Introduction

Ubiquinone (UQ) consists of a quinone ring connected to a polyisoprenoid tail. The quinone ring structure of UQ is conserved among different organisms, but the length of the polyisoprenoid tail is species-dependent (Tsui & Clarke, 2019). In 2007, a UQ compound (antroquinonol, AQ) was isolated from the solid-state fermentation (SSF) products of Antrodia camphorata (Lee et al., 2007). Since then, AQ, 4-acetyl-AQ, AQD and other UQ compounds have also been isolated from the SSF products of A. camphorata (Wang et al., 2014; Yang et al., 2009). The structure of AQ compounds is very similar to UQ3, and they have high biological activity, such as anticancer, anti-inflammatory activity and the ability to alleviate alcoholic liver injury (Kumar et al., 2011; Lee et al., 2015; Lin et al., 2017).

The cultivation method has a major influence on the synthesis of bioactive compounds by A. camphorata. The basswood cultivation of A. camphorata results in fruit bodies that contain a variety of triterpenoids (Lin et al., 2011; Qiao et al., 2015). However, the bioactive compounds in SSF and liquid-state fermentation (LSF) products are quite different from those in the fruit bodies. Under the conditions of SSF, A. camphorata synthesizes a variety of AQ and antroquinone compounds (Xia et al., 2014). However, the mycelia of A. camphorata mainly contain antroquinone compounds and have almost no AQ under LSF conditions (Xia et al., 2019).

Improving the biosynthesis of active ingredients of A. camphorata during LSF has attracted much research attention. In a preliminary study, AQ was induced by adding UQ0 as a precursor during LSF, and oxidative stress was also found to be beneficial to the biosynthesis of AQ (Xia et al., 2017). We analyzed gene expression in A. camphorata at the transcriptional level and showed that genes related to the synthesis pathways of UQ and other terpenoid-quinones were upregulated by adding UQ0 to the isoprenoid side chain. Then, the Agrobacterium-mediated transformation system of A. camphorata was established and the genes for quinone ring modification (coq2-6) and isoprene synthesis (HMGR, fps) were overexpressed. The results showed that overexpression of genes for isoprene side chain synthesis could not increase the yield of AQ, but overexpression of coq2 and coq5 could significantly increase AQ production. This is consistent with the results of the experiment of precursors. It indicated that the A. camphorata lack the ability to modify the quinone ring of AQ during LSF. Of the modification steps, prenylation of UQ0 is the key step of AQ biosynthesis. The result will help us to understand the genetic evidence for the requirements of AQ biosynthesis in A. camphorata.

Keywords: Antrodia camphorata, Antroquinonol, Agrobacterium-mediated transformation system, Liquid-state fermentation, Biosynthesis
**Materials and Methods**

**Strains and Culture Conditions**

*A. camphorata* S-29 was obtained from the Shanghai Engineering Research Center of Food Microbiology (Shanghai, China). The *A. camphorata* S-29 strain was kept at the China General Microbiological Culture Collection Center (preservation number, CGMCC No. 2267) and cultured on potato dextrose agar (PDA) slants at 28 °C for 12 days, and then stored at 4 °C. Spores obtained from mycelium aged 12 days were collected by flooding the surface of the plates with 25 mL of sterile deionized water containing tween 80 (1 g/L). Five milliliters of the spore suspension (contain 1 × 10⁸ spores/mL) was used to inoculate 100 mL of seed culture medium (SCM) in a 500 mL Erlenmeyer flask. The SCM contains glucose 20 g/L, soy bean meal 4 g/L, corn steep liquor 20 mL/L, citric acid 0.5 g/L, MgSO₄·7H₂O 0.5 g/L and Na₂HPO₄ 0.5 g/L, pH 5.0. The 0.3 g/L UQ₀ was added into culture medium at 72 hr of fermentation.

To assess the ability of AQ production, the transformants of *A. camphorata* S-29 was cultured in LSF with the precursor of UQ₀.

**Cloning of the Genes from *A. camphorata***

Total RNA of *A. camphorata* S-29 was isolated from 5-day-old mycelium homogenized with liquid nitrogen and extracted using AxyPrep Total RNA Isolation Kit (Axygen, California, USA) according to the manufacturer’s instructions. cDNA was obtained by reverse transcription with a PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). For cloning of target genes (coq2, coq3, coq5, coq6, fps, HMGR), Oligo software 7.0 were used to design the primers (Table 1) based on the genomic DNA of *A. camphorata* S-29. The NcoI and BamHI restriction sites were introduced in sequence of target genes. The PCR products were purified and cloned into pMD18-T (TaKaRa, Japan) for sequencing (Invitrogen China, Shanghai). Briefly, Taq DNA polymerase (TransGen Biotech, China) was used in a 20 µL reaction mixture including 12.5 µL Taq premix, 1 µL of cDNA, 0.5 12.5 µL primers of target genes.

**Vector Construction and Agrobacterium Electroporation**

The vector pCAMBIA1301 used in this study was kindly provided Doctor Jianhua Li (CAS Center for Excellence in Molecular Plant Sciences). Six *A. tumefaciens* binary vectors based on pCAMBIA1301 were constructed as follows. The coding sequence of genes (coq2, coq3, coq5, coq6, fps, HMGR) were amplified with gene specific primers. To generate recombinant plasmids, pCAMBIA1301 was digested with NcoI and BstEII. Subsequently, the backbone of pCAMBIA1301 and the DNA fragments of target genes were assembled to produce the plasmids using CloneExpress Ultra One Step Cloning Kit (vazyme, Nanjin, China).

All vectors were introduced into *A. tumefaciens* LBA4404 through electroporation (BioRad, USA) and grown on YEP plate containing 50 µg/mL kanamycin and 50 µg/mL rifampin at 28 °C for 2 days. To identify positive clones, 12 single colonies of *A. tumefaciens* were picked and assayed by colony PCR with the primers of target genes.

