AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

PHYSIOLOGY

Ornithine Decarboxylase-Mediated Production of Putrescine Influences Ganoderic Acid Biosynthesis by Regulating Reactive Oxygen Species in *Ganoderma lucidum*

Chen-Gao Wu,[a,b] Jia-Long Tian,[a,b] Rui Liu,[a,b] Peng-Fei Cao,[a,b] Tian-Jun Zhang,[a,b] Ang Ren,[a,b] Liang Shi,[a,b] Ming-Wen Zhao[a,b]

Key Laboratory of Agricultural Environmental Microbiology, Ministry of Agriculture, Nanjing Agricultural University, Nanjing, Jiangsu, People's Republic of China[a]; Department of Microbiology, College of Life Sciences, Nanjing Agricultural University, Nanjing, Jiangsu, People's Republic of China[b]

ABSTRACT Putrescine is an important polyamine that participates in a variety of stress responses. Ornithine decarboxylase (ODC) is a key enzyme that catalyzes the biosynthesis of putrescine. A homolog of the gene encoding ODC was cloned from *Ganoderma lucidum*. In the *ODC*-silenced strains, the transcript levels of the *ODC* gene and the putrescine content were significantly decreased. The *ODC*-silenced strains were more sensitive to oxidative stress. The content of ganoderic acid was increased by approximately 43 to 46% in the *ODC*-silenced strains. The content of ganoderic acid could be recovered after the addition of exogenous putrescine. Additionally, the content of reactive oxygen species (ROS) was significantly increased by approximately 1.3-fold in the *ODC*-silenced strains. The ROS content was significantly reduced after the addition of exogenous putrescine. The gene transcript levels and the activities of four major antioxidant enzymes were measured to further explore the effect of putrescine on the intracellular ROS levels. Further studies showed that the effect of the ODC-mediated production of putrescine on ROS might be a factor influencing the biosynthesis of ganoderic acid. Our study reports the role of putrescine in large basidiomycetes, providing a basis for future studies of the physiological functions of putrescine in microbes.

IMPORTANCE It is well known that ODC and the ODC-mediated production of putrescine play an important role in resisting various environmental stresses, but there are few reports regarding the mechanisms underlying the effect of putrescine on secondary metabolism in microorganisms, particularly in fungi. *G. lucidum* is gradually becoming a model organism for studying environmental regulation and metabolism. In this study, a homolog of the gene encoding ODC was cloned in *Ganoderma lucidum*. We found that the transcript level of the *ODC* gene and the content of putrescine were significantly decreased in the *ODC*-silenced strains. The content of ganoderic acid was significantly increased in the *ODC*-silenced strains. Further studies showed that the effect of the ODC-mediated production of putrescine on ROS might be a factor influencing the biosynthesis of ganoderic acid. Our study reports the role of putrescine in large basidiomycetes, providing a basis for future studies of the physiological functions of putrescine in microbes.

KEYWORDS *Ganoderma lucidum*, ODC, putrescine, ganoderic acid, ROS

Received 9 June 2017 **Accepted** 7 August 2017

Accepted manuscript posted online 11 August 2017

Citation Wu C-G, Tian J-L, Liu R, Cao P-F, Zhang T-J, Ren A, Shi L, Zhao M-W. 2017. Ornithine decarboxylase-mediated production of putrescine influences ganoderic acid biosynthesis by regulating reactive oxygen species in *Ganoderma lucidum*. Appl Environ Microbiol 83:e01289-17. https://doi.org/10.1128/AEM.01289-17.

Editor Daniel Cullen, USDA Forest Products Laboratory

Address correspondence to Ming-Wen Zhao, mwzhao@njau.edu.cn.

Ganoderma lucidum, which is one of the most widely cultivated medicinal basidiomycetes in East Asia, contains a variety of bioactive substances, including ganoderic acid (GA), polysaccharides, fatty acids, and other organic compounds (1, 2). GA is one of the main secondary metabolites of *G. lucidum* and is synthesized via the mevalonate pathway, in which acetyl-coenzyme A (acetyl-CoA) is converted through a series of chemical reactions into intermediates and then finally to GA (3, 4). Pharmacological studies have shown that GA has antiplatelet aggregation effects (5) and the ability to lower cholesterol levels (6). Furthermore, *G. lucidum* is an important white rot fungus. *G. lucidum* has attracted broad attention and research interest in recent years due to its important applications (3, 7, 8). However, the level of basic research on *G. lucidum* is insufficient. In recent years, the complete genome sequence, a genetic transformation system, and reliable reverse genetic methods for *G. lucidum* have been published (9–11). With the application of these developed genetic tools, *G. lucidum* is gradually becoming a model organism for the study of environmental regulation and metabolism.

Ornithine decarboxylase is a key enzyme in the polyamine biosynthetic pathway (12). Previous studies indicate that ornithine decarboxylase (ODC) is related to the stress response. For example, upon oxidative stress, the transcript of the *ODC* gene increased significantly in murine papilloma phosphatidylethanolamine (PE) cells (13). In human hepatoma HUH7 cells, chemically induced oxidative stress can activate the transcript of the *ODC* gene, which is concomitant with an increase in polyamine content (14). These results suggest that the *ODC* gene plays an important role in the stress response in organisms. The effect of ODC is mediated by its production of putrescine. Putrescine, which is a product of ODC, is an important type of polyamine (15). Studies have shown that putrescine can participate in the regulation of growth and metabolism in organisms (16). In plants, putrescine is highly involved in the response to environmental stress. In *Arabidopsis*, the accumulation of putrescine can improve tolerance to dehydration and freezing stress (17). Putrescine treatments increase the intracellular titer of putrescine, which modulates the tolerance of *Nicotiana tabacum* to osmotic stress (18). Putrescine also affects metabolism. Exogenous putrescine alleviates the feedback inhibition of photosynthesis in NaCl-stressed plants by promoting sucrose transport and regulating the activities of enzymes involved in carbohydrate metabolism (19). In animals, putrescine supplementations promote the proliferation of porcine trophectoderm cells, which is mediated by increased protein synthesis via the activation of the mechanistic target of the rapamycin (mTOR) signaling pathway (20). Fewer studies exploring the function of putrescine have been performed in microorganisms than in animals and plants (21). These studies found that the function of putrescine in microorganisms is also related to the environmental stress response. In *Burkholderia cenocepacia*, putrescine can reduce antibiotic-induced oxidative stress (22). Furthermore, putrescine has other reported functions. In *Shewanella oneidensis*, disruption of the biosynthesis of putrescine can enhance biofilm cohesiveness (23). Studies exploring the role of putrescine in fungi are also very limited. Putrescine may play a role in the protection of *Ustilago maydis* against salt and osmotic stress (24). These studies suggest that ODC and the ODC-mediated production of putrescine play an important role in biological growth and metabolism.

To investigate the role of ODC and the effect of its production of putrescine on the growth and secondary metabolism of *G. lucidum*, we cloned the *ODC* gene in *G. lucidum* and used RNA interference (RNAi) technology to construct *ODC*-silenced strains. We found a decrease in the putrescine content and reactive oxygen species (ROS) and an increase in the GA content in the *ODC*-silenced strains compared with those in the wild-type (WT) and empty vector control (CK1 and CK2) strains. Further mechanistic studies indicated that the *ODC* gene influences the biosynthesis of GA by regulating the levels of ROS via the ODC-mediated production of putrescine. Our study will help others better understand the roles ODC and ODC-mediated putrescine production play in fungi.

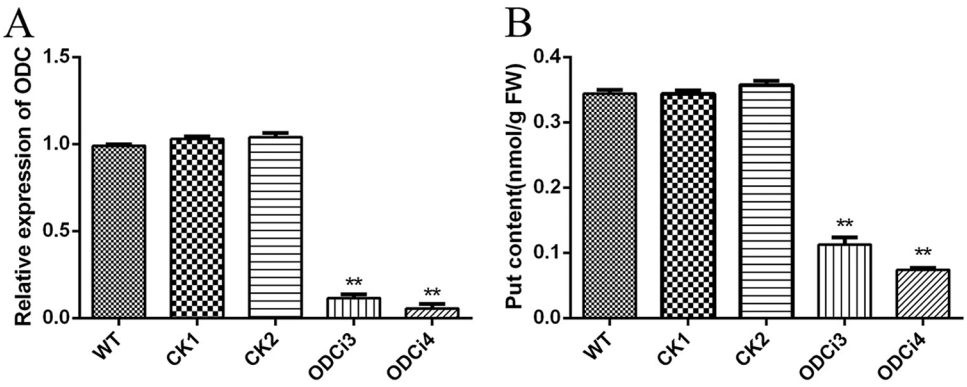

FIG 1 Detection of *ODC* transcript levels and putrescine (Put) content in the *ODC*-silenced strains. (A) Seven-day-old liquid mycelia from control (WT, CK1, and CK2) and ODC-silenced strains were collected to detect the relative mRNA level of the *ODC* gene in *G. lucidum*. The expression level of the *ODC* gene in the WT strain was arbitrarily set to 1. (B) Seven-day-old liquid mycelia from control (WT, CK1, and CK2) and *ODC*-silenced strains were collected to measure the putrescine content. Values are the means ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the control (WT, CK1, and CK2) strains (**, $P < 0.01$, Student's *t* test). FW, fresh weight.

