A Novel Protein AoHPS1 Is Involved In Oxidative Stress and Kojic Acid Synthesis in *Aspergillus Oryzae*

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Abstract

Aspergillus oryzae usually suffers from oxidative stress during the process of aerobic fermentation. However, there is little information on what genes involve in oxidative stress of *A. oryzae*. Here, we found that the expression of a novel gene *Aohps1* was induced during the growth and development of *A. oryzae* with and without oxidative stress. Sequence analysis revealed that *Aohps1* has four transmembrane regions and is conserved in *Aspergillus* species. Overexpression and deletion of *Aohpi1* caused the growth defects with and without oxidative stress, including mycelium growth, conidia formation and biomass. Meanwhile, the *Aohpi1* overexpression strain exhibited more sensitivity to oxidative stress than the *Aohpi1* disrupted mutant. Furthermore, overexpression and disruption of *Aohps1* resulted in the inhibition of kojic acid production with and without oxidative stress, consistent with the reduced expression of *kojA* that directly contributed to the synthesis of kojic acid. Additionally, the yield of kojic acid is less in the *Aohps1* overexpression strain than in the *Aohps1* deletion mutant under oxidative stress. Collectively, the discovery of *Aohps1* provides new insights into oxidative stress and kojic acid synthesis in *A. oryzae*.

Introduction

Aspergillus oryzae as an important filamentous fungus is used in the fermentation industry for more than a thousand years (Kobayashi et al. 2007; Machida et al. 2008). During the course of fermentation, on the one hand, it has to face and deal with a wide variety of environmental challenges, including oxidative stress. For example, during oxidative stress the high levels of reactive oxygen species (ROS) to which *A. oryzae* must rise are produced by neutrophil cells, evoking cell damage. In response to oxidative stress, *A. oryzae* has to rapidly reprogram gene expression and gene products to buffer the lethal elevation in ROS. On the other hand, there is abundant evidence to show that secondary metabolism is closely related to oxidative stress in filamentous fungi (Hong et al. 2013). *A. oryzae* has been widely used to produce several industrially important secondary metabolites, such as kojic acid (Marui et al. 2010; Terabayashi et al. 2010). However, whether and how oxidative stress affects secondary metabolism in *A. oryzae* is not clear yet.

For defence against ROS, cells have developed various antioxidant strategies, including antioxidant enzymatic strategies and non-enzyme antioxidant strategies (Bai et al. 2003; Breitenbach et al. 2015; Finkel 2003; Li et al. 2009). The enzymatic systems involved in ROS detoxification mainly include superoxide dismutases (SODs), catalases (CATs) and peroxidases (PODs), while non-enzyme antioxidants consist of GSH, ascorbate or vitamin E (Herrero et al. 2008; Li et al. 2009). A key feature of the response to oxidative stress is to sense the stress signaling rapidly, in turn leading to a reprogramming of expression of genes and their products to protect cells from oxidative stress. Although fungi can utilize multiple different means to control the expression of genes responsible for oxidative challenge, a common feature is the invocation of a transcriptional response to oxidative stress (Hong et al. 2013; Montibus et al. 2015). In yeast, Yap1 and Skn7 are thought as the main two oxidative stress-related transcription factors. *Yap1* encodes a leucine-zipper (bZip) AP-1 transcriptional factor and

mediates the expression of genes encoding most yeast antioxidants in response to oxidants (Kuge and Jones 1994; Schnell et al. 1992). Disruption of *Yap1* leads to be hypersensitive to H_2O_2 (Schnell et al. 1992). Likely, *Skn7* is identified as a response regulator protein involved in H_2O_2 hypersensitivity and found to regulate the expression of anti-oxidant genes, including *CTT1* (cytosolic catalase), *SOD1* (superoxide dismutases) in response to oxidative stress (Krems et al. 1996; Morgan et al. 1997). Additionally, the stress response element binding protein Msn2p is required for activating the expression of *CTT1* in response to oxidative stress (Schmitt and McEntee 1996).

Kojic acid is a secondary metabolite naturally produced by *Aspergillus oryzae*, which is used widely in the food, pharmaceutical and cosmetic area (Mohamad 2010; Saeedi et al. 2019). The related genes for kojic acid have been identified in recent studies (Arakawa et al. 2019; Chang et al. 2011; Marui et al. 2011; Terabayashi et al. 2010). Firstly, a kojic acid cluster in *A.oryzae* is found, including *kojR*, *kojA* and *kojT* (Terabayashi et al. 2010). *KojR* encoding a zinc finger protein, serves as a positive regulator to promote kojic acid synthesis through activating its target genes, *kojA* and *kojT* (Marui et al. 2011). *KojA* is identified as a FAD-dependent oxidoreductase and catalyzes the conversion of glucose to kojic acid, while *kojT* is proposed to be a kojic acid transporter helping to transport kojic acid to the extracellular (Terabayashi et al. 2010). *LaeA*, a global transcriptional regulator of secondary metabolism, works as the upstream of *kojR* to positively regulate the synthesis of kojic acid (Oda et al. 2014). Recently, another regulatory protein KepA is also found to be involved in kojic acid production through mediating *kojR* and *kojA* expression (Arakawa et al. 2019). Additionally, *MsnA*, homologous to *Msn2*, is proved to be related to kojic acid biosynthesis in *A. parasiticus* and *A. flavus* (Chang et al. 2011). Moreover, disruption of *MsnA* leads to not only elevated amounts of kojic acid but also higher levels of ROS, making cells under increased oxidative stress (Chang et al. 2011). However, the information on what genes involve in oxidative stress and kojic acid production are unknown in *A. oryzae*.

In this study, an uncharacterized gene (Ao090009000298) was induced during the process of growth and development of *A. oryzae*, and it was overexpressed in *A. oryzae*, resulting in the sensitivity of *A. oryzae* to hydrogen peroxide. Therefore, Ao090009000298 was named *Aohps1* (hydrogen peroxide sensitivity). *Aohps1* was overexpressed by the *A. oryzae amyB* promoter and *Aohps1* knock-out strain was constructed by CRIPSR/Cas9 system. The growth and kojic acid production of *Aohps1* overexpression and deletion strains with and without oxidative stress were investigated. The study helps us understand kojic acid synthesis and oxidative stress in *A. oryzae*.