**Sensitivity of *A. camphorata* S-29 to Hygromycin B**

The strain *A. camphorata* S-29 was inoculated on PDA medium supplemented with different hygromycin B concentrations (0, 20, 40, 60 and 80 µg/mL) and cultivated for 30 days at 28 °C. Three replicates were used during this experiment.

**Agrobacterium-Mediated Transformation of *A. camphorata***

The *A. tumefaciens* strains LBA4404, EHA105 and GV3101, which after harboring pCAMBIA1301 or recombinant plasmids were cultivated in YEB medium containing 50 µg/mL kanamycin, 50 µg/mL rifampin and 1 mmol/L acetosyringone to an OD₆₀₀ of 0.6 at 28 °C and 200 r/min. Then, the cells of *A. tumefaciens* were collected by centrifugation (4000 g for 5 min at 4 °C) and resuspended in LFM (containing 50 µg/mL kanamycin, 50 µg/mL rifampin and 1 mmol/L acetosyringone). For transformation, the mycelium of *A. camphorata* were co-cultured with *A. tumefaciens* in LFM for 2 days at 25 °C and plated onto PDA medium containing 20 µg/mL hygromycin and 50 µg/mL spectinomycin and cultured for 5 days at 25 °C.

To establish the optimal transformation conditions of *A. camphorata* S-29, the *A. tumefaciens* strains LBA4404, EHA105 and
GV3101 were selected for agrobacterium-mediated transformation. The cell age of *A. camphorata* S-29 was studied by testing the following time: 5, 10, 15, 20, 25 and 30 days. And the biomass of *A. camphorata* S-29 was set as 0.1, 0.5, 1, 10 and 20 mmol/L. The co-culture temperature of *A. camphorata* S-29 and *A. tumefaciens* was determined by testing different temperatures (22, 25 and 28 °C). And the co-culture time was 0, 1, 2, 3 and 4 days. The concentration of *A. tumefaciens* was set as OD_{600} of 0.25, 0.5, 1.0, 1.5 and 2.0. The duration of oscillation with glass beads was studied by testing the following time: 0.5, 1.0, 1.5 and 2.0 min. Different AS concentrations (0.1, 0.5, 1.0, and 20 mmol/L) were added to the LFM medium. AS-free medium was used as the control. All the experiments were carried out in triplicate.

Validation of Transformants and Stability Test
To confirm the incorporation of *hyg* gene, PCR analysis was performed. The genomic DNA of the transformed *A. camphorata* S-29 was extracted. The amplification of the *hyg* gene from the genome was performed with primers Hyg-F and Hyg-R (Table 1). PCR reaction parameters were as follows: 95 °C for 3 min, followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min, and followed by a final extension of 10 min at 72 °C.

To determine the stability of transformants, the transformants of *A. camphorata* S-29 were randomly selected and cultured on SCM plates containing 20 μg/mL of *hygromycin B* for eight generations to observe whether the transformants still showed resistance to hygromycin.

HPLC Analysis of Antroquinonol and Antrodin C
Dried *A. camphorata* mycelia was extracted with 95% alcohol under sonication (40 kHz) at 50 °C for 1 hr. Analytical HPLC (Waters 2695, USA) was performed to determine the AQ content with a C18 column (5 μm, 4.6 × 250 mm i.d., Sepax Co., Newark, DE, USA) and eluted with a gradient mobile phase at a flow rate of 1.0 mL/min. The signal was monitored with a UV detector set at 254 nm. The injection volume of samples was 20 μL. The mobile phase consisted of A: H_{2}O (pH 3 with TFA) and B: CH_{3}CN. The elution condition started at A:B = 65:35; 0–4 min, linear gradient to A:B = 43:57; 4–10 min, linear gradient to A:B = 30:70; 10–15 min, linear gradient to A:B = 10:90; 15–18 min, linear gradient to A:B = 0:100 and 18–28 min, A:B = 0:100 to A:B = 65:35.

Determination of the Biomass
The mycelia were collected by suction filtration through a filter-paper, followed by washing with distilled water three times and drying at 50 °C until a constant weight was reached. The biomass concentration was expressed as the dry weight of the mycelia per unit volume of culture medium.

Statistical Analysis
All the experiments were carried out in triplicate. Analysis of variance (ANOVA) and regression analyses were conducted by using the SPSS statistical software package. The results are expressed as the mean ± S.D.

Results

Resistance of *A. camphorata* S-29 to Hygromycin B
The susceptibility of *A. camphorata* S-29 to different concentrations of hygromycin B was tested in solid medium. As shown in Fig. 1A and B, the growth of *A. camphorata* S-29 was completely inhibited by hygromycin B at all tested concentrations (20, 40, 60 and 80 μg/mL). Therefore, 20 μg/mL of hygromycin B was selected for screening transformants of *A. camphorata* S-29.

Establishing a System for ATMT of *A. camphorata* S-29
Three agrobacterial strains, LBA4404, GV3101 and EHA105, were tested by co-culturing them with *A. camphorata* S-29 (0.5 g/mL) for 5 days. As shown in Fig. 2A, all three strains could be used to transform *A. camphorata* S-29; however, the transformation efficiencies of the strains were significantly different (p < 0.05). The number of transformants produced by LBA4404 was 1.66- and 2.07-fold higher than the number of strains produced by GV3101 and EHA105, respectively. Thus, strain LBA4404 was selected for subsequent studies.

The effects of different experimental conditions on the transformation efficiency of *A. camphorata* S-29 were investigated. As shown in Fig. 2B, the highest transformation efficiency was observed when *A. camphorata* S-29 was cultured for 5 days. While, with the extension of cell age, the germination time of *A. camphorata* S-29 increased significantly. As shown in Fig. 2C, the maximum number of transformants was obtained with an *A. camphorata* S-29 biomass of 0.5 g/mL. Further increases in biomass resulted in a clear decrease in the number of transformants.