RESULTS

Cloning and analysis of the *ODC* gene. To identify the putative *ODC* genes in *G. lucidum*, the *Dichomitus squalens* (GenBank accession no. XP_007368085.1) *ODC* gene was subjected to a BLAST search against the *G. lucidum* genome database (*G. lucidum* strain 260125-1, NCBI taxid: 1077286) (11). By performing a homology comparison, a homologous gene (GL17083-R1) was found. cDNA primers and genomic DNA primers were designed for the *ODC* gene, and the full-length cDNA and genomic DNA of the *ODC* gene were cloned by PCR (Table 1). The *ODC* genomic sequence was 1,823 bp and contained 2 exons (see Fig. S1A in the supplemental material). The length of the *ODC* cDNA was 1,629 bp and was predicted to encode a protein of 543 amino acid residues.

The domain prediction program SMART found that ODC contains the following two distinct domains, as shown in Fig. S2: an ornithine and arginine decarboxylase domain (Orn_Arg_deC-N, amino acids [aa] 116 to 353) and an ornithine decarboxylase C-terminal sheet domain (Orn_DAP_Arg_deC, aa 357 to 518). These domains are referred to as group IV decarboxylases, which are pyridoxal dependent and act on ornithine, lysine, and arginine. These results implied that the cloned gene belonged to the ODC family. By performing a phylogenetic analysis, we found that the ODCs from different living organisms, including plants, animals, and fungi, all have high homology with *G. lucidum*. As expected, the predicted *G. lucidum* ODC amino acid sequence was most similar to that of other ODCs of basidiomycetes, such as *Dichomitus squalens* (79%) and *Phanerochaete carnosa* (65%) (Fig. S1B).

Construction of the *G. lucidum ODC*-silenced strains and measurement of putrescine content. To explore the function of ODC in *G. lucidum*, *ODC*-silenced strains were constructed using a previously established dual-promoter system (Fig. S3) (9). We screened several positive transformants on hygromycin plates and then used quantitative real-time PCR (qRT-PCR) to determine the transcript levels of the *ODC* gene in the randomly selected resistant transformants. Based on the qRT-PCR analysis, we screened 15 independent positive transformants with a high silencing efficiency, and the phenotypes of these transformants are highly similar. The *ODCi3* and *ODCi4* transformants were randomly selected from the 15 transformants, and the transcript levels of these two *ODC*-silenced strains were decreased by 88% and 94%, respectively, compared with the WT strain (Fig. 1A). The two *ODCi* transformants were used in the subsequent gene function studies. The *ODC* gene is a key gene in the synthesis of putrescine; therefore, we measured the putrescine content in the two *ODC*-silenced strains. The putrescine content in *G. lucidum* was decreased by approximately 67% and 79% in the *ODCi3* and *ODCi4* mutant strains, respectively, compared with that in the WT strain (Fig. 1B). This

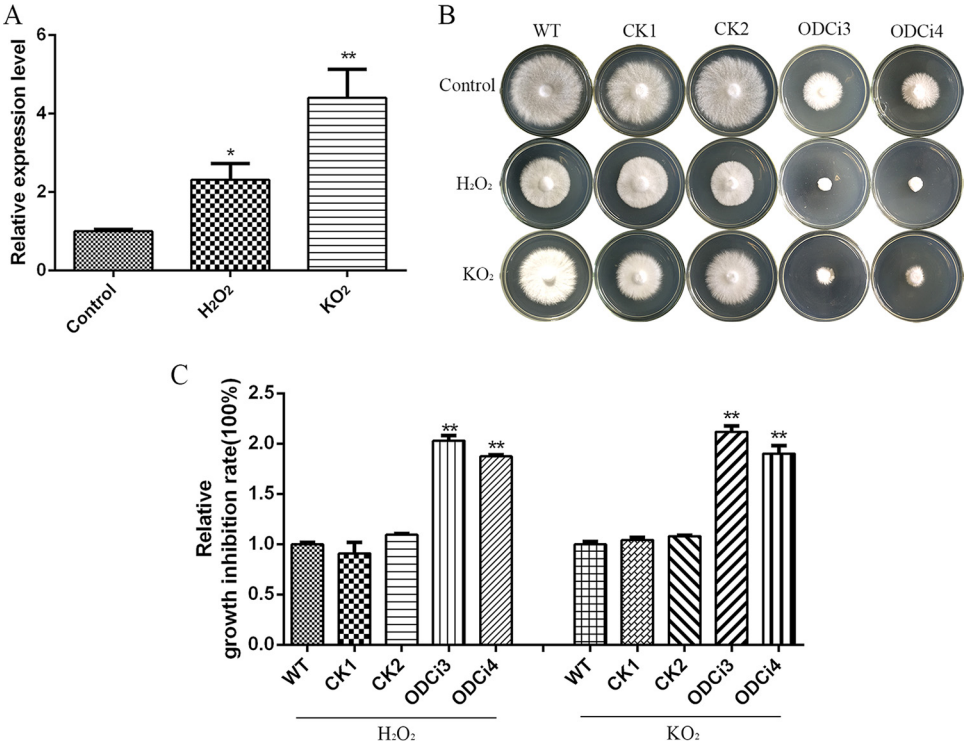

FIG 2 Phenotypic assays of the *ODC*-silenced strains exposed to oxidative stress and the relative mRNA level of the *ODC* gene in the WT strains. (A) The relative mRNA level of the *ODC* gene in the WT strains was measured after treatment with the oxidants (8 mM H_2O_2 and 3.5 mM KO_2). (B) *G. lucidum* strains were grown on a CYM solid agar medium at 28°C and treated with or without 8 mM H_2O_2 or 3.5 mM KO_2 for 5 days. (C) Quantitative analysis of the relative inhibitory rate in the control (WT, CK1, and CK2) and *ODC*-silenced strains under oxidative stress and antioxidative stress tolerance conditions. The growth inhibition rate in each strain was calculated as follows: [diameter (untreated) − diameter (treated)]/diameter (untreated). Then, the growth inhibition rate in the WT strains was arbitrarily set to 1, and the growth inhibition rates in all other strains were compared with those in the WT strains. Values are the means ± standard deviations (SD) of the results of three independent experiments. Asterisks indicate significant differences from the control (WT, CK1, and CK2) strains (*, $P < 0.05$; **, $P < 0.01$, Student's *t* test).

result was consistent with the trend of the *ODC* gene expression at the transcript level. The decrease in the putrescine content further indicated that the cloned gene was a functional *ODC* gene.

The *ODC* gene contributes to resistance against oxidative stress. In a previous study, putrescine was found to be involved in responses to environmental stress (18, 22, 25). Therefore, to further study the function of the *ODC* gene in *G. lucidum*, the transcript levels of the *ODC* gene were measured in *G. lucidum* following the addition of exogenous hydrogen peroxide (H_2O_2) and potassium superoxide (KO_2) (H_2O_2 and KO_2 are the most typical oxidants in cells under oxidative stress conditions). As shown in Fig. 2A, compared with the untreated *G. lucidum*, the transcript of the *G. lucidum ODC* gene increased by 1.3-fold and 3.4-fold, respectively, after the addition of exogenous H_2O_2 or KO_2. This result is consistent with the trends of the results of *CYP*, *PP2A*, and *TIF* as the internal reference genes to detect *ODC* transcript levels (Fig. S4). These results implied that the *ODC* gene may play a role in the regulation of *G. lucidum* under oxidative stress. We further studied the relative rate of growth inhibition in the *ODC*-silenced strains under H_2O_2 and KO_2 stress. As shown in Fig. 2B and C, the results showed that *G. lucidum* was more sensitive to the exogenous H_2O_2 and KO_2 when *ODC* gene expression was silenced. Compared with the WT strain, the relative growth inhibition was increased by approximately 100% and 87% in the *ODCi3* and *ODCi4* mutant strains, respectively, after the addition of 8 mM exogenous H_2O_2 and increased by approximately 110% and 90% in the *ODCi3* and *ODCi4* mutant strains, respectively, after the addition of 3.5 mM exogenous KO_2. These results suggest that the *ODC* gene plays an important role in oxidative stress resistance in *G. lucidum*.

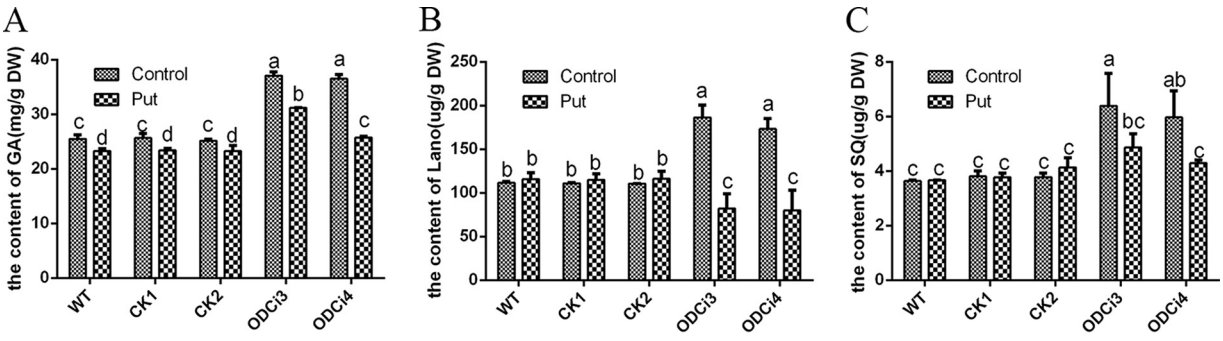

FIG 3 The effect of the putrescine treatment on GA biosynthesis. (A) Measurement of the total GA content in the control (WT, CK1, and CK2) and *ODC*-silenced strains with or without the putrescine treatment in *G. lucidum*. (B and C) The cellular levels of lanosterol (Lano) (B) and the cellular level of squalene (SQ) (C) in these strains with or without the 1 mM putrescine treatment. Three repeated experiments were performed with similar results. The values are the means ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$, according to Duncan's multiple-range test). DW, dry weight.