Materials And Methods

Microorganisms and cultivations

In this study, the *A. oryzae* 3.042 strain (CICC 40092) served as wild type (WT). The strain Δ3.042 (Δ*pyrG*) was derived from 3.042 strain by the CRISPR/Cas9 system (Fan et al. 2020). Czapek-Dox (CD) medium (2% soluble starch, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.05% NaCl, 0.002% $FeSO_4$ pH 5.5) was used to grow the *A. oryzae* strains. CD medium with 0.02% H_2O_2 (v/v) was used for oxidative

stress. The modified CD medium (2% soluble starch, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.05% NaCl, 0.002% $FeSO_4$, 500 μM $FeCl_3$, 200 μM $ZnSO_4$ pH 5.5) was used to observe kojic acid. All fungal strains were cultured at 30°C.

Sequence analysis of *Aohps1*

The “Secondary structure consensus prediction” tool was used to predict α-helices, random coil, and other protein features. The protein sequence of *Aohps1* was submitted to TMHMM Server v. 2.0 to predict transmembrane helices. For multiple alignment, amino acid sequences of *Aohps1* and its orthologous genes from different species were retrieved from the Ensembl Genome Databases and aligned with ClustalW program.

Construction of *Aohps1* disruption and overexpression strains

To obtain *Aohps1* disruption strain, a 20 bp protospacer sequence targeted to *Aohps1* was obtained using CRISPRdirect (https://crispr.dbcls.jp/). The U6 promoter attached with the protospacer sequence for *Aohps1* was amplified from our previous vector pPTRII-Cas9-AoGld3 (Fan et al. 2020) using the forward primer PU6-F and reverse primer PU6-Aohps1-R with the protospacer sequence for *Aohps1*, generating the DNA fragment PU6-Aohps1. The U6 terminator and sgRNA sequence were obtained by PCR using the vector pPTRII-Cas9-AoGld3 as a temple with the forward primer containing the protospacer sequence for *Aohps1* and reverse primer Aohps1-TU6-R, producing the fragment Aohps1-TU6. The two fragments with the protospacer sequence for *Aohps1* were fused by overlapping PCR and inserted into the SmaI-digested pPTRII-Cas9 vector, generating the plasmid pPTRII-Cas9-Aohps1. The resulting plasmid was introduced into the strain 3.042 using the PEG–protoplast method as described previously (Maruyama and Kitamoto 2011), and then screened through the pyrithiamine resistance marker *ptrA* and DNA sequencing.

To construct *Aohps1* overexpression strain, the open read frame (ORF) of *Aohps1* was amplified using the primers listed in Table S2, and cloned into the AflII/BamHI-digested pEX2B vector under the control of *amyB* promoter. The sequenced plasmid was transformed into the strain Δ3.042 through a standard protoplast method. The positive transformants were examined by PCR.

Expression analysis

Total RNA extraction from grounded mycelia was performed using a Fungal Total RNA Extraction Kit (Omega) according to the manufacturer’s instructions. The genomic DNA contamination in the RNA samples was eliminated using gDNA Eraser (TaKaRa). The total RNA (1 μg) was taken as a template for a first-strand cDNA synthesis using a PrimeScript™ RT reagent Kit following the instruction manual. The CFX96 Real-Time PCR Detection System (Bio-Rad) was used to detect gene expression levels using a SYBR Green PCR Kit (TaKaRa) with the primers listed in Table S2. Histone H1 (AO090012000496) serves as a reference gene. Each gene expression was normalized with the Histone H1. Three independent biological replicates were carried out. Relative expression levels were calculated with the previously described method (Livak and Schmittgen 2001).

Morphological analysis and determination of kojic acid

The fungal strains were point inoculated on CD agar plates and cultured at 30°C. The fungal colonies were photographed and their diameters were measured after fungal strains were cultured for three days. To measure dry cell weight, spore stock suspension (10^8 spores per mL) was spread on CD agar medium covered with cellophane and incubated at 30°C for three days, then the mycelia on the cellophanes were harvested and dried at 65°C overnight. To quantify the number of conidia, the conidia cultured on CD agar medium for three days were collected by washing solution (0.025% Tween 80, 0.8% NaCl) and counted using a hemocytometer. To detect the kojic acid on the modified CD agar plates, the fungal strains were harvested from three 3 cm cores together with the medium, and extracted with 3 mL absolute ethanol, then the supernatant was collected to determine the content of kojic acid by the colorimetric method with modification after centrifugation at 10,000 rpm for 5 min.

Results

Expression analysis of *Aohps1* in *A. oryzae*

To explore the expression pattern of *Aohps1*, samples from three different stages of *A. oryzae* were taken, which included that stage I mycelia expansion spans about 24 h; stage II early sporulation spans about 48 h and stage III mature sporulation spans about 72 h. RT-qPCR analysis showed that the expression of *Aohps1* was induced in three development stages of *A. oryzae* (Fig. 1). After three days of growth under H_2O_2 treatment, the transcription level of *Aohps1* had no significant changes compared to that without H_2O_2 treatment (Fig. 1).

Characterization of *Aohps1*

The *Aohps1* gene encodes an uncharacterized protein of 306 amino acid residuals according to Aspergillus Genome Database (Fig. 2A). In silico analysis of *Aohps1* protein showed that AoHPS1 had no given domains and contained 54% random coil, 29% α-helix and 10% extended strand (Fig. 2A). The prediction of transmembrane region of AoHPS1 sequence had four distinct hydrophobic regions (Fig. 2B). Multiple alignments showed that AoHPS1 is conserved in *Aspergillus* species (Fig. 2C).

Generation of *Aohps1* overexpression and deletion mutants

To reveal the function of *Aohps1*, *Aohps1* overexpression and deletion mutants were constructed using *A. oryzae amyB* gene promoter and the CRISPR/Cas9 system, respectively (Fig. 3). In the *Aohps1*-overexpressing strain (OE-Aohps1), qPCR analysis showed that the transcription level of *Aohps1* increased by 5-fold relative to the control WT strain on the starch-containing medium (Fig. 3C). For the *Aohps1*-disrupted strain (Δ*Aohpi1*), one base-pair deletion in the second exon of *Aohps1* led to the termination of translation (Fig. 3A). But disruption of *Aohps1* didn't result in the reduced expression of *Aohps1* (Fig. 3C).

Phenotypic characterization of *Aohps1* overexpression and disruption strains

After cultivation for three days on CD agar medium, the growth of *Aohps1* overexpression and disruption strains slightly decreased compared with the WT strain, while the *Aohps1* overexpression strain exhibited a more severe growth defect than the *Aohps1* deletion mutant and the WT strain under H_2O_2 treatment (Fig. 4A, B). Moreover, *Aohps1* overexpression and disruption strains exhibited severe conidiation defects (Fig. 4C). Additionally, the biomass of *Aohps1* overexpression and disruption strains markedly reduced relative to the WT strain with H_2O_2 stress, whereas the *Aohps1* deletion mutant had no changes in the biomass compared with the WT strain without H_2O_2 treatment (Fig. 4D).