Co-culture time and temperature also had significant effects on transformation efficiency. The transformation efficiency increased as the co-culture time increased to 2 days, beyond which,
a decrease in efficiency was observed (Fig. 2D). The maximum transformants number was obtained with a co-culture temperature of 25 °C (Fig. 2E) and when A. tumefaciens LBA4404 concentration was 0.5 (Fig. 2F). The transformants markedly increased as the LBA4404 concentration increased from 0.25 to 0.5. However, a further increase in the LBA4404 concentration led to a notable decrease in the number of transformants. As shown in Fig. 2G, the maximum number of transformants was obtained at an acetosyringone (AS) concentration of 1.0 mmol/L. An increase or decrease in the AS concentration from 1.0 mmol/L led to a reduction in the number of transformants. The maximum number of transformants was attained when the oscillation duration was 1.0 min (Fig. 2H). Further increases in the oscillation duration led to a notable decrease in the number of transformants.

Finally, after A. camphorata S-29 (0.5 g/mL) was cultured for 5 days, the culture was oscillated with for 1 min, and then co-cultured with A. tumefaciens LBA4404 (at an OD600 of 0.5) at 25°C for 2 days in a culture medium supplemented with 1 mmol/L AS. Under these conditions, the plasmid pCAMBIA1301 was integrated into the genome of A. camphorata S-29, resulting in stable hygromycin B resistance (Fig. 2I).

**Effect of Exogenous Addition of a Quinone Ring and Side Chain Precursors on AQ Synthesis**

The cultivation method has a major influence on the synthesis of bioactive compounds by A. camphorata. As shown in Fig. 3A and B, few bioactive compounds were synthesized during LSF, and UQ0 and AQ were not detected. However, we detected UQ0, AQ, antrodin C and other bioactive compounds after SSF. As shown in Fig. 3C, AQ is a structural analog of UQ3. Thus, we hypothesized that AQ biosynthesis is closely related to the UQ biosynthesis pathway in A. camphorata. To test this hypothesis, we selected three compounds (UQ0, farnesol and farnesyl diphosphate, FPP) to use as precursors for the quinone ring and side chain, to induce AQ biosynthesis during LSF. The results showed that UQ0 successfully induced AQ production; however, farnesol and FPP did not play a role in producing AQ (Fig. 3D–H). Further analysis showed that the combined induction of fermentation by UQ0 and farnesol or FPP did not significantly increase the yield of AQ (p > 0.05, Fig. 3I).

Several studies have shown that precursors induce the biosynthesis of AQ-like compounds during LSF (Chiang et al., 2013). However, the mechanism of AQ production during LSF remains
unclear. According to the above results, the precursor of the quinone ring plays an active role in the biosynthesis of AQ under LSF conditions. However, the precursor of the side chain may not affect AQ biosynthesis in *A. camphorata* under LSF conditions. This phenomenon may be due to the lack of appropriate substrates or poor absorption of the substrates by *A. camphorata*.

In a preliminary study, we analyzed the effect of UQ0 on gene transcription (Liu et al., 2020). Genes related to the biosynthesis pathways of UQ and other terpenoid-quinones (coq2, coq5, wrbA and aro8) were significantly upregulated when UQ0 was added to the fermentation broth at 72 hr. Thus, the addition of UQ0 seems to activate the synthesis of AQ during LSF. To increase the endogenous production of AQ precursors, we overexpressed genes involved in quinone ring modification and side chain synthesis for AQ biosynthesis, using a previously established ATMT system. We used this system to further verify the function of precursors in AQ biosynthesis by *A. camphorata* S-29.

**Construction of Expression Plasmid in *A. camphorata* S-29**

*A. camphorata* S-29 was inoculated in a seed culture medium for 4 days at 28 °C, and the mycelia were then collected for total RNA extraction. As shown in Fig. 4, the target genes coq2, coq3, coq5, HMGCR and fps were obtained by PCR amplification, after which, the target fragments were recovered and cloned (Fig. 4A). The expression plasmids pCAMBIA1301-coq2, pCAMBIA1301-coq3, pCAMBIA1301-coq5, pCAMBIA1301-coq6, pCAMBIA1301-HMGCR and pCAMBIA1301-fps were then constructed by seamless cloning of the target fragments into the respective vectors (Fig. 4B). The constructs were then transformed into competent *A. tumefaciens* LBA4404, and bacterial colony PCR verification was performed. The positively cloned strains were subsequently sent to Sangon Biotech Co., Ltd. (Shanghai, China) for sequence verification. Sequencing results showed that the recombinant vector was successfully constructed (Fig. 4C).

**A. tumefaciens-Mediated Genetic Transformation**

The expression plasmids were introduced into the *A. camphorata* S-29 genome using an *A. tumefaciens* LBA4404-mediated transformation system. Twelve transformants were randomly selected, and the wild-type strain was used as a control. The hygromycin resistance gene (*hyg*) was amplified by PCR, and as shown in Fig. 5A, it was found to be successfully inserted into the *A. camphorata* S-29 genome. The transformants of *A. camphorata* S-29 could stably contain hygromycin B 20 mg/L (Fig. 5B).
Effect of Overexpression of Quinone Ring Modification Genes on AQ Biosynthesis

The genes coq2, coq3, coq5 and coq6 encode a series of enzymes for quinone ring modifications, such as methylation, methoxylation and isoprenyl linking. These modifications play an important role in the biosynthesis of UQ (Stefely & Pagliarini, 2017). As shown in Fig. 6A, some of these genes had a significant impact on the efficiency of AQ synthesis. Compared to the control group (containing plasmid pCAMBIA 1301), overexpression of coq2 or coq5 significantly increased the yield of AQ from 0.34 mg/g biomass to 1.39 and 1.29 mg/g biomass, respectively. Overexpression of coq6 only slightly increased AQ production, whereas overexpression of coq3 had no effect. Overexpression of coq5 significantly increased antrodin C (Ac) yield from 1.39 to 2.38 mg/g biomass. The chromatograms of transformants overexpressing different target genes are shown in Fig. 6B–F. As shown in Table 2, compared with the control group (pC), overexpression of genes related to quinone ring modification has no inhibitory effect on growth of A. camphorata (except coq6). Thus, the genes coq2 and coq5 play important roles in the production of bioactive compounds, especially AQ. Differences in colony morphology were also noted between the wild-type strain and the transformants.