GA content is increased in the *ODC*-silenced strains and can be reverted by the addition of exogenous putrescine. GA is an important pharmacologically active compound that is synthesized by the mevalonate (MVA) pathway in *G. lucidum* (3). To study the effect of the *ODC* gene on GA biosynthesis, the GA content in the *ODC*-silenced strains was measured. Compared with the WT strain, the GA content in both *ODC*-silenced strains was significantly increased. As shown in Fig. 3A, the GA content increased by approximately 46% and 43% in the *ODCi3* and *ODCi4* mutant strains, respectively. Lanosterol and squalene are the two key intermediate metabolites of the GA biosynthesis pathway. The lanosterol content was increased by approximately 67% and 55% in the *ODCi3* and *ODCi4* mutant strains, respectively, compared with the content in the WT strain (Fig. 3B). The squalene content was increased by approximately 76% and 64% in the *ODCi3* and *ODCi4* mutant strains, respectively, compared with the content in the WT strain (Fig. 3C). These results suggest that the *ODC* gene affects the biosynthesis of GA.

The putrescine content decreased significantly in the *ODC*-silenced strains. We sought to determine whether the increased GA content was due to the reduced putrescine content in the *ODC*-silenced strains. Therefore, 1 mM putrescine dihydrochloride (1,4-diaminobutane dihydrochloride) was added to the *ODC*-silenced strains, and the GA content in the *ODC*-silenced strains was measured and compared with the GA content in the WT strain. We found that the GA content in the *ODC*-silenced strains was restored nearly to WT levels (Fig. 3A). The change in the contents of squalene and lanosterol, which are involved in GA biosynthesis, was consistent with the change in GA (Fig. 3B and C). These results suggest that the ODC-mediated production of putrescine is involved in the regulation of GA biosynthesis.

ROS are increased in the *ODC*-silenced strains and can be reverted by the addition of exogenous putrescine. Previous exploration has demonstrated the protective effects of putrescine against antibiotic-induced ROS formation in *Burkholderia cenocepacia* (22). Our earlier reports have also shown that the biosynthesis of GA is related to the levels of intracellular ROS (26, 27); therefore, we measured the levels of ROS in the *ODC*-silenced strains. An ROS fluorescent probe (2′,7′-dichlorodihydrofluorescein diacetate [DCFH-DA]) was used to determine whether the production of ROS was changed in the *ODC*-silenced strains. As shown in Fig. 4A and B, the fluorescence intensity analysis revealed that the ROS fluorescence value was upregulated by approximately 1.44-fold and 1.39-fold in the *ODCi3* and *ODCi4* mutant strains, respectively, compared with that in the WT strain. The intracellular H_2O_2 content was also quantitatively measured in the *ODC*-silenced strains (Fig. 4C) and was upregulated by approximately 65% and 60% in the *ODCi3* and *ODCi4* mutant strains, respectively. These results indicate that the *ODC*-silenced strains accumulated significantly more ROS than the WT strain. These findings are consistent with previous reports,

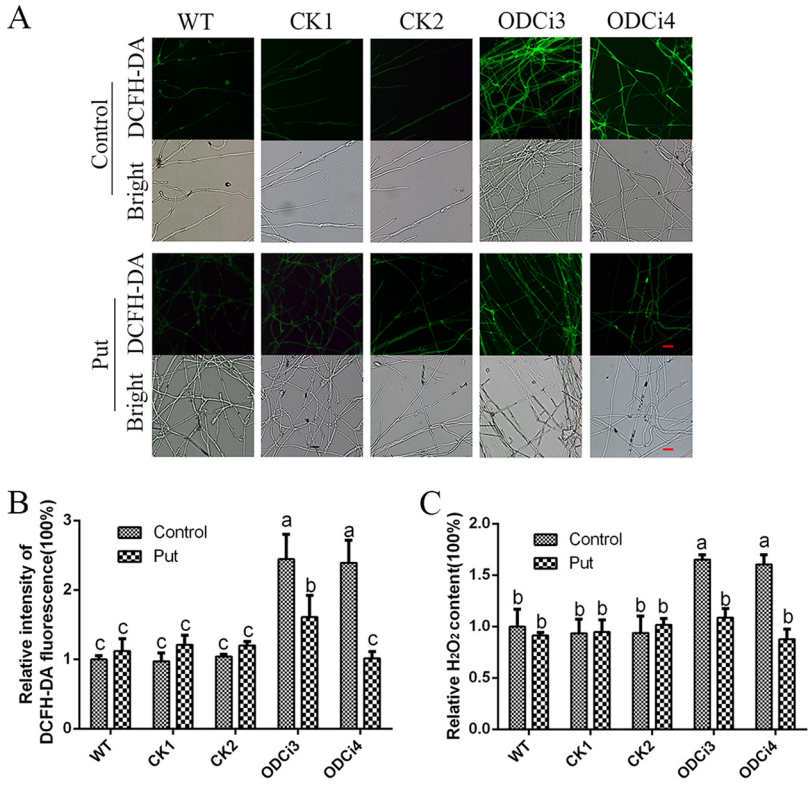

FIG 4 The ROS levels in *G. lucidum* after the putrescine treatment. (A) The control (WT, CK1, and CK2) and *ODC*-silenced *G. lucidum* strains were cultured at 28°C on a CYM solid agar medium with or without 1 mM putrescine for 4 days. The change in the ROS levels was measured by DCFH-DA staining after the 1 mM putrescine treatment. Scale bar = 100 μm. (B) Changes in the ROS fluorescence ratio in the hyphal regions of the control (WT, CK1, and CK2) and *ODC*-silenced strains with or without the 1 mM putrescine (Put) treatment. The *y* axis represents the ROS fluorescence ratio, as measured by confocal laser scanning microscopy (CLSM), and the *x* axis represents the different strains. The values in each column represent the captured fluorescence ratio in each observation. (C) Measurement of the amount of H_2O_2 in *G. lucidum* with or without the 1 mM putrescine treatment. The values are the means ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$, according to Duncan's multiple-range test).

suggesting that ODC is involved in the regulation of the ROS signaling pathway. To further explore the effect of ODC on ROS signaling, we wanted to know whether ROS signaling is related to the ODC-mediated production of putrescine. After the addition of 1 mM putrescine dihydrochloride, the ROS fluorescence value of the *ODC*-silenced strains decreased to WT levels (Fig. 4A and B). The intracellular H_2O_2 content also decreased to WT levels (Fig. 4C). These results indicate that the ODC-mediated production of putrescine affects the ROS content.

ODC-mediated production of putrescine affects the gene transcript levels and the activities of antioxidant enzymes. Under cold stress, putrescine treatments increase the antioxidant response in zucchini fruit (28). To explore the mechanism by which putrescine affects ROS in *G. lucidum*, we further assessed the gene transcript levels and the activities of four major antioxidant enzymes (i.e., catalase [CAT], superoxide dismutase [SOD], ascorbate peroxidase [APX], and glutathione peroxidase [GPX]). In our studies, there were no significant effects on the transcript levels of the *CAT1*, *SOD1*, and *SOD2* genes in the *ODCi3* and *ODCi4* mutant strains compared with those in the WT strain (Fig. 5A, C, and D). In contrast, the transcript level of the *CAT2* gene was upregulated by approximately 72% and 56%, and the *SOD4* gene was upregulated by approximately 30% and 55% in the *ODCi3* and *ODCi4* mutant strains, respectively (Fig. 5B and E). The transcript level of the *APX* gene was downregulated by approximately 54% and 60%, and the *GPX* gene was downregulated by approximately 55% and 50% in the *ODCi3* and *ODCi4* mutant strains, respectively (Fig. 5F and G). After the addition