The effects of overexpression and disruption of *Aohps1* on kojic acid synthesis

To study the effects of *Aohps1* on secondary metabolism of *A. oryzae*, the control WT, OE-Aohps1 and Δ*Aohpi1* strains were cultivated on CD agar medium with ferric ions chelated by kojic acid, showing a red color. The control WT, OE-Aohps1 and Δ*Aohpi1* strains all displayed a visible red color on the agar medium after cultivation for three days (Fig. 5A). But the control WT strains showed a red color much more intense than OE-Aohps1 and Δ*Aohpi1* strains (Fig. 5A). Quantitative analysis displayed that the yield of kojic acid in the control WT was about 1.7 and 2.1 times higher than those in the OE-Aohps1 and Δ*Aohpi1* strains without H_2O_2 treatment, respectively (Fig. 5A). Under H_2O_2 treatment, the production of kojic acid in the WT, OE-Aohps1 and Δ*Aohpi1* strains was down-regulated and kojic acid production was significantly lower in the OE-Aohps1 and Δ*Aohpi1* strains than in the control strain (Fig. 5). Moreover, the yield of kojic acid in the OE-Aohps1 strain decreased more than that in Δ*Aohpi1* strains compared with WT (Fig. 5). To clarify whether the declined production of kojic acid is related to transcription levels, the expression levels of *LaeA*, *kojA*, *kojR* and *kojT* involved in kojic acid synthesis were analyzed. We found that *kojA* but not *kojR* and *kojT* was decreased significantly in the OE-Aohps1 and Δ*Aohpi1* strains without H_2O_2 treatment (Fig. 6B, C and D). However, the expression profiles of *LaeA*, *kojR* and *kojT* in the OE-Aohps1 and Δ*Aohpi1* strains were up-regulated markedly under H_2O_2 treatment relative to those without H_2O_2 treatment (Fig. 6A, B and C). Impressively, the transcription level of *kojA* in the OE-Aohps1 strain had no significant changes with and without H_2O_2 treatment whereas *kojA* expression in the Δ*Aohpi1* strain was increased significantly after H_2O_2 treatment (Fig. 6B), consistent with the fact that kojic acid production was significantly less in the OE-Aohps1 strain than in the Δ*Aohpi1* mutant under H_2O_2 treatment (Fig. 5B).

Discussion

A. oryzae is widely used in the fermentation industry to produce fermented food, enzymes and secondary metabolites (Abe et al. 2006; Gomi 2019; Ichishima 2016). During the fermentation process, the aeration and agitation contribute to not only the growth of *A. oryzae* but also the development of oxidative stress (Li et al. 2009). However, there is little information on the involvement of *A. oryzae* in oxidative stress. In this study, an uncharacterized gene *Aohps1* was found to be involved in oxidative stress. At the

transcription level, *Aohps1* expression didn't be inhibited by H_2O_2 (Fig. 1). In terms of the growth and development of *A. oryzae*, the overexpressing *Aohps1* strain became more susceptible to H_2O_2 than WT, and *Aohps1* overexpression and deletion strains exhibited obvious growth inhibition in mycelium growth, conidia formation and biomass compared with the control WT under oxidative stress (Fig. 4). These results indicate that *Aohps1* has a role in oxidative stress and the growth and development of *A. oryzae*.

Aohps1 encodes a hypothetical protein according to the *Aspergillus* genome database. To explore its possible function, we attempted to search its homologous proteins to provide research clues, but there were no known proteins homologous with AoHPS1 to be found in other species. Here, sequence analysis showed AoHPS1 contains four transmembrane regions and the transmembrane domains are mainly located at the N terminus of AoHPS1 protein (Fig. 2B, C), suggesting that AoHPS1 might function in cell surface. In addition, multiple alignments revealed that AoHPS1 and its homologous proteins are conserved in *Aspergillus* species (Fig. 2C).

Kojic acid is widely used in food and cosmetic fields as anti-oxidant (El-Kady et al. 2014; Mohamad 2010; Saeedi et al. 2019). Previous study has been revealed that disruption of *MsnA* encoding a zinc finger protein, increased the production of kojic acid followed by higher levels of reactive oxygen species (ROS) in *Aspergillus parasiticus* and *Aspergillus flavus* (Chang et al. 2011), indicating kojic acid might be used to combat oxidative stress in cells. However, it remains unclear what genes involve in oxidative stress by affecting the synthesis of kojic acid in *A. oryzae*. In this present study, overexpression and deletion of *Aohps1* inhibited the synthesis of kojic acid under no oxidative stress (Fig. 5). Furthermore, under oxidative stress, kojic acid production is significantly less in the *Aohps1* overexpression strain than in the *Aohps1* disrupted mutant, while the *Aohps1*-overexpressing strain is more sensitive to H_2O_2 than the *Aohps1* deletion mutant (Fig. 4, 5), suggesting that *Aohps1* is involved in protecting *A. oryzae* from oxidative stress through affecting the synthesis of kojic acid. How *Aohps1* affects kojic acid synthesis in *A. oryzae* ? We found that oxidative stress contributed to the expression of *LaeA*, *kojR* and *kojT* in the *Aohps1* overexpression and deletion strains (Fig. 6A, C and D), inconsistent with the reduced production of kojic acid in the *Aohps1* overexpression and deletion (Fig. 5). This is probably because *LaeA* and *kojR* as the regulatory proteins are indirectly related to kojic acid and there are other genes as the targets of *LaeA* and *kojR* involved in the response to oxidative stress. It has been reported that *kojA* is proposed to directly contribute to kojic acid synthesis as an oxidoreductase (Terabayashi et al. 2010), which is also supported by the *kojA* expression and kojic acid production in the *Aohps1* overexpression and deletion strains. Without oxidative stress, overexpression and disruption of *Aohps1* inhibited the expression of *kojA* (Fig. 6B), resulting in the reduced production of kojic acid (Fig. 5). Under oxidative stress, the transcription level of *kojA* in the *Aohps1* deletion mutant was up-regulated whereas that in the *Aohps1* overexpression strain had no changes relative to the control WT without oxidative stress (Fig. 6B), in agreement with the yield of kojic acid is more in the *Aohps1* deletion mutant than the *Aohps1* overexpression strain (Fig. 5). These data indicate that *Aohps1* might be involved in kojic acid synthesis by affecting the expression of *kojA*.