Effect of Overexpression of Side Chain Synthesis Genes on AQ Biosynthesis

Previous experiments showed that the addition of exogenous side chain precursors did not promote AQ synthesis during LSF. This may be because there are sufficient quantities of side chain precursors in A. camphorata S-29 during the process of LSF, or because the utilization efficiency of exogenously added precursors is extremely low. The genes fps and HMGR encode enzymes for the synthesis of isoprene side chains. Therefore, we overexpressed these two genes to increase the content of endogenous precursors, and further investigated the role of side chain precursors in the synthesis of AQ. As shown in Fig. 7A, overexpression of fps and HMGR genes did not increase AQ biosynthesis. In fact, the synthesis of AQ by A. camphorata S-29 decreased slightly. The biomass of A. camphorata was decreased by overexpression of HMGR (Table 2). This was consistent with the results of the fermentation experiments. However, it could be seen from the chromatogram that overexpression of the side chain synthesis genes promoted the generation of new compounds (retention time at 12.30 min, Fig. 7B–D). The chromatograms of the transformants overexpressing the fps and HMGR genes were different from those of the transformants overexpressing coq genes and those of the wild-type strain.

Discussion

The growth of A. camphorata is slow, and there are few reports on genetic transformation systems for this fungus. The genetic transformation methods for filamentous fungi mainly include polyethylene glycol-mediated transformation, electroporation, biolistics, restriction enzyme-mediated integration and ATMT (Kim & Song, 2004; Liu & Friesen, 2012; Szewczyk et al., 2013; Wang et al., 2014). The single-copy of A tumefaciens T-DNA has a high
integration efficiency and is easy to produce a large number of genetic-stable transformants (Ruiz-Díez, 2002).

We constructed an ATMT system for *A. camphorata* and used it to achieve stable expression of the *hyg* gene. The strain of *A. tumefaciens*, size of the target gene, induction conditions and concentration of the inducer have a significant impact on the transformation efficiency. *A. tumefaciens* could be divided into four types according to the toxicity, and the toxicity of the strain has a certain relationship with the transformation efficiency (Vladimirov et al., 2015). Besides, the transformation efficiency of fungi by *A. tumefaciens* is highly strain specific (Abello et al., 2008). The concentration ratio of *A. tumefaciens* to fungi will significantly affect the infection efficiency of *A. tumefaciens*. A low concentration of *A. tumefaciens* will lead to insufficient infection of the fungus and reduce the transformation rate, while, a high concentration will cause serious pollution and affect the growth of the fungus (Vladimirov et al., 2015). In the meantime, co-cultivation time will also affect the number of T-DNA insertions, thereby affecting the transformation efficiency. During the process of transformation, the chemotaxis of *A. tumefaciens* can be induced by phenolic compounds. Acetosyringone (AS) is a commonly used inducer, which can activate the *vir* region of the Ti plasmid to promote the transfer of T-DNA (Winans, 1992). A low concentration cannot activate the *vir* region, and too high will cause an increase in the number of copies of T-DNA inserted (Kemppainen et al., 2005). Consistent with other studies, we found that AS was necessary for the ATMT of *A. camphorata* and was able to induce the expression of *vir* region of T-DNA (Malonek & Meinhardt, 2001). The ATMT system was demonstrated to be a suitable method for the genetic transformation of *A. camphorata*. Exogenous genes were effectively integrated into the genome of *A. camphorata* using this system.

*A. camphorata* has attracted much attention because it contains a variety of bioactive compounds (Lu et al., 2013). However, the cultivation method has a major influence on the synthesis of bioactive compounds by *A. camphorata*. Previous studies have shown that AQ can only be synthesized by *A. camphorata* under SSF conditions, and there are few bioactive compounds synthesized during LSF (Xia et al., 2019). There are significant differences between SSF and LSF in dissolved oxygen, nutrient concentration distribution, etc. Compared with LSF, SSF has lower catabolite repression, resulting in higher yields of enzyme and other metabolites (Hölker et al., 2004). More important, the substrate of SSF has a large specific surface area, and the biological interface (Fungus hyphae and water) formed on it is conducive to the free diffusion of oxygen (Yovita et al., 2006). *A. camphorata* also grows on the surface of grains, and the free diffusion of oxygen could significantly increase the level of dissolved oxygen at the biological interface. Our further research shows that the higher level of dissolved oxygen is beneficial to the synthesis of AQ by *A. camphorata*. While, only through dissolved oxygen regulation (pure oxygen fermentation, hydrogen peroxide feeding, etc.), the yield of AQ is still low in LSF.

