxide in improvement of growth and arsenic-induced toxicity in mustard seedlings. Scientific Reports. 2020, 10:6900.
7. Gu RX, Zhu SH, Zhou J, Liu N, Shi JY. Inhibition on brown rot disease and induction of defence response in harvested peach fruit by nitric oxide solution. European Journal of Plant Pathology. 2014, 139:369-378.
8. Song LL, Ding W, Zhao MG, Sun BT, Zhang LX. Nitric oxide protects against oxidative stress under heat stress in the calluses from two ecotypes of reed. Plant Science. 2006, 171:449-458.
9. Peto A, Lehotai N, Lozano-Juste J, Leon J, Tari I, Erdei L, Kolbert Z. Involvement of nitric oxide and auxin in signal transduction of copper-induced morphological responses in *Arabidopsis* seedlings. Annals of Botany. 2011, 108:449-457.
10. Liu R, Shi L, Zhu T, Yang T, Ren A, Zhu J, Zhao MW. Cross-talk between nitric oxide and calcium-calmodulin regulate ganoderic acid biosynthesis in *Ganoderma lucidum* under heat stress. Applied and Environmental Microbiology. 2018, 84:e00043-00018.
11. Hou LD, Zhao MR, Huang CY, Wu XL, Zhang JX. Nitric oxide improves the tolerance of *Pleurotus ostreatus* to heat stress by inhibiting mitochondrial aconitase. Applied and Environmental Microbiology. 2020, 86:e02303-02319.
12. Rogov AG, Zvyagilskaya RA. Physiological role of alternative oxidase (from yeasts to plants). Biochemistry. 2015, 80:400-407.
13. Siedow JN, Umbach AL. The mitochondrial cyanide-resistant oxidase: structural conservation amid regulatory diversity. Biochimica et Biophysica Acta. 2000, 1459:432-439.
14. Chae MS, Lin CC, Kessler KE, Nargang CE, Tanton LL, Hahn LB, Nargang FE. Identification of an alternative oxidase induction motif in the promoter region of the *aod-1* gene in *Neurospora crassa*. Genetics. 2007, 175:1597-1606.
15. Del-Saz NF, Ribas-Carbo M, McDonald AE, Lambers H, Fernie AR, Florez-Sarasa I. An in vivo perspective of the role(s) of the alternative oxidase pathway. Trends in Plant Science. 2018, 23:206-219.

16. Maxwell DP, Wang Y, McIntosh L. The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *PNAS*. 1999, 96:8271-8276.
17. Popov VN. Possible role of free oxidation processes in the regulation of reactive oxygen species production in plant mitochondria. *Biochemical Society Transactions*. 2003, 31:1316-1317.
18. Rhoads DM, Subbaiah CC. Mitochondrial retrograde regulation in plants. *Mitochondrion*. 2007, 7:177-194.
19. Vanlerberghe GC, Martyn GD, Dahal K. Alternative oxidase: a respiratory electron transport chain pathway essential for maintaining photosynthetic performance during drought stress. *Physiol Plant*. 2016, 157:322-337.
20. Jian W, Zhang DW, Zhu F, Wang SX, Pu XJ, Deng XG, Luo SS, Lin HH. Alternative oxidase pathway is involved in the exogenous SNP-elevated tolerance of *Medicago truncatula* to salt stress. *Journal of Plant Physiology*. 2016, 193:79-87.
21. Borovik OA, Grabelnych OI. Mitochondrial alternative cyanide-resistant oxidase is involved in an increase of heat stress tolerance in spring wheat. *Journal of Plant Physiology*. 2018, 231:310-317.
22. Luevano Martinez LA, Caldeira da Silva CC, Nicastro GG, Schumacher RI, Kowaltowski AJ, Gomes SL. Mitochondrial alternative oxidase is determinant for growth and sporulation in the early diverging fungus *Blastocladiella emersonii*. *Fungal Biology*. 2019, 123:59-65.
23. Magnani T, Soriani FM, Martins VdP, Policarpo ACdF, Sorgi CA, Faccioli LH, Curti C, Uyemura SA. Silencing of mitochondrial alternative oxidase gene of *Aspergillus fumigatus* enhances reactive oxygen species production and killing of the fungus by macrophages. *Journal of Bioenergetics and Biomembranes*. 2008, 40:631-636.
24. Vanlerberghe GC. Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *International Journal of Molecular Sciences*. 2013, 14:6805-6847.
25. McIntosh V. Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. *Plant Physiology*. 1996, 111:589-595.
26. Fouquerel E, Sobol RW. ARTD1 (PARP1) activation and NAD(+) in DNA repair and cell death. *DNA Repair (Amst)*. 2014, 23:27-32.
27. Tamai S, Iida H, Yokota S, Sayano T, Kiguchiya S, Ishihara N, Hayashi JI, Mihara K, Oka T. Characterization of the mitochondrial protein LETM1, which maintains the mitochondrial tubular shapes and interacts with the AAA-ATPase BCS1L. *Journal of Cell Science*. 2008, 121:2588-2600.
28. Yao X, Li JJ, Liu JP, Liu K. An *Arabidopsis* mitochondria-localized RRL protein mediates abscisic acid signal transduction through mitochondrial retrograde regulation involving ABI4. *Journal of Experimental Botany*. 2015, 66:6431-6445.
29. Saha B, Borovskii G, Panda SK. Alternative oxidase and plant stress tolerance. *Plant Signal Behav*. 2016, 11:e1256530.
30. Li H, Chang JJ, Zheng JX, Dong YC, Liu QY, Yang XZ, Wei CH, Zhang Y, Ma JX, Zhang X. Local melatonin application induces cold tolerance in distant organs of *Citrullus lanatus* L. via long