In summary, a novel gene *Aohps1* was found to be involved in oxidative stress and kojic acid synthesis in *A. oryzae*. *Aohps1* overexpression strain exhibited reduced oxidative stress resistance. Meanwhile, Overexpression and deletion of *Aohpi1* led to growth defects, including mycelium growth, conidia formation and biomass with and without oxidative stress. Furthermore, overexpression and disruption of *Aohps1* caused the inhibition of kojic acid production, consistent with the declined expression of *kojA* that was directly involved in the production of kojic acid. Additionally, *Aohps1* overexpression strain is more susceptible to oxidative stress than *Aohps1* disrupted mutant, consistent with the yield of kojic acid is less in the *Aohps1* overexpression strain than in the *Aohps1* deletion mutant. These data suggest that *Aohps1* plays an important role in oxidative stress and kojic acid synthesis in *A. oryzae*.

Declarations

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals experiment.

References

1. Abe K, Gomi K, Hasegawa F, Machida M (2006) Impact of Aspergillus oryzae genomics on industrial production of metabolites. Mycopathologia 162:143–153. doi:10.1007/s11046-006-0049-2
2. Arakawa GY et al (2019) A unique $Zn(II)_2$-Cys_6-type protein, KpeA, is involved in secondary metabolism and conidiation in *Aspergillus oryzae*. Fungal Genet Biol 127:35–44. doi:10.1016/j.fgb.2019.02.004
3. Bai Z, Harvey LM, McNeil B (2003) Oxidative stress in submerged cultures of fungi. Crit Rev Biotechnol 23:267–302. doi:10.1080/07388550390449294
4. Breitenbach M, Weber M, Rinnerthaler M, Karl T, Breitenbach-Koller L (2015) Oxidative stress in fungi: its function in signal transduction, interaction with plant hosts, and lignocellulose degradation. Biomolecules 5:318–342. doi:10.3390/biom5020318

5. Chang PK et al (2011) Loss of *msnA*, a putative stress regulatory gene, in *Aspergillus parasiticus* and *Aspergillus flavus* increased production of conidia, aflatoxins and kojic acid. Toxins 3:82–104 doi:10.3390/toxins3010082
6. El-Kady IA, Zohri AN, Hamed SR (2014) Kojic Acid production from agro-industrial by-products using fungi. Biotechnol Res Int 2014:642385. doi:10.1155/2014/642385
7. Fan J, Zhang Z, Long C, He B, Hu Z, Jiang C, Zeng B (2020) Identification and functional characterization of glycerol dehydrogenase reveal the role in kojic acid synthesis in *Aspergillus oryzae*. World J Microb Biot 36:136. doi:10.1007/s11274-020-02912-4
8. Finkel T (2003) Oxidant signals and oxidative stress. Curr Opin Cell Biol 15:247–254. doi:10.1016/s0955-0674(03)00002-4
9. Gomi K (2019) Regulatory mechanisms for amylolytic gene expression in the koji mold *Aspergillus oryzae*. Biosci Biotech Bioch 83:1385–1401. doi:10.1080/09168451.2019.1625265
10. Herrero E, Ros J, Belli G, Cabiscol E (2008) Redox control and oxidative stress in yeast cells. Biochim Biophys Acta 1780:1217–1235. doi:10.1016/j.bbagen.2007.12.004
11. Hong SY, Roze LV, Linz JE (2013) Oxidative stress-related transcription factors in the regulation of secondary metabolism. Toxins 5:683–702. doi:10.3390/toxins5040683
12. Ichishima E (2016) Development of enzyme technology for *Aspergillus oryzae*, *A. sojae*, and *A. luchuensis*, the national microorganisms of Japan. Biosci Biotech Bioch 80:1681–1692 doi:10.1080/09168451.2016.1177445
13. Kobayashi T et al (2007) Genomics of *Aspergillus oryzae*. Biosci Biotech Bioch 71:646–670. doi:10.1271/bbb.60550
14. Krems B, Charizanis C, Entian KD (1996) The response regulator-like protein Pos9/Skn7 of *Saccharomyces cerevisiae* is involved in oxidative stress resistance. Curr Genet 29:327–334. doi:10.1007/BF02208613
15. Kuge S, Jones N (1994) YAP1 dependent activation of TRX2 is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. EMBO J 13:655–664
16. Li Q, Harvey LM, McNeil B (2009) Oxidative stress in industrial fungi. Crit Rev Biotechnol 29:199–213. doi:10.1080/07388550903004795
17. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25:402–408 doi. DOI 10.1006/meth.2001.1262
18. Machida M, Yamada O, Gomi K (2008) Genomics of *Aspergillus oryzae*: learning from the history of Koji mold and exploration of its future. DNA Res 15:173–183. doi:10.1093/dnares/dsn020
19. Marui J, Ohashi-Kunihiro S, Ando T, Nishimura M, Koike H, Machida M (2010) Penicillin biosynthesis in *Aspergillus oryzae* and its overproduction by genetic engineering. J Biosci Bioeng 110:8–11. doi:10.1016/j.jbiosc.2010.01.001

20. Marui J et al (2011) Kojic acid biosynthesis in *Aspergillus oryzae* is regulated by a $Zn(II)_2Cys_6$ transcriptional activator and induced by kojic acid at the transcriptional level. J Biosci Bioeng 112:40–43. doi:10.1016/j.jbiosc.2011.03.010
21. Maruyama J, Kitamoto K (2011) Targeted gene disruption in Koji mold *Aspergillus oryzae*. Methods Mol Biol 765:447–456. doi:10.1007/978-1-61779-197-0_27
22. Mohamad RM, Mohd S, Suhaili N, Mohd Salleh M, Ariff AB (2010) Kojic acid: Applications and development of fermentation process for production. Biotechnol Mol Biol Rev 5:24–37
23. Montibus M, Pinson-Gadais L, Richard-Forget F, Barreau C, Ponts N (2015) Coupling of transcriptional response to oxidative stress and secondary metabolism regulation in filamentous fungi. Crit Rev Microbiol 41:295–308. doi:10.3109/1040841X.2013.829416
24. Morgan BA, Banks GR, Toone WM, Raitt D, Kuge S, Johnston LH (1997) The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cerevisiae*. EMBO J 16:1035–1044. doi:10.1093/emboj/16.5.1035
25. Ntana F, Mortensen UH, Sarazin C, Figge R (2020) Aspergillus: A powerful protein production platform. Catalysts 10:1064. doi:10.3390/catal10091064
26. Oda K, Kobayashi A, Ohashi S, Sano M (2014) *Aspergillus oryzae* laeA regulates kojic acid synthesis genes. Biosci Biotech Bioch 75:1832–1834. doi:10.1271/bbb.110235
27. Saeedi M, Eslamifar M, Khezri K (2019) Kojic acid applications in cosmetic and pharmaceutical preparations. Biomed Pharmacother 110:582–593. doi:10.1016/j.biopha.2018.12.006
28. Schmitt AP, McEntee K (1996) Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. P Natl Acad Sci USA 93:5777–5782. doi:10.1073/pnas.93.12.5777
29. Schnell N, Krems B, Entian KD (1992) The PAR1 (YAP1/SNQ3) gene of *Saccharomyces cerevisiae*, a c-jun homologue, is involved in oxygen metabolism. Curr Genet 21:269–273. doi:10.1007/BF00351681
30. Terabayashi Y et al (2010) Identification and characterization of genes responsible for biosynthesis of kojic acid, an industrially important compound from *Aspergillus oryzae*. Fungal Genet Biol 47:953–961. doi:10.1016/j.fgb.2010.08.014