Through structural comparisons and precursor-addition experiments, the synthesis of AQ was shown to be closely related to the synthesis pathway of UQ. The biosynthesis of UQ begins with the fusion of the benzoquinone ring and the polyisoprenoid tail. Thus, common natural aromatic precursors of UQ are 4-hydroxybenzoic acid (4-HB) and para-aminobenzoic acid (pABA) (Marbois et al., 2010; Payet et al., 2016; Pierrel et al., 2010). Several
Fig. 6. Effect of overexpression of quinone ring modification genes from A. camphorata S-29 on AQ biosynthesis. A: Yield of AQ and Ac in different transformants. B: The colony morphology and chromatogram of transformant with pCAMBIA1301. C: The colony morphology and chromatogram of transformant with pCAMBIA1301-coq2. D: The colony morphology and chromatogram of transformant with pCAMBIA1301-coq3. E: The colony morphology and chromatogram of transformant with pCAMBIA1301-coq5. F: The colony morphology and chromatogram of transformant with pCAMBIA1301-coq6.

studies have shown that precursors of the benzoquinone ring induce the biosynthesis of AQ-like compounds, such as UQ0 and orsellinic acid, by A. camphorata during LSF (Chiang et al., 2013; Chou et al., 2017). However, according to our results, the efficiency of these precursors differs.

Precursors of UQ biosynthesis, such as tyrosine, 4-HB and pABA, have very low efficiency for, or are incapable of, inducing AQ synthesis. However, UQ0 efficiently induced the biosynthesis of AQ in A. camphorata under LSF conditions. UQ0 and AQ were not detected in the mycelia of A. camphorata under LSF conditions, but they were detected under SSF conditions. The ring structure of AQ is very similar to that of UQ0, which may reduce the number of ring modifications needed to yield AQ. Our results indicate that the addition of a benzoquinone ring is critical to the biosynthesis
### Table 2 Effect of Overexpression of Genes on Productivity of AQ and Biomass of *A. camphorata*

| Target gene | pC | coq2 | coq3 | coq5 | coq6 | fps | HMGR |
|-------------|----|------|------|------|------|-----|------|
| Biomass (g/L) | 9.10 ± 0.72abc | 8.11 ± 0.52ab | 9.83 ± 0.83c | 8.65 ± 0.55abc | 7.47 ± 0.83a | 7.87 ± 0.80abc | 7.07 ± 0.69a |
| Aq (mg/L) | 3.10 ± 0.26a | 11.25 ± 0.56c | 2.62 ± 0.17a | 11.17 ± 0.83c | 4.36 ± 0.34b | 2.25 ± 0.12a | 2.48 ± 0.13a |
| Ac (mg/L) | 12.36 ± 0.49b | 17.15 ± 0.64c | 8.42 ± 0.44a | 20.50 ± 1.71d | 16.74 ± 0.79c | 12.59 ± 1.25a | 11.97 ± 0.63a |

Results of experiments are express as the mean ± SEM for each experimental group (n=3). The significance of differences between the data was assessed using One-way ANOVA by Dunnett’s tests, with the level of significance set at *P* < 0.05.

![Graph A](image1.png)
![Graph B](image2.png)
![Graph C](image3.png)
![Graph D](image4.png)

**Fig. 7.** Effect of overexpression of side chain genes from *A. camphorata* S-29 on AQ biosynthesis. A: Yield of AQ and Ac in different transformants. B: The colony morphology and chromatogram of transformant with pCAMBIA1301. C: The colony morphology and chromatogram of transformant with pCAMBIA1301-fps. D: The colony morphology and chromatogram of transformant with pCAMBIA1301-HMGR.

The biosynthesis of UQ requires multiple conserved mitochondrial matrix enzymes (CoQ1–CoQ9) (Hughes et al., 2017; Tran & Clarke, 2007). Deletion of any one of the coq1–coq9 genes in yeast leads to total loss of UQ6 production (Do et al., 2001; Marbois et al., 2009). In this study, we cloned four genes related to the modification of the benzoquinone ring during UQ biosynthesis by *A. camphorata*. Of these genes, coq2 was found to play an important role in the biosynthesis of AQ in *A. camphorata* and overexpression of coq2 increased the yield of AQ after LSF. CoQ2, a membrane-bound prenyltransferase, is responsible for prenyl transfer reactions using aromatic substrates in the initial stages of UQ biosynthesis (Cheng & Li, 2014; Quinzii et al., 2006). Therefore, the efficient FPP transfer ability of the COQ2 enzyme in *A. camphorata* is relatively unique. Generally, the activity of polyprenyltransferases is not specific to the length of the polyisoprenoid tail during UQ biosynthesis (Ashby et al., 1992; Gin & Clarke, 2005). Overexpression of coq5 significantly increased the yield of AQ, while overexpression of AQ during LSF. The mycelia of *A. camphorata* lack the ability to synthesize the quinone rings of AQ during LSF. However, the mechanism of inducing AQ synthesis during LSF remains unclear.
of coq3 and coq6 genes had no significant effect. CoQ5 is responsible for the methylation of benzene rings (Barkovich et al., 1997). CoQ3 is a type of O-methyltransferase that is responsible for two methoxylation reactions of benzene rings. CoQ6 is responsible for the C5-hydroxylation of the quinone ring of UQ. The AQ content was also increased by overexpression of coq5. Generally, the length of the side chain of UQ does not affect the substrate-recognition ability of the COQ5 protein. More importantly, the COQ5 protein is required for the stability of the supramolecular complex involved in UQ biosynthesis (Baba et al., 2004). During LSF, UQ0 may be utilized as a precursor of the quinone ring for AQ biosynthesis, thereby reducing the subsequent number of modifications required to synthesize the quinone ring of AQ. However, the function of coq5 in this process remains unclear.