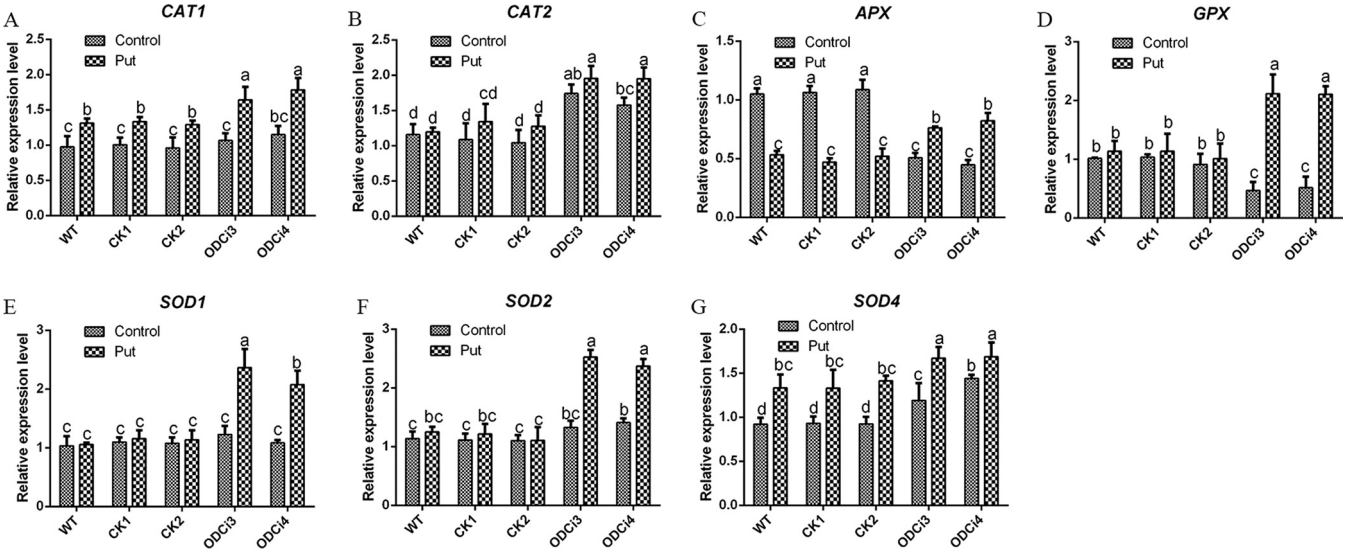

FIG 5 The effect of the putrescine treatment on the transcript levels of four major antioxidant enzyme-related genes in the *ODC*-silenced strains. Seven-day-old liquid mycelia from control (WT, CK1, and CK2) and *ODC*-silenced strains were collected to detect the relative mRNA level of four major antioxidant enzyme-related genes in *G. lucidum*. The transcript levels of the CAT-related (including *CAT1* [A] and *CAT2* [B]), SOD-related (including *SOD1* [C], *SOD2* [D], and *SOD4* [E]), APX-related [F], and GPX-related [G] genes were measured in strains treated without or with 1 mM putrescine (Put). The values are the means ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$, according to Duncan's multiple-range test).

of 1 mM putrescine dihydrochloride, the transcript levels of the *CAT1*, *CAT2*, *SOD1*, *SOD2*, *SOD4*, and *GPX* genes were all significantly upregulated in the *ODCi3* and *ODCi4* mutant strains compared with those in the WT strain (without the addition of 1 mM putrescine dihydrochloride) (Fig. 5A to E and G). However, there were no significant effects on the transcript level of the *APX* gene after the addition of putrescine in the *ODCi3* and *ODCi4* mutant strains (Fig. 5F). We further determined the activities of four major antioxidant enzymes. As shown in Fig. 6A and B, compared with the WT strains, the activity of CAT increased by approximately 38% and 23%, and the activity of SOD increased by approximately 16% and 12% in the *ODCi3* and *ODCi4* mutant strains, respectively. There was no significant change in the activity of APX (Fig. 6C). In contrast, the activity of GPX decreased by approximately 51% and 47% in the *ODCi3* and *ODCi4* mutant strains, respectively (Fig. 6D). After the addition of 1 mM putrescine dihydrochloride, the activities of CAT, SOD, and GPX were all significantly increased in the *ODCi3* and *ODCi4* mutant strains compared with those in the WT strain (without the addition of 1 mM putrescine dihydrochloride) (Fig. 6A, B, and D). However, the activity of APX was decreased by approximately 20% and 23% in the *ODCi3* and *ODCi4* mutant strains, respectively (Fig. 6C). These results imply that the ODC-mediated production of putrescine affects the intracellular antioxidant enzyme activity, which influences the ROS levels.

ODC-mediated production of putrescine affects the biosynthesis of GA by ROS. It has been reported that ROS signaling regulates the biosynthesis of GA following the application of exogenous methyl jasmonate (26) and hydrogen-rich water (27). To further determine whether the ODC-mediated production of putrescine affects the biosynthesis of GA via ROS, 5 mM *N*-acetyl cysteine (NAC; which is an ROS scavenger) was added to the CYM solid agar medium. As shown in Fig. 7A and B, the ROS fluorescence value of the *ODC*-silenced strains decreased to WT levels. The intracellular H_2O_2 content in the *ODC*-silenced strains also decreased to WT levels (Fig. 7C). To determine whether the increase in the GA content in the *ODC*-silenced strains was due to the increased ROS content, 5 mM NAC was added to the CYM liquid culture medium, and the GA content was measured. Compared with the GA content in the WT strain, the GA content in the *ODC*-silenced strains was restored nearly to WT levels (Fig. 8A), and the changes in the contents of lanosterol and squalene, which are involved in GA

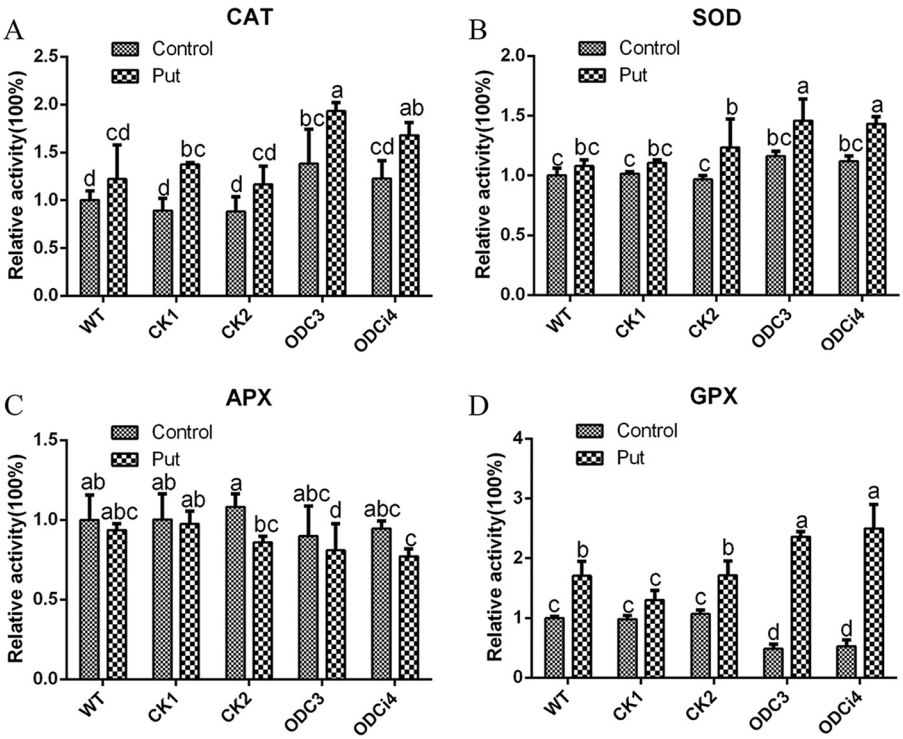

FIG 6 The effect of the putrescine treatment on four major antioxidant enzymes in the *ODC*-silenced strains. Seven-day-old liquid mycelia from control (WT, CK1, and CK2) and *ODC*-silenced strains were collected to detect the activities of four major antioxidant enzymes in *G. lucidum*. Measurement of the activities of CAT (A), SOD (B), APX (C), and GPX (D) in strains treated without or with 1 mM putrescine (Put). The values are the means ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$, according to Duncan's multiple-range test).

biosynthesis, were consistent with the change in GA (Fig. 8B and C). Furthermore, we measured the expression of genes encoding three key enzymes in GA biosynthesis, i.e., *hmgr*, *sqs*, and *osc*. The transcript levels of the *hmgr* gene were upregulated by approximately 120% and 77%, the transcript levels of the *sqs* gene were upregulated by approximately 62% and 91%, and the transcript levels of the *osc* gene were upregulated by approximately 44% and 30% in the *ODCi3* and *ODCi4* mutant strains, respectively, compared with those in the WT strain (Fig. 9A to C). As shown in Fig. 9A to C, compared with the WT strain (without the addition of 5 mM NAC), the transcript levels of the *hmgr*, *sqs*, and *osc* genes were all significantly downregulated in the *ODCi3* and *ODCi4* mutant strains following the addition of NAC. These results imply that the ODC-mediated production of putrescine regulates the ROS levels and then influences GA biosynthesis by affecting the expression of genes encoding key enzymes in GA biosynthesis.

DISCUSSION

Putrescine is an important type of polyamine. The *ODC* gene is required for the synthesis of putrescine. The *ODC* gene and putrescine play an important role in the response to various environmental stresses. To date, many studies have explored the *ODC* gene and putrescine in plants and animals, but very few studies have been conducted in fungi, and no studies have been performed in large basidiomycetes. In this paper, we report the cloning of the *ODC* gene in *G. lucidum* and a study of the function of the *ODC* gene and putrescine in *G. lucidum* using RNAi technology. Our results show that the *ODC* gene and putrescine negatively influence the biosynthesis of GA. This study investigated the functions of the *ODC* gene and putrescine in large basidiomycetes.