Figures

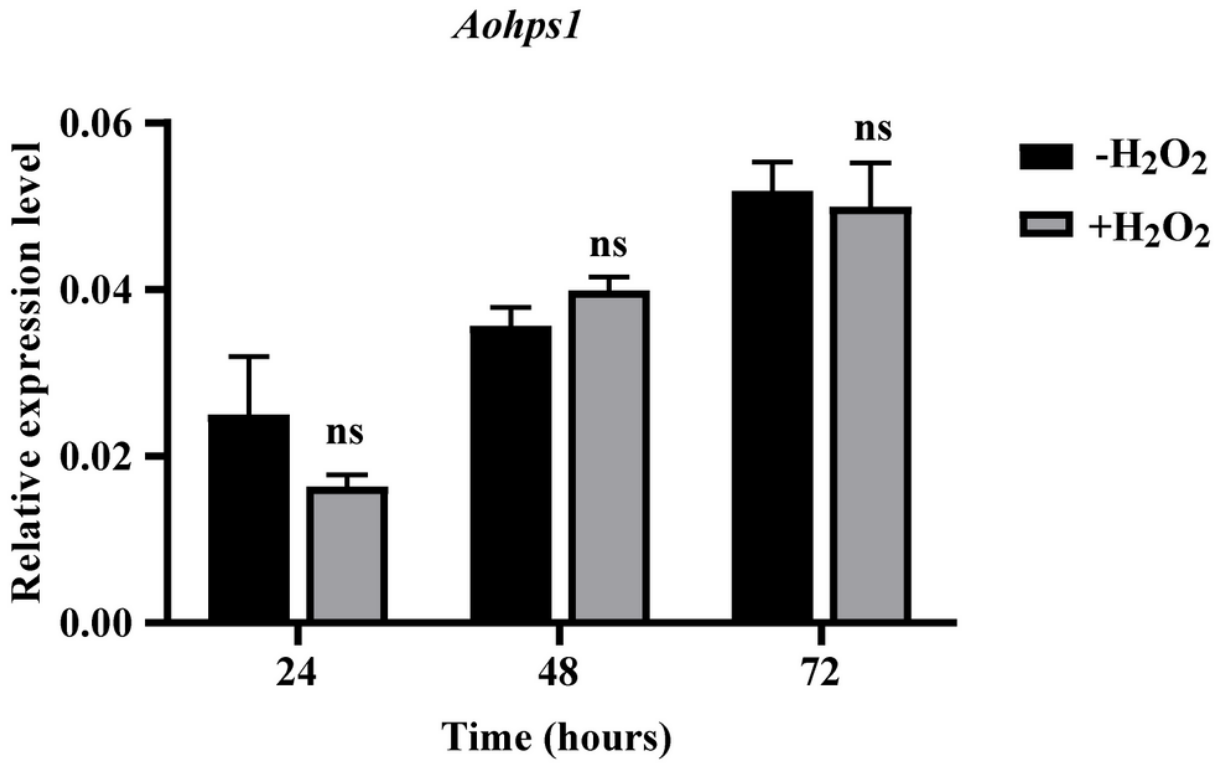

Figure 1

Expression analysis of Aohps1. Relative expression level of Aohps1 in the wild type strain cultured on the agar CD medium with or without H2O2 treatment for different times. The mycelia were collected at the time points indicated. Total RNA was extracted, and qPCR for Aohps1 was performed. Error bars indicate standard error of the mean (n = 3). No significant difference (ns), *p < 0.05, and ** p < 0.01 by Student's t-test.