The precursors of the polyisoprenoid tail failed to induce AQ synthesis during LSF. Thus, we hypothesize that the quantity of the isoprenoid side chain in the system was sufficient, and it may not be a limiting factor for AQ biosynthesis by A. camphorata under LSF conditions. In eukaryotes, the isoprenoid side chain used for UQ biosynthesis is generated via the mevalonate pathway (Grunler et al., 1994). Therefore, we selected two genes involved in this pathway and investigated their effect on AQ production. The results showed that overexpressing the fps and HMGR genes did not increase the yield of AQ under LSF conditions. However, overexpression of these two genes induced the synthesis of an unknown compound visualized on the chromatogram at a retention time of 12.30 min. These results indicated that increasing the quantity of the polyisoprenoid tail failed to promote the synthesis of AQ. This is consistent with the results of precursor addition in previous experiments. The HMGR gene encodes an enzyme that catalyzes the synthesis of mevalonate from HMG-CoA, which is the first rate-limiting step of terpenoid biosynthesis (Ayte et al., 1990). Many terpenoid compounds are produced by A. camphorata (Geethangili & Tzeng, 2011). Therefore, overexpression of HMGR may promote the flow of products of the mevalonate pathway to terpenoid synthesis, rather than to UQ synthesis. The fps gene encodes a type of prenyltransferase that is responsible for the condensation reaction of isopentenyl pyrophosphate with dimethylallyl pyrophosphate, to produce FPP (Chappell, 1995). Although the overexpression of fps does not increase the yield of AQ, it has a key effect on the length of the isoprene side chain (Okada et al., 1996; Okada et al., 1998). The fps gene is important for the synthesis of precursors of various types of metabolites in microorganisms and plants, such as
triterpenoids, sterols and UQs (Newman & Chappell, 1999). As shown in Fig. 8, there are three pathways for quinone ring synthesis of AQ (Chou et al. 2017; Yang et al. 2017). However, there are still many unclear steps in the AQ synthesis pathway, especially the quinone ring modification process. As a precursor, UQ0 could significantly reduce the synthesis steps of AQ. It is helpful to study the role of isoprene side chain and quinone ring in the synthesis of AQ.

Conclusion

A. camphorata is a unique medicinal fungus in Taiwan, containing a variety of active compositions, of which AQ, a ubiquinone compound, has significant antitumor activities. While, the AQ could not be synthesized in the LSF. To study the biosynthesis of AQ and its regulation mechanism, the ATMT system of A. camphorata S-29 was established. The genes related to modification of benzoquinone ring and isoprenoid side chain synthesis were overexpressed using ATMT system in A. camphorata S-29. Overexpression of coq2 and coq3 increased the yield of AQ significantly, while overexpression of fps and HMGR could not promote the biosynthesis of AQ in A. camphorata under the condition of LSF. Combine the results of precursors, the addition of benzoquinone ring is critical to the biosynthesis of AQ in LSF. The mycelium of A. camphorata lack the ability to synthesize or modification quinone rings of AQ in LSF.

Funding

This work was supported by the Natural Science Foundation of China (grant number 31871757), the Shanghai Agriculture Applied Technology Development Program (grant number 2019-02-08-00-07-F01152), the Shanghai Engineering Research Center of food microbiology program (grant number 19DZ2281100).

Data Availability

All data analyzed in this study are included in this published article.

Conflict of Interest

The authors declare no conflict of interest.

References

Abello, J., Kelemu, S., & Garcia, C. (2008) Agrobacterium-mediated transformation of the endophytic fungus Acremonium implicatum associated with Brachiaria grasses. Mycological Research, 112, 407–413.

Ashby, M.N., Kutsunai, S.Y., Ackerman, S., Tzagoloff, A., & Edwards, P.A. (1992) COQ2 is a candidate for the structural gene encoding parahydroxybenzoate: Polyprenyltransferase. Journal of Biological Chemistry, 267, 4128–4136.

Ayte, J., Gil-Ghmez, G., Haro, D., Marrero, P.F., & Hegardt, F.G. (1990) Rat mitochondrial and cytosolic 3-hydroxy-3-methylglutaryl-CoA synthases are encoded by two different genes. Proceedings of the National Academy of Sciences, 87, 3874–3878.

Baba, S.W., Belogrudov, G.I., Lee, J.C., Lee, P.T., Strahan, J., Shepherd, J.N., & Clarke, C.F. (2004) Yeast coq5 C-methyltransferase is required for stability of other polypeptides involved in coenzyme q biosynthesis. Journal of Biological Chemistry, 279, 10052–10059.

Barkovich, R.J., Shtanko, A., Shepherd, J.A., Lee, P.T., Myles, D.C., Tzagoloff, A., & Clarke, C.F. (1997) Characterization of the Coq5 gene from Saccharomyces cerevisiae. Evidence for a C-methyltransferase in ubiquinone biosynthesis. Journal of Biological Chemistry, 272, 9182–9188.

Bourras, S., Rouxel, T., & Meyer, M. (2015) Agrobacterium tumefaciens gene transfer: How a plant pathogen hacks the nuclei of plant and nonplant organisms. Phytopathology, 105, 1288–1301.

Chappell, J. (1995) Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Annual Review of Plant Physiology and Plant Molecular Biology, 46, 521–547.

Cheng, W. & Li, W. (2014) Structural insights into ubiquinone biosynthesis in membranes. Science, 343, 878–881.

Chiang, C.C., Huang, T.N., Lin, Y.W., Chen, K.H., & Chiang, B.H. (2013) Enhancement of 4-acetylantiroquinonol B Production by Supplementation of Its Precursor during Submerged Fermentation of Antrodia cinnamomea. Journal of Agricultural and Food Chemistry, 61, 9160–9165.

Chou, C.C., Yang, S.H., Wu, H.L., Lin, P.Y., Chang, T.L., Sheu, F., Chen, K.H., & Chiang, B.H. (2017) Biosynthesis of Antroquinonol and 4-Acylantiroquinonol B via a Polyketide Pathway Using Osrellinic Acid as a Ring Precursor in Antrodia cinnamomea. Journal of Agricultural and Food Chemistry, 65, 74–86.

Do, T.Q. (2001) A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in Saccharomyces cerevisiae abc1 mutants. Journal of Biological Chemistry, 276, 18161–18168.

Geethangili, M. & Tseng, Y.M. (2011) Review of pharmacological effects of antrodia camphorata and its bioactive compounds. Evidence Based Complementary and Alternative Medicine, 1741–427X, 212641.