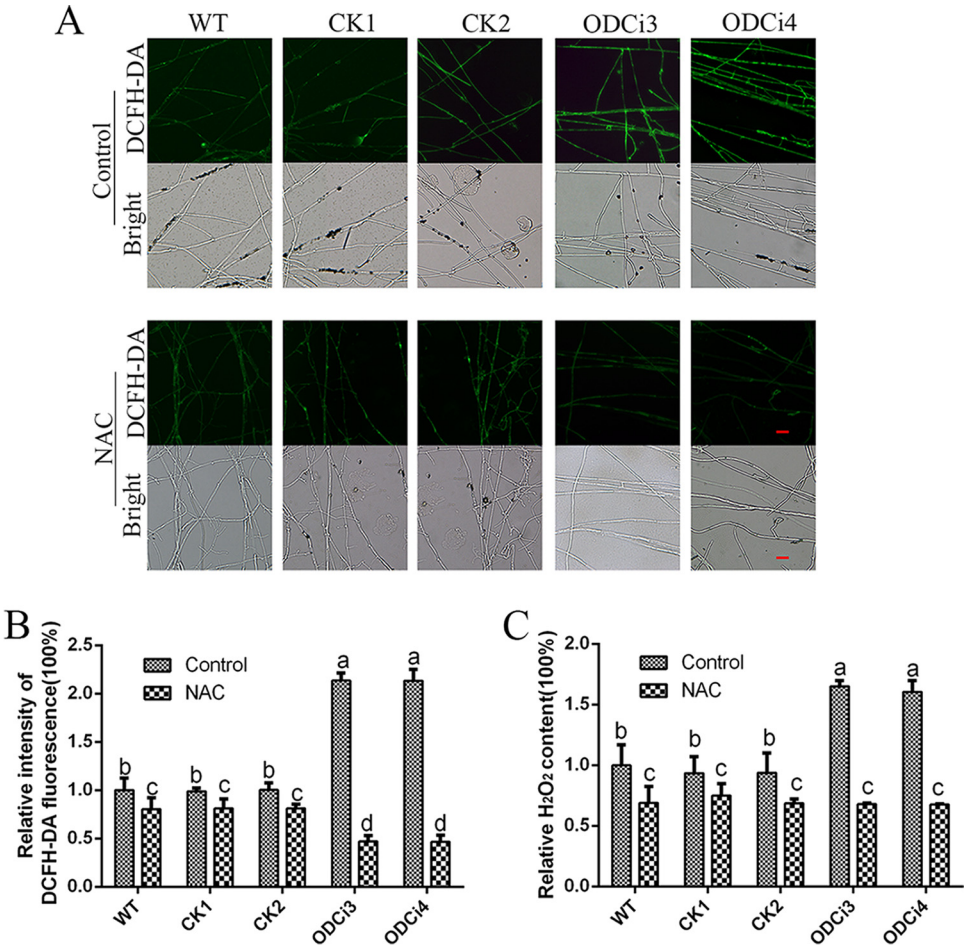

FIG 7 The ROS levels in *G. lucidum* after the ROS scavenger (NAC) treatment. (A) The control (WT, CK1, and CK2) and *ODC*-silenced *G. lucidum* strains were cultured at 28°C on a CYM solid agar medium with or without 5 mM NAC for 4 days. The change in the ROS levels was measured by DCFH-DA staining after the 1 mM putrescine treatment. Scale bar = 100 μm. (B) Changes in the ROS fluorescence ratio in the hyphal regions of the control (WT, CK1, and CK2) and *ODC*-silenced strains with or without the 5 mM NAC treatment. The *y* axis represents the ROS fluorescence ratio, as measured by CLSM, and the *x* axis represents the different strains. The values in each column are the captured fluorescence ratios in each observation. (C) Measurement of the amount of H_2O_2 in *G. lucidum* with or without the 5 mM NAC treatment. The values are the means ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$, according to Duncan's multiple-range test).

ROS are important signaling molecules. ROS play an important role in the regulation of cell growth and development, the stress response, and the regulation of cell injury in animals and plants (29). ROS have also been reported to act as signaling molecules for the regulation of the biosynthesis of secondary metabolites in fungi. In our earlier explorations, ROS regulate the biosynthesis of GA (30, 31). ODC and putrescine play an important role in organisms by regulating the levels of ROS. Under oxidative stress, *ODC* transcript was significantly upregulated and may have also undergone posttranscriptional regulation in murine papilloma PE cells (13). In the tomato (*Lycopersicon esculentum* Mill.), under chilling stress conditions, exogenous putrescine can improve the tolerance to chilling by inhibiting the production of H_2O_2 and activating the antioxidant system (25). In zucchini, exogenous putrescine can increase the antioxidant response to alleviate damage due to low temperatures (28). In the present study, we found that *G. lucidum* was more sensitive to oxidative stress when the *ODC* gene was silenced (Fig. 3A and B). Further studies showed that putrescine affects the transcript levels of four major antioxidant enzyme-related genes and the activities of four major antioxidant enzymes (Fig. 5 and 6). As shown in Fig. 7, the ROS fluorescence value and the

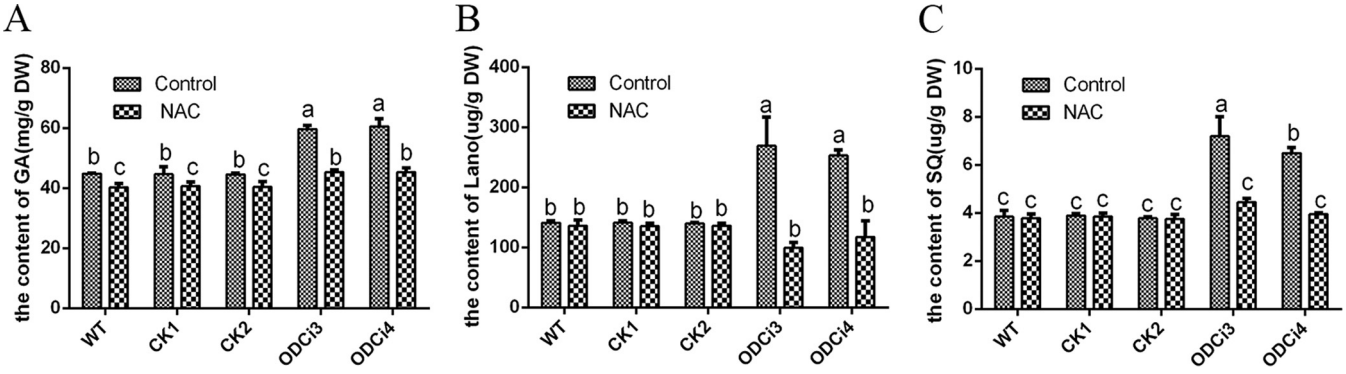

FIG 8 The effect of ROS scavenger (NAC) treatment on GA biosynthesis. (A) Measurement of the amount of total GA content in the control (WT, CK1, and CK2) and *ODC*-silenced strains with or without the putrescine treatment in *G. lucidum*. (B and C) The cellular levels of lanosterol (Lano) (B) and the cellular levels of squalene (SQ) (C) in these strains with or without the 5 mM NAC treatment. The values are the means ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$, according to Duncan's multiple-range test).

intracellular H_2O_2 content significantly increased in the *ODC*-silenced strains. Further studies showed that the ROS fluorescence value and the intracellular H_2O_2 content in the *ODC*-silenced strains were decreased to WT levels after the addition of 5 mM NAC, which is an ROS scavenger. These results indicate that effects of ODC and the production of putrescine on ROS may be due to their effect on the intracellular ROS content and intracellular antioxidant enzyme activity. Previous research has reported similar results in tomato and zucchini, in which exogenous putrescine was shown to activate the antioxidant system and improve tolerance to low-temperature stress (25, 28).

ODC and putrescine regulate the content of not only ROS but also secondary metabolites. In *Nicotiana tabacum* L., silencing the *ODC* gene causes a significant decrease in the nicotine and nornicotine levels and a significant increase in anatabine compared with the vector-only controls. These substances are important secondary metabolites in *Nicotiana tabacum* L. Silencing of the *ODC* gene also led to significantly reduced concentrations of polyamines (including putrescine) (16). After subjecting *Cucumis sativus* L. to NaCl stress, exogenous putrescine alleviated the feedback inhibition of photosynthesis by promoting sucrose transport and regulating the activities of enzymes involved in carbohydrate metabolism (19). Putrescine treatments lead to an induction of gamma-aminobutyric acid transaminase (GABA-T; an enzyme in the GABA shunt pathway), which causes the proline and betaine content to increase in zucchini fruit under cold stress conditions (32). In this study, we found that by silencing the *ODC* gene, the GA content and the levels of the two key intermediate metabolites (i.e.,

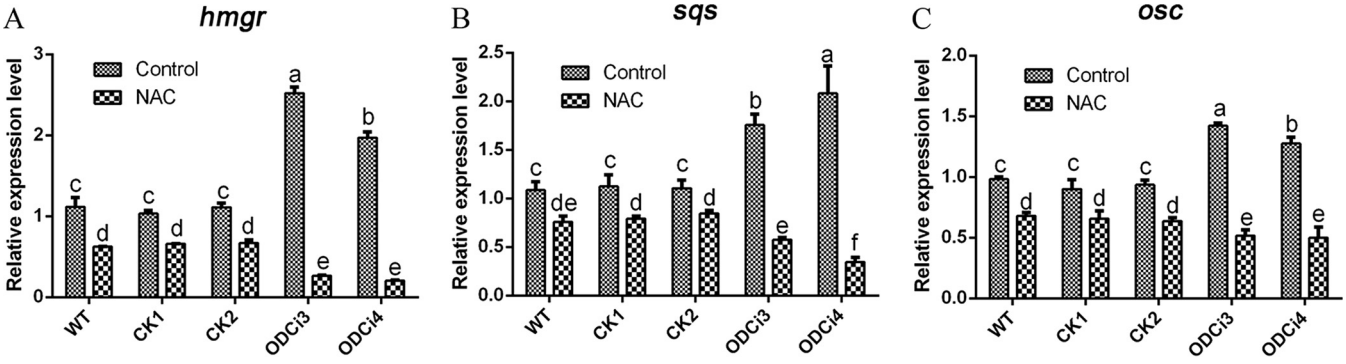

FIG 9 The effect of ROS scavenger (NAC) treatment on the transcript levels of three key enzyme-encoding genes in GA biosynthesis, i.e., *hmgr* (encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase) (A), *sqs* (encoding squalene synthase) (B), and *osc* (encoding lanosterol synthase) (C) in the *ODC*-silenced strains. Seven-day-old liquid mycelia from control (WT, CK1, and CK2) and *ODC*-silenced strains were collected to detect the relative mRNA levels of three key enzyme-encoding genes in GA biosynthesis in *G. lucidum*. Relative expression of key enzyme genes in the GA biosynthetic pathway was measured in strains treated without or with 5 mM NAC. The values are the means ± standard deviations (SD) of the results of three independent experiments. Different letters indicate significant differences between the lines ($P < 0.05$, according to Duncan's multiple-range test).