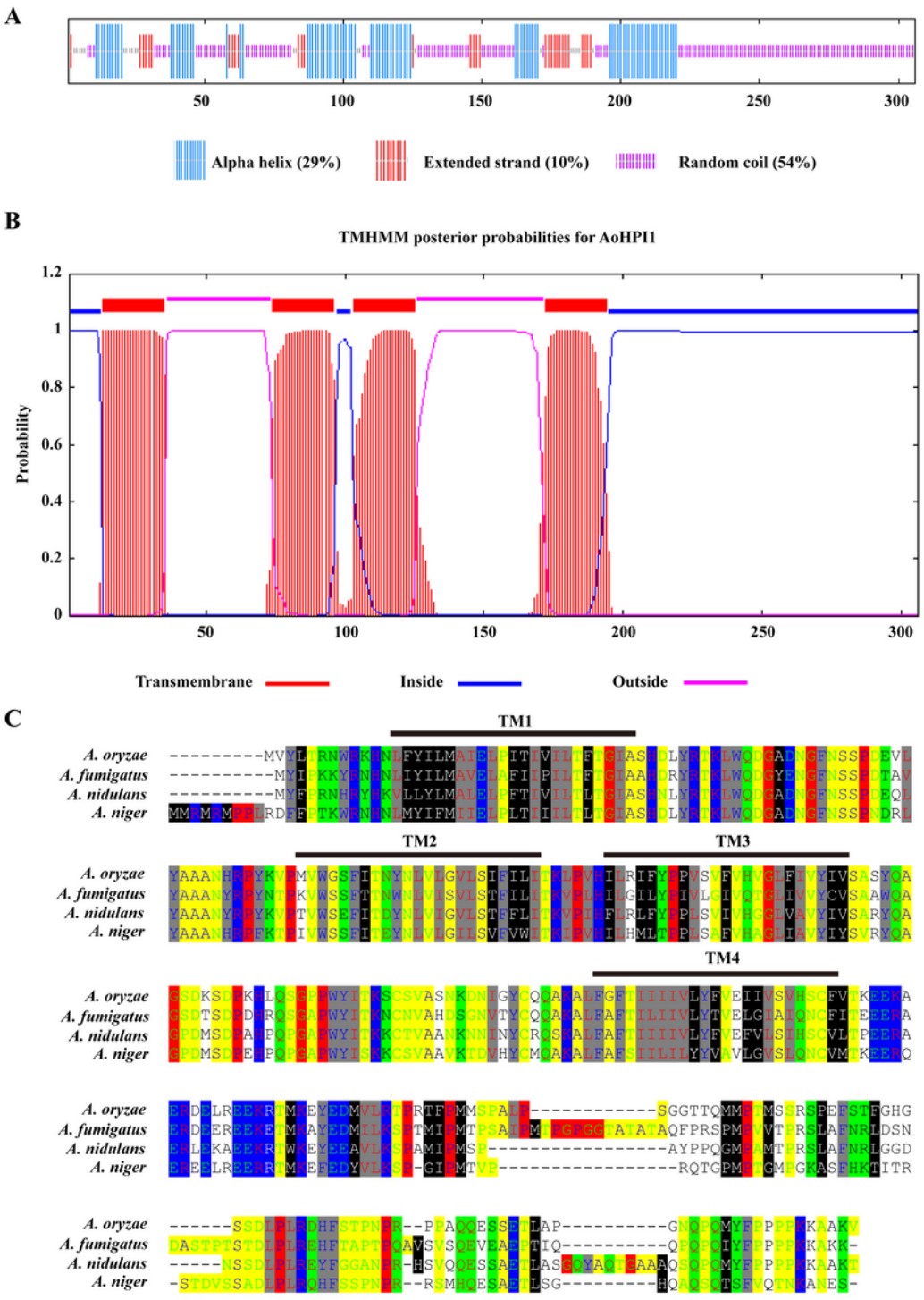

Figure 2

Characterization of Aohps1. (A) Analysis of secondary structure of AoHPS1 protein. The “secondary structure consensus prediction” tool was used to predict α-helices, random coil, and other protein features. (B) The transmembrane helices in AoHPI1 protein were obtained by using the TMHMM Server. (C) Alignment of amino acid sequences of AoHPI1 and its homologous proteins in Aspergillus species and transmembrane domains (TM) predicted by SMART.

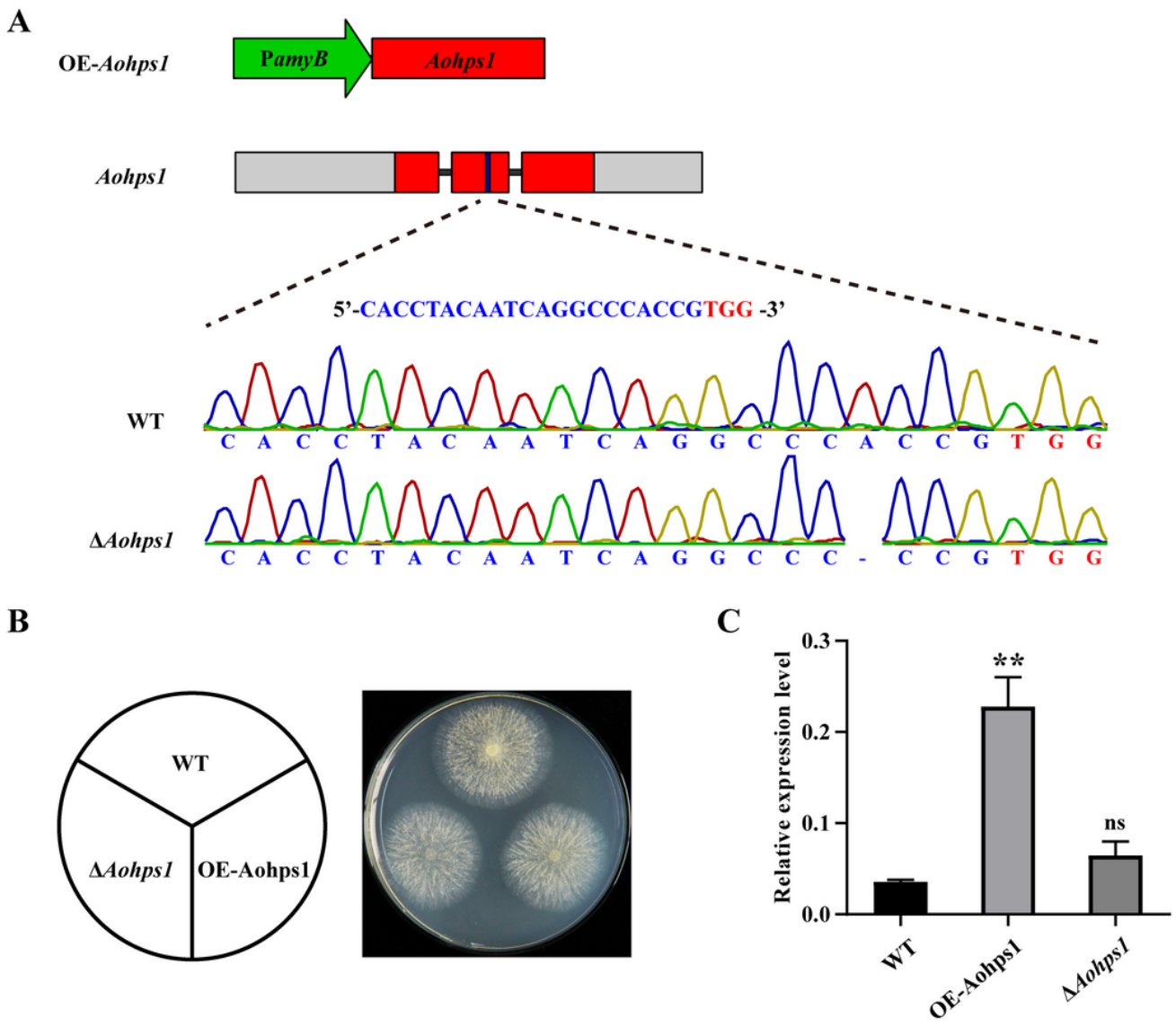

Figure 3

Construction of Aohps1 overexpression and disruption strains. (A) Strategy for generation of Aohps1 overexpression and disruption mutants (OE-Aohps1 and ΔAohps1). The A. oryzae amyB promoter was used to overexpress Aohps1. For Aohps1 disrupted strain (ΔAohps1), the target sequence for Aohps1 was targeted at the second exon of Aohps1. A 1-bp deletion occurred in the target sequence within Aohps1. Red sequence and blue sequence represent target sequence and protospacer adjacent motif (PAM), respectively. (B) The WT, OE-Aohps1 and ΔAohps1 strains were grown on CD agar medium for three days. (C) The expression levels of Aohps1 in WT, OE-Aohps1 and ΔAohps1 strains were determined using qPCR, and the Histone H1 (AO090012000496) was used as an internal control. The data are shown as mean ± SD of three replicates.