Gin, P. & Clarke, C.F. (2005) Genetic evidence for a multi-subunit complex in coenzyme Q biosynthesis in yeast and the role of the Coq1 hexaprenyl diphosphate synthase. Journal of Biological Chemistry, 280, 2676–2681.

Grunler, J., Ericsson, J., & Dallner, G. (1994) Branch-point reactions in the biosynthesis of cholesterol, dolichol, ubiquinone and prenylated proteins. Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism, 1212, 259–277.

Hölker, U., Höfer, M., & Lenz, J. (2004) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Applied Microbiology and Biotechnology, 64, 175–186.

Hu, Y.D., Zhang, B.B., Xu, G.R., Liao, X.R., & Cheung, P.C.K. (2016) A mechanistic study on the biosynthetic regulation of bioactive metabolite Antroquinonol from edible and medicinal mushroom Antrodia camphorata. Journal of Functional Foods, 25, 70–79.

Hughes, B.G., Harrison, P.M., & Hekimi, S. (2017) Estimating the occurrence of primary ubiquinone deficiency by analysis of large-scale sequencing data. Scientific Reports, 7, 17744.

Kemppainen, M., Circosta, A., Tagu, D., Martin, F., & Pardo, AG. (2005) Agrobacterium mediated transformation of the ectomycorrhizal symbiont Laccaria bicolor S238N. Mycorrhiza, 16, 19–22.

Kim, S. & Song, J.H. (2004) Genetic transformation and mutant isolation in Ganoderma lucidum by restriction enzyme-mediated integration. FEMS Microbiology Letters, 233, 201–204.

Kumar, K.J.S., Chu, F.H., Hsieh, H.W., Liao, J.W., Li, W.H., Lin, C.C., Shaw, J.F., & Wang, S.Y. (2011) Antroquinonol from ethanolic extract of mycelium of Antrodia cinnamomea protects hepatic cells from ethanol-induced oxidative stress through Nrf-2 activation. Journal of Ethnopharmacology, 136, 168–177.

Lee, T.H., Lee, C.K., Tsou, W.L., Liu, S.Y., & Wen, W.C. (2007) A new cytotoxic agent from solid-state fermented mycelium of Antrodia camphorata. Planta Medica, 73, 1412–1415.
Lee, W.T., Lee, T.H., Cheng, C.H., Chen, K.C., Chen, Y.C., & Lin, C.W. (2015) Antroquinol from Antrodia Camphorata suppresses breast tumor migration/invasion through inhibiting ERK-AP-1 and Akt-NF-kappaB-dependent MMP-9 and epithelial-mesenchymal transition expressions. *Food and Chemical Toxicology*, 78, 33–41.

Lin, H.C., Lin, C.W., Wu, C.H., Cheng, C.H., Mi, F.L., Liao, J.H., Chen, K.C., Lin, M.H., & Wu, T.H. (2017) Antroquinol, a Ubiquinone Derivative from the Mushroom Antrodia camphorata, Inhibits Colon Cancer Stem Cell-like Properties: Insights into the Molecular Mechanism and Inhibitory Targets. *Journal of Agricultural and Food Chemistry*, 65, 51–59.

Lin, T.Y., Chen, C.Y., Chien, S.C., Hsiao, W.W., & Wang, S.Y. (2011) Metabolite Profiles for Antrodia cinnamomea Fruiting Bodies Harvested at Different Culture Ages and from Different Wood Substrates. *Journal of Agricultural and Food Chemistry*, 59, 7626–7635.

Liu, X.F., Xia, Y.J., Zhang, Y., Sang, K.K., Xiong, Z.Q., Liu, X.X., & Ai, L.Z. (2020) RNA-Seq transcriptomic analyses of Antrodia camphorata to determine antroquinol and antrodin C biosynthetic mechanisms in the in situ extractive fermentation. *Journal of the Science of Food and Agriculture*, 100, 4252–4262.

Liu, X.F., Xia, Y.J., Zhang, Y., Liang, L.H., Xiong, Z.Q., Song, X., & Ai, L.Z. (2021) Enhancement of antroquinol production via the overexpression of 4-hydroxybenzoate polyphenyltransferase biosynthesis-related genes in Antrodia cinnamomea. *Phytochemistry*, 184, 112677.

Liu, Z. & Friesen, T.L. (2012) Polyethylene glycol (PEG)-mediated transformation in filamentous fungal pathogens. Methods in *Molecular Biology*, 835, 365–375.

Lu, M.C., El-Shazy, M., Wu, T.Y., Du, Y.C., Chang, T.T., Chen, C.F., Hsu, Y.M., Lai, K.H., Chiu, C.P., & Chang, F.R. (2013) Recent research and development of Antrodia cinnamomea. *Pharmacology & Therapeutics*, 139, 124–156.

Malonek, S. & Meinhardt, F. (2001) *Agrobacterium* tumefaciens-mediated genetic transformation of the phytopathogenic ascomycete *Calonectria morganii*. *Current Genetics*, 40, 152–155.

Marbois, B., Gin, P., Gulemezian, M., & Clarke, C.F. (2009) The yeast Coq4 polypeptide organizes a mitochondrial protein complex essential for coenzyme Q biosynthesis. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1791, 69–75.

Marbois, B., Xie, L.X., Choi, S., Hirano, K., Hyman, K., & Clarke, C.F. (2010) para-Aminobenzoic acid is a precursor in coenzyme Q6 biosynthesis in Saccharomyces cerevisiae. *Journal of Biological Chemistry*, 285, 27827–27838.

Newman, J.D. & Chappell, J. (1999) Isoprenoid biosynthesis in plants: Carbon partitioning within the cytoplasmic pathway. *Critical Reviews in Biochemistry and Molecular Biology*, 34, 95–106.