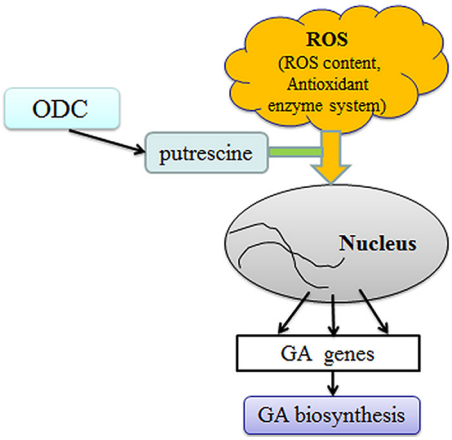

FIG 10 Schematic representation showing that ODC mediates the production of putrescine to influence GA biosynthesis via ROS in *G. lucidum*. The decreased putrescine content in the *ODC*-silenced strains influences the ROS content and the homeostasis of the intracellular antioxidant enzyme system, affecting the biosynthesis of GA. Putrescine biosynthesis is catalyzed by ODC. In the hypothetical model, the black and yellow solid arrows and the green solid line indicate data supported by experiments reported in this work.

lanosterol and squalene) significantly increased. Further studies found that the addition of exogenous putrescine restored the GA levels and the levels of lanosterol and squalene to WT levels. These studies were consistent with previous reports. These results suggest that ODC and the ODC-mediated production of putrescine can influence the biosynthesis of GA.

GA is an important secondary metabolite in *G. lucidum*, and ROS are important signaling molecules that regulate the biosynthesis of GA. Exogenous methyl jasmonate is related to the biosynthesis of GA via ROS generated by NADPH oxidase in *G. lucidum*. Hydrogen-rich water affects GA biosynthesis by regulating the effects of ROS balance via glutathione peroxidase in *G. lucidum* (26, 27). Furthermore, our previous study had shown that the depletion of ROS via reduced NOX expression acts via calcium to affect GA production (30). In the present study, we found that both the ROS and GA levels were significantly increased in the *ODC*-silenced strains. Further studies showed that the ROS and GA levels were significantly decreased in the *ODC*-silenced strains after the addition of NAC (Fig. 7 and 8). At the same time, we examined the effect of calcium levels in the process of ROS regulation of GA biosynthesis in the *ODC*-silenced strains (Fig. S5). Compared with the WT strains, the GA content was significantly decreased in the *ODC*-silenced strains when $CaCl_2$ or EGTA was added (Fig. S6). These results imply that calcium does not directly regulate the process of GA biosynthesis in the *ODC*-silenced strains. The regulation mechanism of calcium involvement in the *ODC*-silenced strains is complex and may be different from that in *NOX*-silenced strains. This remains to be explored in future studies. Furthermore, as shown in Fig. 9, the expression of the genes encoding three key enzymes in GA biosynthesis, i.e., *hmgr*, *sqs*, and *osc*, was also decreased after the addition of 5 mM NAC. These results imply that ODC and the production of putrescine affect the ROS levels, and ROS partially influences GA biosynthesis by affecting the expression of genes encoding key enzymes in GA biosynthesis.

In summary, these data indicate that ODC and the ODC-mediated production of putrescine regulate the intracellular ROS levels, and ROS influences GA biosynthesis and the gene expression of three key enzymes in GA biosynthesis (Fig. 10). We investigated the *ODC* gene and putrescine function in *G. lucidum*, and the mechanism of GA biosynthesis was preliminarily elucidated. This work also serves as a basis for further investigations of the function of the *ODC* gene, the role of putrescine in secondary metabolism, and the ROS regulation system.

TABLE 1 Oligonucleotide primers used

Primer	Sequence (5′ to 3′)[a]	Description	GenBank accession no.
ODCi-F	ACTGggtaccACCTCCCTTGTGGACTTTA	Gets fragment of *G. lucidum ODC* gene	MF581924
ODCi-R	ACTGactagtCCGTTCGTGCTGCCTGTA		
GL17083-F	ATGGCTCAACTCGAGATCTTC	Clones the *ODC* gene	
GL17083-R	TCACGCGTCCAGGTGGCTGT		
RT-17083-F	GCGGCTGATGTTGCGTGTC	Detects *ODC* expression	
RT-17083-R	CGGCAGAGCGGATGAAG		
RT-CAT1-F	TACGGTATTCAGCTCTTGT	Detects *CAT1* expression	MF581925
RT-CAT1-R	TGTTGTAACGGAACTTCTC		
RT-CAT2-F	GAAGAGGCGATCACTACCG	Detects *CAT2* expression	MF581926
RT-CAT2-R	GAAACCGATGCCAGGAACA		
RT-SOD1-F	ATCGCCGTCTTCGTCGTTT	Detects *SOD1* expression	MF581927
RT-SOD1-R	GTGACTGGTGCGGTAGGGA		
RT-SOD2-F	AATGCTGGAGCGTGCTGGGTC	Detects *SOD2* expression	KF700094
RT-SOD2-R	CGGATGCGTATGGAGTGGTC		
RT-SOD4-F	CTCACCGCGACGCGATTAC	Detects *SOD4* expression	MF581928
RT-SOD4-R	CCCTTGCCCTCAGACTTGG		
RT-APX-F	TTTTCGGTGCCCTTGGTGC	Detects *APX* expression	MF581929
RT-APX-R	ATGTCCGACGCAGGATTCTACT		
RT-GPX-F	GGAAGCCGAGAATGACGAAG	Detects *GPX* expression	MF581930
RT-GPX-R	GTAGTTCTTATCAGCCGCCT		
18s-F	TATCGAGTTCTGACTGGGTTGT	Detects the 18S rRNA gene expression	KM229678
18s-R	ATCCGTTGCTGAAAGTTGTAT		
RT-Hmgr-F	GTCATCCTCCTATGCCAGAC	Detects *hmgr* expression	EU263989
RT-Hmgr-R	GGGCGTAGTCGTAGTCCTTC		
RT-Sqs-F	CTGCTTATTCTACCTGGTGCTACG	Detects *sqs* expression	DQ494674
RT-Sqs-R	GGCTTCACGGCGAGTTTGT		
RT-Osc-F	AGGGAGAACCCGAAGCATT	Detects *osc* expression	GQ169528
RT-Osc-R	CGTCCACAGCGTCGCATAAC		
RT-CYP-F	CGTCGTCTTCGGCGAGGTT	Detects *CYP* expression	KF682413
RT-CYP-R	TGCTCCGTAGCGGTGGTC		
RT-PP2A-F	CCTTATGGGCGACAACC	Detects *PP2A* expression	KF233764
RT-PP2A-R	GCATTACCTCGTCAAACAGAT		
RT-TIF-F	CATCGGTCAGACGCTGTT	Detects *TIF* expression	KF682406
RT-TIF-R	CATGAGGATCTTCGGCAC		

[a]The sequences with lowercase letters ggtacc and actagt indicate KpnI and SpeI restriction sites, respectively.

MATERIALS AND METHODS

Fungal strains and growth conditions. The *Escherichia coli* DH5α strain was used for the plasmid amplification and was grown in Luria-Bertani (LB) medium, containing 100 μg · ml^{-1} ampicillin, as required, at 37°C. The *G. lucidum* ACCC53264 strain was obtained from the Agricultural Culture Collection of China. The *G. lucidum* wild-type strain (ACCC53264) and the silenced transformants were routinely maintained on potato dextrose agar (PDA) medium at 28°C. For the liquid fermentation, the *G. lucidum* strains were inoculated in a modified CYM medium (9) and placed on a rotary shaker incubator at 150 rpm at 28°C for 7 days.

Gene cloning and bioinformatic sequence analysis. The entire *ODC* coding sequence was amplified by PCR using *G. lucidum* cDNA as the template with the primers listed in Table 1, which were designed based on the *G. lucidum* sequenced genome. The fragments were cloned into the pMD18-T vector (TaKaRa, China) for sequencing. The open reading frame (ORF) and exon/intron positions were deduced by comparing the genomic and cDNA sequences. Multiple-sequence alignments were performed using the DNAMAN program. Moreover, a phylogenetic tree was generated with the MEGA 6.0

program using the neighbor-joining (NJ) method. A bootstrap consensus tree with 1,000 bootstrap replications represents the evolutionary history.