- distance transport. *Scientific Reports*. 2017, 7:40858.
31. Qu JB, Zhao MY, Tom H, Feng X, Zhang JX, Huang CY. Identification and characterization of small noncoding RNAs in genome sequences of the edible fungus *Pleurotus ostreatus*. *Biomed Research International*. 2016, 2016:1-9.
 32. Schonbaum G, Bonner W, Storey B, Bahr J. Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. *Plant Physiology*. 1971 47:124-128.
 33. Jarmuszkiewicz W, Sluse-Goffart CM, Vercesi AE, Sluse FE. Alternative oxidase and uncoupling protein: thermogenesis versus cell energy balance. *Bioscience Reports*. 2001, 21:213-222.
 34. Lorenzo L, Carlos G-M, Magdalena G, Gabriela P. Nitric oxide: the versatility of an extensive signal molecule. *Annual Review of Plant Biology*. 2003, 54:109-136.
 35. Knott AB, Bossy-Wetzel E. Nitric oxide in health and disease of the nervous system. *Antioxidants Redox Signaling*. 2009, 11:541-554.
 36. Fancy NN, Bahlmann AK, Loake GJ. Nitric oxide function in plant abiotic stress. *Plant Cell and Environment*. 2016, 40:462-472.
 37. Baidya S, Cary JW, Grayburn WS, Calvo AM. Role of nitric oxide and flavohemoglobin homolog genes in *Aspergillus nidulans* sexual development and mycotoxin production. *Applied and Environmental Microbiology*. 2011, 77:5524-5528.
 38. Prats E, Carver TL, Mur LA. Pathogen-derived nitric oxide influences formation of the appressorium infection structure in the phytopathogenic fungus *Blumeria graminis*. *Research in Microbiology*. 2008, 159:476-480.
 39. Gong XY, Fu YP, Jiang DH, Li GQ, Yi XH, Peng YL. L-arginine is essential for conidiation in the filamentous fungus *Coniothyrium minitans*. *Fungal Genetics and Biology*. 2007, 44:1368-1379.
 40. Dong JF, Zhang M, Lu L, Sun LN, Xu MJ. Nitric oxide fumigation stimulates flavonoid and phenolic accumulation and enhances antioxidant activity of mushroom. *Food Chemistry*. 2012, 135:1220-1225.
 41. Kong WW, Huang CY, Chen Q, Zou YJ, Zhang JX. Nitric oxide alleviates heat stress-induced oxidative damage in *Pleurotus eryngii* var. *tuoliensis*. *Fungal Genetics and Biology*. 2012, 49:0-20.
 42. Yu Y, Yang ZJ, Guo K, Li Z, Zhou HZ, Wei Y, Li JS, Zhang XJ, Harvey P, Yang HT. Oxidative damage induced by heat stress could be relieved by nitric oxide in *Trichoderma harzianum* LTR-2. *Current Microbiology*. 2015, 70:618-622.
 43. Hasanuzzaman M, Nahar K, Hossain MS, Anee TI, Parvin K, Fujita M. Nitric oxide pretreatment enhances antioxidant defense and glyoxalase systems to confer PEG-induced oxidative stress in rapeseed. *Journal of Plant Interactions*. 2017, 12:323-331.
 44. Rahimian Boogar A, Salehi H, Jowkar A. Exogenous nitric oxide alleviates oxidative damage in turfgrasses under drought stress. *South African Journal of Botany*. 2014, 92:78-82.
 45. Domingos P, Prado AM, Wong A, Gehring C, Feijo JA. Nitric oxide: a multitasked signaling gas in plants. *Molecular Plant*. 2015, 8:506-520.