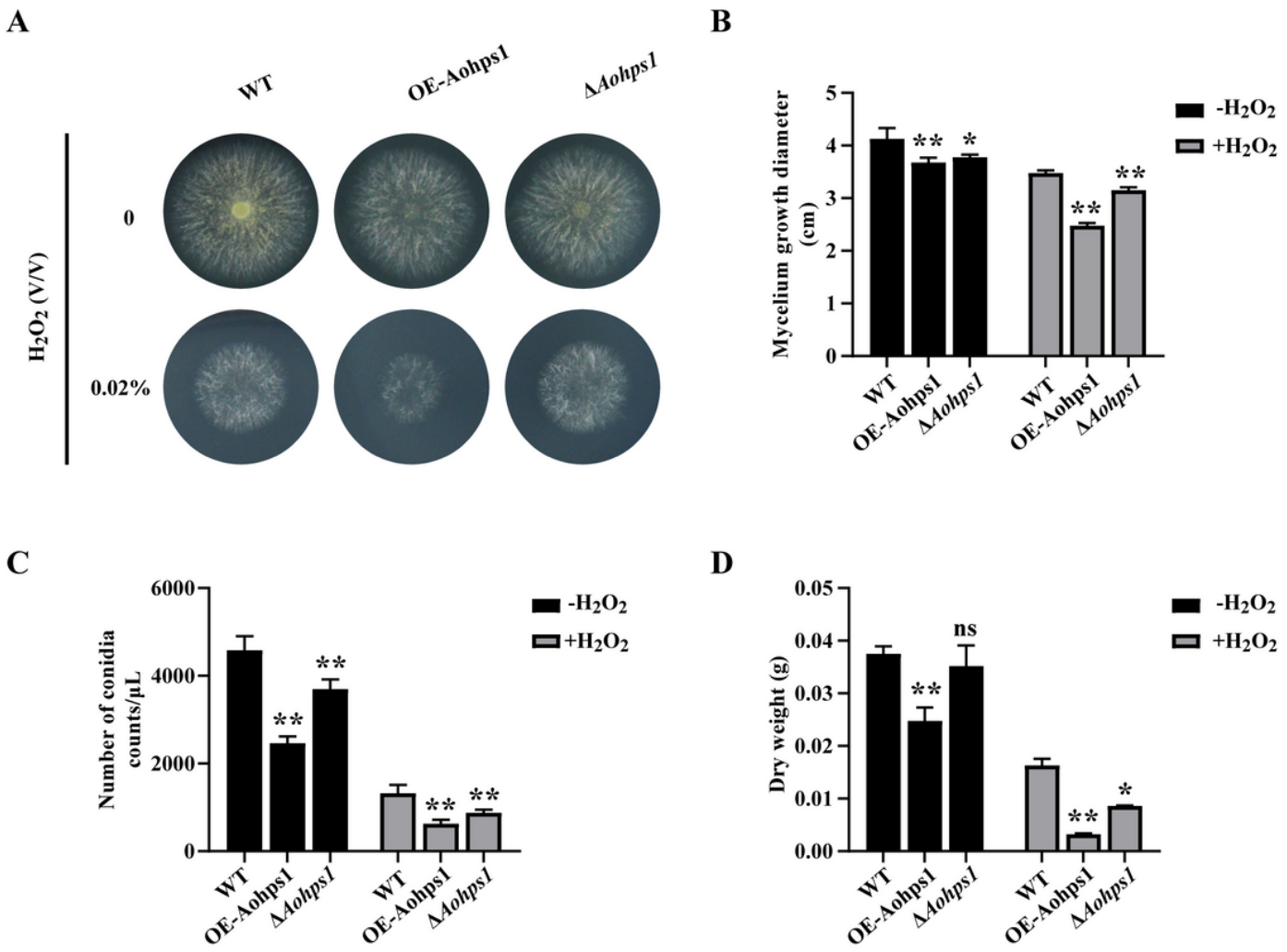

Figure 4

Phenotypes of Aohps1 overexpression and deletion strains compared with WT. (A) Growth of the WT, OE-Aohps1 and ΔAohps1 strains on CD agar medium with or without H2O2 for three days. (B, C, D) Comparison of mycelium growth diameter (B), conidia number (C), and biomass (D) in the WT, OE-Aohps1 and ΔAohps1 strains cultured on CD agar medium with or without H2O2 for three days.