Construction of RNAi plasmids and strains. The construction of the RNAi vector and the empty vector control (CK), transformation of the *G. lucidum* strain, and random screening of the transformants were performed as previously described (9). As shown in Fig. S3, the dual-promoter-silencing vector pAN7-dual, which is driven by the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and the 35S promoter, was used to suppress the expression of *ODC*. The coding region of *ODC* was amplified by PCR using *G. lucidum* cDNA as the template with the primers listed in Table 1. The *ODC* fragment was doubly digested with KpnI and SpeI and inserted into the pAN7-dual plasmid after the KpnI/SpeI restriction digestion. The RNA interference (RNAi)-silencing vector pAN7-dual-ODCi, which contained the hygromycin phosphotransferase-encoding gene (*hph*), was transferred into *G. lucidum* via electroporation, and the transformants were selected on a CYM medium, containing 100 μg/ml hygromycin B from the transformants that were selected randomly; real-time PCR analyses were performed to determine the silencing efficiency of these transformants. We screened 15 independent positive transformants with a high silencing efficiency, and the phenotypes of these transformants are highly similar. The *ODCi3* and *ODCi4* transformants were randomly selected from the 15 transformants. The construction of CK was similar to the construction of the RNAi vector. CK did not contain the ODC fragment.

Real-time PCR analysis of gene expression. Total RNA was extracted using an RNA isolation kit (TaKaRa, China), according to the manufacturer's instructions. The expression levels of gene-specific mRNA in WT and isolated RNAi transformants were assessed using quantitative real-time PCR, according to our previous study (33). The 18S rRNA housekeeping gene was used to normalize the gene expression. The 18S rRNA is relatively stable under our experimental conditions. Furthermore, three additional housekeeping genes (i.e., *CYP* [cyclophilin], *PP2A* [protein phosphatase 2A regulatory subunit], and *TIF* [translation initiation factor]) were used to detect the *ODC* transcript levels under different oxidative stresses. The primers used are listed in Table 1.

Analysis of putrescine content by HPLC. The putrescine extraction, derivatization, and analysis by high-performance liquid chromatography (HPLC) were performed as described previously (34, 35), with some modifications. In brief, liquid fermentation samples from *G. lucidum* mycelium (1 g per sample) were pulverized with a mortar and pestle under liquid nitrogen. Five milliliters per g of the *G. lucidum* sample with 5% (vol/vol) cold perchloric acid were added to the resulting fine powders. The mixtures were transferred to plastic tubes and kept on ice for 1 h. After centrifugation at 12,000 $\times$ *g* and 4°C for 30 min, 0.5 ml of the supernatant fluid was collected by a liquid moving device and transferred to another 10-ml plastic tube. Then, 1 ml of 2 N NaOH was added to 0.5 ml of the supernatant fluid, the mixture was vortexed, 10 μl of benzoyl chloride were added and mixed, the mixture was incubated in a water bath at 37°C for 30 min, and then 2 ml of saturated NaCl was added. After the addition of 3 ml of diethyl ether, the samples were vigorously mixed and then centrifuged at 3,000 $\times$ *g* for 10 min at 4°C for the phase separation. An aliquot (1.5 ml) of the organic solvent phase was dried with nitrogen, and the residue was resuspended in 100 μl of methanol. The benzoylated putrescine was analyzed with a programmable Agilent 1200 liquid chromatograph using a reverse-phase column (4.6 mm by 250 mm, TSK-GEL ODS-80Ts; Tosoh, Tokyo, Japan) and measured at 254 nm, as previously described (35).

Detection of ROS and calcium. The ROS content and calcium content were measured as previously described (30). For the fluorescence detection of ROS, the structures were stained with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min to visualize H_2O_2 under a Zeiss Axio Imager A1 fluorescence microscope. For the fluorescence detection of calcium, the structures were stained with Fluo-3AM, an acetoxymethyl ester of a fluorescent calcium indicator dye (Invitrogen), for 30 min to visualize calcium under a Zeiss Axio Imager A1 fluorescence microscope. ROS level and calcium level quantification were performed using all regions of hyphae in each photo to detect the fluorescence intensity. For each treatment, a Zeiss Axio Imager A1 fluorescence microscope was used to take 20 pieces of photo for fluorescence quantification. The average fluorescence intensities of DCFH and Fluo-3AM in the mycelia were analyzed using ZEN lite (Zeiss software). For the fermentation experiment, the H_2O_2 content in liquid hyphae was assessed using a commercial hydrogen peroxide assay kit (Nanjing Jiancheng Bioengineering Institute, China). H_2O_2 bound to molybdic acid forms a complex, which is measured at 405 nm, and the content of H_2O_2 in the complex was calculated.

Detection of ganoderic acid, squalene, and lanosterol content. The method used for detecting the GA, squalene, and lanosterol content was performed as previously described (26).

Enzymatic activity assays. To measure the enzymatic activity, the mycelia were frozen in liquid N_2. The frozen mycelia (0.3 g) were homogenized in 5 ml of 50 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 15,000 $\times$ *g* for 20 min at 4°C, and the supernatant was immediately used in the enzyme assays. The protein content was determined using the Bradford method, and bovine serum albumin (BSA) was used as a standard.

CAT activity was assayed by measuring the rate of the decomposition of H_2O_2 at 240 nm, as previously described (36). SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), as previously described (37). APX activity was assessed using a commercial ascorbate peroxidase assay kit (Nanjing Jiancheng Bioengineering Institute, China). APX catalyzes the oxidation of ascorbic acid by H_2O_2, and APX activity was assessed by calculating the rate of ascorbic acid oxidation. The enzyme activity of GPX was measured as previously described (27).

Statistical analysis. All experiments were repeated at least three times with identical or similar results. The mean values in the individual experiments are expressed as the mean $\pm$ standard deviations

(SD). Appropriate statistical tests were used for the data in each figure. Student's *t* test was used to compare two samples. A Duncan's multiple-range test was used for multiple comparisons. A *P* value of <0.05 was considered significant.

Accession number(s). The GenBank accession numbers for the various genes are shown in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01289-17.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grants 31400035 and 31672212), the earmarked fund for China Agriculture Research System (CARS-20), the Fundamental Research Funds for the Central Universities (grant KJQN201527), the Science & Technology Pillar Program of Jiangsu Province, China (grant BE2015361), the Jiangsu Planned Projects for Postdoctoral Research Funds (grant 1402020B), and the Postdoctoral Science Foundation of China (grant 2015M570457).