Okada, K., Kainou, T., Matsuda, H., & Kawamukai, M. (1998) Methodological significance of the side chain length of ubiquinone in *Saccharomyces cerevisiae*. *FEBS Letters*, 431, 241–244.

Okada, K., Suzuki, K., Kamiya, Y., Zhu, X., Fujisaki, S., Nishimura, Y., Nishino, T., Nakagawa, T., Kawamukai, M., & Matsuda, H. (1996) Polypropenyl diprophosphate synthase essentially defines the length of the side chain of ubiquinone. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*, 1302, 217–223.

Payet, L.A., Leroux, M., Willson, J., Khara, A., Pelosi, L., & Pierrel, F. (2016) Mechanistic details of early steps in coenzyme Q biosynthesis pathway in yeast. *Cell Chemical Biology*, 23, 1241–1250.

Pierrel, F., Hamelin, O., Douki, T., Kieffer-Jaquino, S., Mühlenhoff, U., Ozeir, M., Lill, R., & Fontecave, M. (2010) Involvement of mitochondrial feredoxin and para-aminobenzoic acid in yeast coenzyme Q biosynthesis. *Chemistry & Biology*, 17, 449–459.

Qiao, X., Wang, Q., Ji, S., Huang, Y., Liu, K.D., Zhang, Z.X., Bo, T., Tzeng, Y.M., Guo, D.A., & Ye, M. (2015) Metabolites identification and multi-component pharmacokinetics of ergostane and lanostane triterpenoids in the anticancer mushroom Antrodia camphorata. *Journal of Pharmaceutical and Biomedical Analysis*, 111, 266–276.

Quinzii, C., Naini, A., Salvioni, I., Trevison, E., Navas, P., Dimaro, S., & Hirano, M. (2006) A mutation in para-hydroxybenzoate polyphenyltransferase (CoQ2) causes primary coenzyme Q10 deficiency. *The American Journal of Human Genetics*, 78, 345–349.

Ruiz-Díez, B. (2002) Strategies for the transformation of filamentous fungi. *Journal of Applied Microbiology*, 92, 189–195.

Sambrook, J. & Russell, D.W. (2001) *Molecular cloning*. (3rd ed.) Cold Spring Harbor.

Stefely, J.A. & Pagliarini, D.J. (2017) Biochemistry of mitochondrial coenzyme Q biosynthesis. *Trends in Biochemical Sciences*, 42, 824–843.

Szewczyk, E., Kasuga, T., & Fan, Z. (2013) Efficient sequential repetitive gene deletions in *Neurospora crassa* employing a self-excising β-recombinase/six cassette. *Journal of Microbiological Methods*, 92, 236–243.

Tran, U.C. & Clarke, C.F. (2007) Endogenous synthesis of coenzyme Q in eukaryotes. *Mitochondrion*, 7, 562–571.

Tsui, H.S. & Clarke, C.F. (2019) Ubiquinone Biosynthetic Complexes in Prokaryotes and Eukaryotes. *Cell Chemical Biology*, 26, 465–467.

Vladimirov, I.A., Matveeva, T.V., & Lutova, I.A. (2015) Oxip biosynthesis and catabolism genes of *Agrobacterium* tumefaciens and *Agrobacterium rhizogenes*. *Russian Journal of Genetics*, 51, 121–129.

Wang, D., Dan, H., Li, G., Song, G., & Li, W. (2014) An efficient tool for random insertional mutagenesis: *Agrobacterium* tumefaciens-mediated transformation of the filamentous fungus *Aspergillus terreus*. *Journal of Microbiological Methods*, 98, 114–118.

Wang, S.C., Lee, T.H., Hsu, C.H., Chang, Y.J., Chang, M.S., Wang, Y.C., Ho, Y.S., Wen, W.C., & Lin, R.K. (2014) Antroquinol D isolated from *Antrodia camphorata*, with DNA demethylation and anticancer potential. *Journal of Agricultural and Food Chemistry*, 62, 5625–5635.

Winans, SC. (1992) Two-way chemical signaling in *Agrobacterium*-plant interactions. *Microbiological Reviews*, 56, 12–31.

Xia, Y.J., Wang, Y.L., Zhang, B.B., Xu, G.R., & Li, W. (2014) Effect of cultural conditions on antrodin C production by basidiomycete *Antrodia camphorata* in solid state fermentation. *Biotechnology and Applied Biochemistry*, 61, 724–732.

Xia, Y.J., Chen, Y., Liu, X.F., Zhou, X., Wang, Z.C., Wang, G.Q., Xiong, Z.Q., & Ai, L.Z. (2019) Enhancement of Antroquinol production via a pathfreder detailed pathway during batch fermentation using pH control coupled with an oxygen vector. *Journal of the Science of Food and Agriculture*, 99, 449–456.

Xia, Y.J., Zhou, X., Wang, G.Q., Zhang, B.B., Xu, G.R., & Ai, L.Z. (2017) Induction of Antroquinol Production by Addition of Hydrogen Peroxide in the Fermentation of Antrodia camphorata S-29. *Journal of the Science of Food and Agriculture*, 97, 595–599.

Yang, S.S., Wang, G.J., Wang, S.Y., Lin, Y.Y., Kuo, Y.H., & Lee, T.H. (2009) New constituents with iNOS inhibitory activity from mycelium of *Antrodia camphorata*. *Planta Medica*, 75, 512–516.

Yang, S.H., Lin, Y.W., & Chiang, B.H. (2017) Biosynthesis of 4-acetylantroquinol B in *Antrodia cinnamomea* via a pathway related to coenzyme Q synthesis. *Biochemical Engineering Journal*, 117, 23–29.

Yovita, SP. & Arjen, R. (2006) Modeling conversion and transport phenomena in solid-state fermentation: A review and perspectives. *Biotechnology Advances*, 24, 161–179.