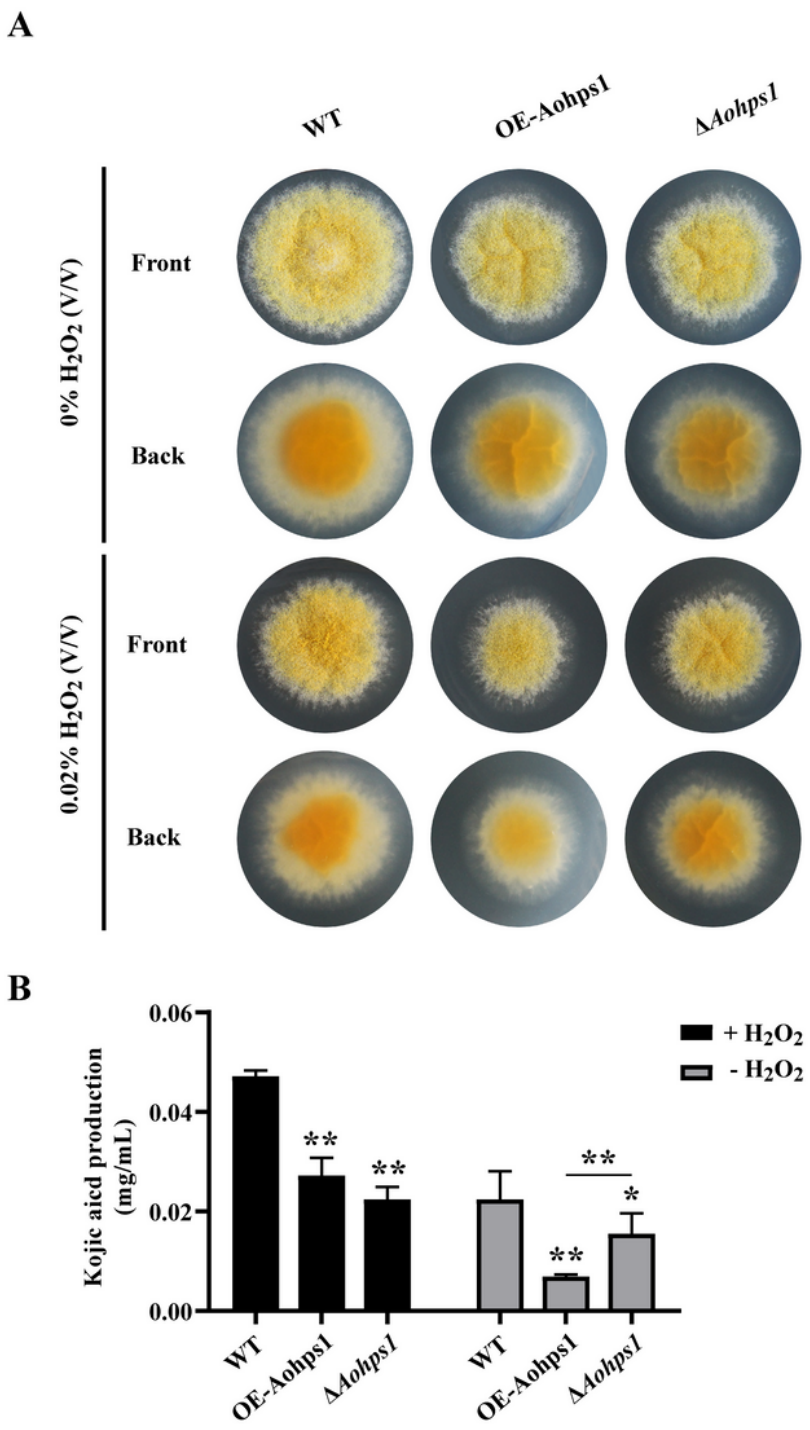

Figure 5

Effects of overexpression and disruption of Aohps1 on kojic acid synthesis. (A) Growth of WT, OE-Aohps1 and ΔAohps1 strains on modified CD agar medium with 500 μM FeCl3 for three days. Kojic acid forms a chelated compound with ferric ions, showing a red color. The modified CD medium supplemented with 2 % H2O2 (V/V) was used for oxidative stress. (B) Determination of the yield of kojic acid secreted into the

CD agar medium by WT, OE-Aohps1 and ΔAohps1 strains after cultivation for three days with and without H2O2 treatment. No significant difference (ns), *p < 0.05, and ** p < 0.01 by Student's t-test.

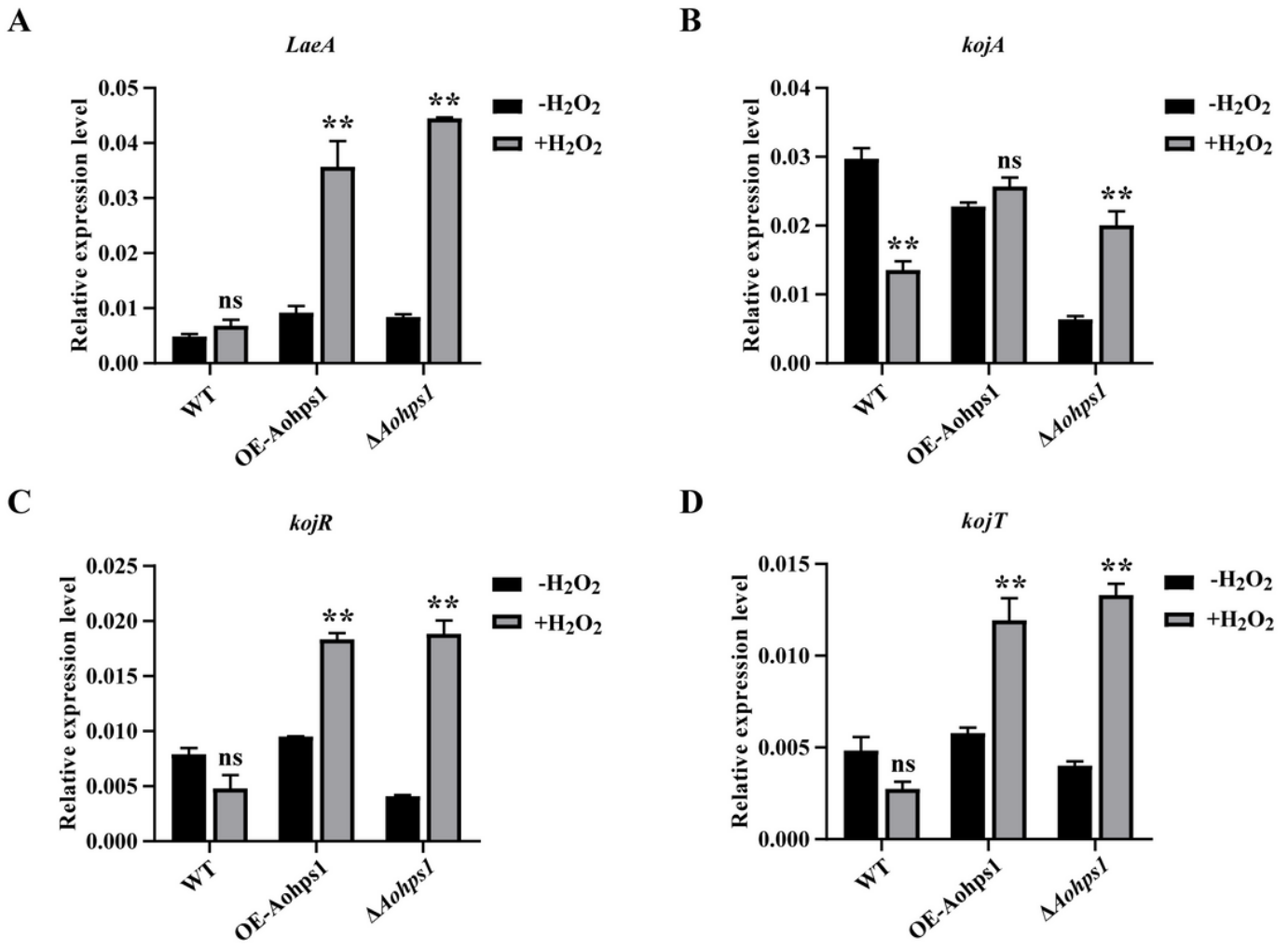

Figure 6

Expression analysis of kojic acid synthesis related genes in WT, OE-Aohps1 and ΔAohps1 strains. The strains were cultured in CD agar medium with or without H2O2 treatment for three days. Gene expression levels of LaeA (A), kojA (B), kojR (C) and kojT (D) involved in the synthesis of kojic acid were normalized to A.oryzae Histone H1gene expression levels. Error bars indicate standard error of the mean (n = 3).

Supplementary Files

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- Supplementarydata.docx