REFERENCES

1. Chang YH, Yang JS, Yang JL, Wu CL, Chang SJ, Lu KW, Lin JJ, Hsia TC, Lin YT, Ho CC, Wood WG, Chung JG. 2009. *Ganoderma lucidum* extracts inhibited leukemia WEHI-3 cells in BALB/c mice and promoted an immune response *in vivo*. Biosci Biotechnol Biochem 73:2589–2594. https://doi.org/10.1271/bbb.90357.
2. Gao Y, Gao H, Chan E, Tang W, Xu A, Yang H, Huang M, Lan J, Li X, Duan W, Xu C, Zhou S. 2005. Antitumor activity and underlying mechanisms of ganopoly, the refined polysaccharides extracted from *Ganoderma lucidum*, in mice. Immunol Invest 34:171–198. https://doi.org/10.1081/IMM-55813.
3. Shi L, Ren A, Mu D, Zhao M. 2010. Current progress in the study on biosynthesis and regulation of ganoderic acids. Appl Microbiol Biotechnol 88:1243–1251. https://doi.org/10.1007/s00253-010-2871-1.
4. Xu JW, Xu YN, Zhong JJ. 2012. Enhancement of ganoderic acid accumulation by overexpression of an N-terminally truncated 3-hydroxy-3-methylglutaryl coenzyme A reductase gene in the basidiomycete *Ganoderma lucidum*. Appl Microbiol Biotechnol 78:7968–7976.
5. Xu JW, Zhao W, Zhong JJ. 2010. Biotechnological production and application of ganoderic acids. Appl Microbiol Biotechnol 87:457–466. https://doi.org/10.1007/s00253-010-2576-5.
6. Hajjaj H, Mace C, Roberts M, Niederberger P, Fay LB. 2005. Effect of 26-oxygenosterols from *Ganoderma lucidum* and their activity as cholesterol synthesis inhibitors. Appl Environ Microbiol 71:3653–3658. https://doi.org/10.1128/AEM.71.7.3653-3658.2005.
7. Sliva D, Loganathan J, Jiang JH, Jedinak A, Lamb JG, Terry C, Baldridge LA, Adamec J, Sandusky GE, Dudhgaonkar S. 2012. Mushroom *Ganoderma lucidum* prevent colitis-associated carcinogenesis in mice. PLoS One 7:e47873. https://doi.org/10.1371/journal.pone.0047873.
8. Zhang X, Ren A, Li MJ, Cao PF, Chen TX, Zhang G, Shi L, Jiang AL, Zhao MW. 2016. Heat stress modulates mycelium growth, heat shock protein expression, ganoderic acid biosynthesis, and hyphal branching of Ganoderma lucidum via cytosolic Ca^{2+}. Appl Environ Microbiol 82:4112–4125. https://doi.org/10.1128/AEM.01036-16.
9. Mu D, Shi L, Ren A, Li M, Wu F, Jiang A, Zhao M. 2012. The development and application of a multiple gene co-silencing system using endogenous *URA3* as a reporter gene in *Ganoderma lucidum*. PLoS One 7:e43737. https://doi.org/10.1371/journal.pone.0043737.
10. Shi L, Fang X, Li MJ, Mu DS, Ren A, Tan Q, Zhao MW. 2012. Development of a simple and efficient transformation system for the basidiomycetous medicinal fungus *Ganoderma lucidum*. World J Microbiol Biotechnol 28:283–291. https://doi.org/10.1007/s11274-011-0818-z.
11. Chen S, Xu J, Liu C, Zhu Y, Nelson DR, Zhou S, Li C, Wang L, Guo X, Sun Y, Luo H, Li Y, Song J, Henrissat B, Levasseur A, Qian J, Li J, Luo X, Shi L, He L, Xiang L, Xu X, Niu Y, Li Q, Han MV, Yan H, Zhang J, Chen H, Lv A, Wang Z, Liu M, Schwartz DC, Sun C. 2012. Genome sequence of the model medicinal mushroom *Ganoderma lucidum*. Nat Commun 3:913. https://doi.org/10.1038/ncomms1923.
12. Choi H, Kyeong HH, Choi JM, Kim HS. 2014. Rational design of ornithine decarboxylase with high catalytic activity for the production of putrescine. Appl Microbiol Biotechnol 98:7483–7490. https://doi.org/10.1007/s00253-014-5669-8.
13. Otieno MA, Kensler TW. 2000. A role for protein kinase C-δ in the regulation of ornithine decarboxylase expression by oxidative stress. Cancer Res 60:4391–4396.
14. Smirnova OA, Isaguliants MG, Hyvonen MT, Keinanen TA, Tunitskaya VL, Vepsalainen J, Alhonen L, Kochetkov SN, Ivanov AV. 2012. Chemically induced oxidative stress increases polyamine levels by activating the transcription of ornithine decarboxylase and spermidine/spermine-N1-acetyltransferase in human hepatoma HUH7 cells. Biochimie 94:1876–1883. https://doi.org/10.1016/j.biochi.2012.04.023.
15. Theiss C, Bohley P, Voigt J. 2002. Regulation by polyamines of ornithine decarboxylase activity and cell division in the unicellular green alga *Chlamydomonas reinhardtii*. Plant Physiol 128:1470–1479. https://doi.org/10.1104/pp.010896.
16. Dalton HL, Blomstedt CK, Neale AD, Gleadow R, DeBoer KD, Hamill JD. 2016. Effects of down-regulating ornithine decarboxylase upon putrescine-associated metabolism and growth in *Nicotiana tabacum* L. J Exp Bot 67:3367–3381. https://doi.org/10.1093/jxb/erw166.
17. Alet AI, Sanchez DH, Cuevas JC, del Valle S, Altabella T, Tiburcio AF, Marco F, Ferrando A, Espasandín FD, González ME, Ruiz OA, Carrasco P. 2014. Putrescine accumulation in *Arabidopsis thaliana* transgenic lines enhances tolerance to dehydration and freezing stress. Plant Signal Behav 6:278–286. https://doi.org/10.4161/psb.6.2.14702.
18. Kotakis C, Theodoropoulou E, Tassis K, Oustamanolakis C, Ioannidis NE, Kotzabasis K. 2014. Putrescine, a fast-acting switch for tolerance against osmotic stress. J Plant Physiol 171:48–51. https://doi.org/10.1016/j.jplph.2013.09.015.
19. Yuan YH, Zhong M, Shu S, Du NS, He LZ, Yuan LY, Sun J, Guo SR. 2015. Effects of exogenous putrescine on leaf anatomy and carbohydrate metabolism in cucumber (*Cucumis sativus* L.) under salt stress. J Plant Growth Regul 34:451–464. https://doi.org/10.1007/s00344-015-9480-2.
20. Kong XF, Wang XQ, Yin YL, Li XL, Gao HJ, Bazer FW, Wu GY. 2014. Putrescine stimulates the mTOR signaling pathway and protein synthesis in porcine trophectoderm cells. Biol Reprod 91:106. https://doi.org/10.1095/biolreprod.113.113977.
21. Valdés-Santiago L, Ruiz-Herrera J. 2013. Stress and polyamine metabolism in fungi. Front Chem 1:42. https://doi.org/10.3389/fchem.2013.00042.
22. El-Halfawy OM, Valvano MA. 2014. Putrescine reduces antibiotic-induced oxidative stress as a mechanism of modulation of antibiotic resistance in *Burkholderia cenocepacia*. Antimicrob Agents Chemother 58:4162–4171. https://doi.org/10.1128/AAC.02649-14.
23. Ding YZ, Peng N, Du YH, Ji LH, Cao B. 2014. Disruption of putrescine biosynthesis in Shewanella oneidensis enhances biofilm cohesiveness and performance in Cr(VI) immobilization. Appl Environ Microbiol 80:1498–1506. https://doi.org/10.1128/AEM.03461-13.
24. Valdés-Santiago L, Guzman-de-Pena D, Ruiz-Herrera J. 2010. Life without putrescine: disruption of the gene-encoding polyamine oxidase in *Usti-*

lago maydis odc mutants. FEMS Yeast Res 10:928–940. https://doi.org/10.1111/j.1567-1364.2010.00675.x.
25. Song YJ, Diao QN, Qi HY. 2014. Putrescine enhances chilling tolerance of tomato (*Lycopersicon esculentum* Mill.) through modulating antioxidant systems. Acta Physiol Plant 36:3013–3027. https://doi.org/10.1007/s11738-014-1672-z.
26. Shi L, Gong L, Zhang X, Ren A, Gao T, Zhao M. 2015. The regulation of methyl jasmonate on hyphal branching and GA biosynthesis in *Ganoderma lucidum* partly via ROS generated by NADPH oxidase. Fungal Genet Biol 81:201–211. https://doi.org/10.1016/j.fgb.2014.12.002.
27. Ren A, Liu R, Miao ZG, Zhang X, Cao PF, Chen TX, Li CY, Shi L, Jiang AL, Zhao MW. 2016. Hydrogen-rich water regulates effects of ROS balance on morphology, growth and secondary metabolism via glutathione peroxidase in *Ganoderma lucidum*. Environ Microbiol 19:566–583. https://doi.org/10.1111/1462-2920.13498.
28. Palma F, Carvajal F, Jamilena M, Garrido D. 2016. Putrescine treatment increases the antioxidant response and carbohydrate content in zucchini fruit stored at low temperature. Postharvest Biol Technol 118:68–70. https://doi.org/10.1016/j.postharvbio.2016.03.009.
29. Suzuki N, Miller G, Morales J, Shulaev V, Torres MA, Mittler R. 2011. Respiratory burst oxidases: the engines of ROS signaling. Curr Opin Plant Biol 14:691–699. https://doi.org/10.1016/j.pbi.2011.07.014.
30. Mu D, Li C, Zhang X, Li X, Shi L, Ren A, Zhao M. 2014. Functions of the nicotinamide adenine dinucleotide phosphate oxidase family in *Ganoderma lucidum*: an essential role in ganoderic acid biosynthesis regulation, hyphal branching, fruiting body development, and oxidative-stress resistance. Environ Microbiol 16:1709–1728. https://doi.org/10.1111/1462-2920.12326.
31. Li C, Shi L, Chen D, Ren A, Gao T, Zhao M. 2015. Functional analysis of the role of glutathione peroxidase (GPx) in the ROS signaling pathway, hyphal branching and the regulation of ganoderic acid biosynthesis in *Ganoderma lucidum*. Fungal Genet Biol 82:168–180. https://doi.org/10.1016/j.fgb.2015.07.008.
32. Palma F, Carvajal F, Ramos JM, Jamilena M, Garrido D. 2015. Effect of putrescine application on maintenance of zucchini fruit quality during cold storage: contribution of GABA shunt and other related nitrogen metabolites. Postharvest Biol Technol 99:131–140. https://doi.org/10.1016/j.postharvbio.2014.08.010.
33. Ren A, Qin L, Shi L, Dong X, Mu da S, Li YX, Zhao MW. 2010. Methyl jasmonate induces ganoderic acid biosynthesis in the basidiomycetous fungus *Ganoderma lucidum*. Bioresour Technol 101:6785–6790. https://doi.org/10.1016/j.biortech.2010.03.118.
34. Naka Y, Watanabe K, Sagor GH, Niitsu M, Pillai MA, Kusano T, Takahashi Y. 2010. Quantitative analysis of plant polyamines including thermospermine during growth and salinity stress. Plant Physiol Biochem 48:527–533. https://doi.org/10.1016/j.plaphy.2010.01.013.
35. Sagor GH, Chawla P, Kim DW, Berberich T, Kojima S, Niitsu M, Kusano T. 2015. The polyamine spermine induces the unfolded protein response via the MAPK cascade in *Arabidopsis*. Front Plant Sci 6:687. https://doi.org/10.3389/fpls.2015.00687.
36. Aebi H. 1984. Catalase *in vitro*. Methods Enzymol 105:121–126. https://doi.org/10.1016/S0076-6879(84)05016-3.
37. Giannopolitis CN, Ries SK. 1977. Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol 59:309–314.