46. Wahid A, Gelani S, Ashraf M, Foolad M. Heat tolerance in plants: an overview. *Environmental and Experimental Botany*. 2007, 61:199-223.
47. Moller IM. Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annual Review of Plant Physiology and Plant Molecular Biology*. 2001, 52:561-591.
48. Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*. 2012, 2012:1-26.
49. Mittler R, Finka A, Goloubinoff P. How do plants feel the heat? *Trends in Biochemical Sciences*. 2012, 37:118-125.
50. Liu R, Zhu T, Yang T, Yang ZY, Ren A, Shi L, Zhu J, Yu HS, Zhao MW. Nitric oxide regulates ganoderic acid biosynthesis by the S-nitrosylation of aconitase under heat stress in *Ganoderma lucidum*. *Environmental Microbiology*. 2020.
51. Karpets YV, Kolupaev YE, Yastreb TO. Effect of sodium nitroprusside on heat resistance of wheat coleoptiles: dependence on the formation and scavenging of reactive oxygen species. *Russian Journal of Plant Physiology*. 2011, 58:1027-1033.
52. Song LL, Yue LL, Zhao HQ, Hou MF. Protection effect of nitric oxide on photosynthesis in rice under heat stress. *Acta Physiologiae Plantarum*. 2013, 35:3323-3333.
53. Hirayama T, Shinozaki K. Research on plant abiotic stress responses in the post-genome era: past, present and future. *The Plant Journal*. 2010, 61:1041-1052.
54. Wu ZJ, Li XH, Liu ZW, Li H, Wang YX, Zhuang J. Transcriptome-based discovery of AP2/ERF transcription factors related to temperature stress in tea plant (*Camellia sinensis*). *Funct Integr Genomics*. 2015, 15:741-752.
55. Tang S, Dong Y, Liang D, Zhang ZJ, Ye CY, Shuai P, Han X, Zhao Y, Yin W, Xia XL. Analysis of the drought stress-responsive transcriptome of black cottonwood (*Populus trichocarpa*) using deep RNA sequencing. *Plant Molecular Biology Reporter*. 2014, 33:424-438.
56. Fu LJ, Shi K, Gu M, Zhou YH, Dong DK, Liang WS, Song FM, Yu JQ. Systemic induction and role of mitochondrial alternative oxidase and nitric oxide in a compatible tomato–*Tobacco mosaic virus* interaction. *MPMI*. 2010, 23:39-48.
57. Huang X, von Rad U, Durner J. Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in *Arabidopsis* suspension cells. *Planta*. 2002, 215:914-923.
58. Sun YJ, Liu XH, Zhai H, Gao HY, Yao YX, Du YP. Responses of photosystem II photochemistry and the alternative oxidase pathway to heat stress in grape leaves. *Acta Physiologiae Plantarum*. 2016, 38:232.
59. Analin B, Mohanan A, Bakka K, Challabathula D. Cytochrome oxidase and alternative oxidase pathways of mitochondrial electron transport chain are important for the photosynthetic performance of pea plants under salinity stress conditions. *Plant Physiology and Biochemistry*. 2020, 154:248-259.

60. Wang J, Vanlerberghe GC. A lack of mitochondrial alternative oxidase compromises capacity to recover from severe drought stress. *Physiologia Plantarum*. 2013, 149:461-473.
61. Vishwakarma A, Kumari A, Mur LAJ, Gupta KJ. A discrete role for alternative oxidase under hypoxia to increase nitric oxide and drive energy production. *Free Radical Biology and Medicine*. 2018, 122:40-51.
62. Poyton RO, Ball KA, Castello PR. Mitochondrial generation of free radicals and hypoxic signaling. *Trends in Endocrinology and Metabolism*. 2009, 20:332-340.
63. Giraud E, Van Aken O, Ho LH, Whelan J. The transcription factor ABI4 is a regulator of mitochondrial retrograde expression of alternative oxidase1a. *Plant Physiology*. 2009, 150:1286-1296.
64. Rustin P, Jacobs HT. Respiratory chain alternative enzymes as tools to better understand and counteract respiratory chain deficiencies in human cells and animals. *Physiologia Plantarum*. 2009, 137:362–370.
65. Zhang RY, Hu DD, Zhang YY, Goodwin PH, Huang CY, Chen Q, Gao W, Wu XL, Zou YJ, Qu JB, Zhang JX. Anoxia and anaerobic respiration are involved in “spawn-burning” syndrome for edible mushroom *Pleurotus eryngii* grown at high temperatures. *Scientia Horticulturae*. 2016, 199:75-80.
66. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 2011, 12:323.
67. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Biogeosciences*. 2010, 26:139-140.
68. Wang LN, Wu XL, Gao W, Zhao MR, Zhang JX, Huang CY. Differential expression patterns of *Pleurotus ostreatus* catalase genes during developmental stages and under heat stress. *Genes*. 2017, 8:335.

Tables

Table 1. List of NO-regulated genes under HS related to the respiration.

Gene ID	Gene Description
g3097	Succinate dehydrogenase iron-sulfur subunit
g6590	-
g11376	Alternative oxidase
g12148	Hypothetical protein PLEOSDRAFT_1096427 (<i>P. ostreatus</i> PC15)
g12952	Mitochondrial chaperone bcs1
g10914	-

Figures

Figure 1

The addition of exogenous NO reduced the degree of mitochondrial damage and the accumulation of ROS in mycelia under HS. A. Effect of exogenous NO on mycelial growth after HS. The red lines represent the radius of mycelial growth throughout the growth period, and the blue lines represent the radius of mycelial growth during recovery from HS (40 °C for 48 h). The red arrow points to mycelia that experienced growth recovery. B. Effect of exogenous NO on the H₂O₂ content in mycelia after HS. C. O₂⁻ content. D. O₂⁻ production rate. E. NADH content. F. NAD⁺/NADH ratio. G. Total respiratory rate. H. ATP content. The values are presented as the means ± SEs from three independent experiments. Different letters indicate significant differences among the samples ($P < 0.05$ according to Duncan's test).

Figure 2

Number of up and downregulated genes among the CK, HS, SNP (SNP_HS) and cPTIO (cPTIO_HS) groups.

Figure 3

Functional classification of GO terms assigned to the DEGs. A. Upregulated DEGs identified from the SNP_cPTIO comparison. B. Downregulated DEGs identified from the SNP_cPTIO comparison. The y-axis on the left represents the percentage of DEGs assigned the GO term, and the y-axis on the right shows the number of DEGs.

Figure 4

Significantly enriched pathways obtained for the DEGs and heatmap of oxidoreductase activity. A. Significantly enriched pathways obtained for the DEGs identified from the SNP_cPTIO comparison. B. Heatmap of the antioxidant system (oxidation-reduction process and oxidoreductase activity) consisting of the DEGs identified from the SNP_cPTIO comparison.

Figure 5

Heatmap of NO-regulated genes in the respiratory chain under HS.

Figure 6

NO induced aox gene expression in *P. ostreatus* under HS. A. Expression of the aox gene after exposure to HS for different times. B. Effect of NO on the expression of the aox gene under HS. The values are presented as the means $\pm$ SEs from three independent experiments. Different letters indicate significant differences among the samples ($P < 0.05$ according to Duncan's test).

Figure 7

aox affects ROS production and promotes mycelial growth by regulating the respiratory pathway under HS. A. Recovery of WT, OE-aox and RNAi-aox strains after HS. The red lines represent the radius of

mycelial growth throughout the growth period, and blue lines represent the radius of mycelial growth during 48 h of recovery after HS. B. H₂O₂ content. C. O₂⁻ content. D. O₂⁻ production rate. E. NADH content. F. NAD⁺/NADH ratio. G. ATP content. The values are presented as the means ± SEs from three independent experiments. Different letters indicate significant differences among the samples (P<0.05 according to Duncan's test).

Figure 8

Expression of key antioxidant enzyme-encoding genes in *aox*-transformed strains under HS. A. Relative expression of *cat1*. B. Relative expression of *cat2*. C. Relative expression of *TrxR*. D. Relative expression of *GSH-PX*. E. Relative expression of *SOD1*. F. Relative expression of *SOD2*. G. Relative expression of *SOD3*. H. Relative expression of *SOD4*. The values are presented as the means ± SEs from three independent experiments. Different letters indicate significant differences among the samples (P<0.05 according to Duncan's test).

Figure 9

Exogenous BHAM regulates the expression of antioxidant enzymes under HS. A. Exogenous BHAM inhibited the growth of mycelia after HS. The red arrow points to the mycelia that experienced growth recovery. B. H₂O₂ content. C. O₂⁻ content. D. O₂⁻ production rate. E. Relative expression of *cat1*. F. Relative expression of *cat2*. G. Relative expression of *TrxR*. H. Relative expression of *GSH-PX*. I. Relative expression of *SOD1*. J. Relative expression of *SOD2*. K. Relative expression of *SOD3*. L. Relative

expression of SOD4. The values are presented as the means $\pm$ SEs from three independent experiments. Different letters indicate significant differences among the samples ($P < 0.05$ according to Duncan's test).

Figure 10

Schematic representation of the mechanism through which NO alleviates ROS production by inducing *aox* gene expression under HS. I, II, III, IV, and V: Complexes 1-5, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementaryinformation.docx](#)
- [Additionalfile1.docx](#)