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Genetic evidence for the requirements of antroquinonol biosynthesis by *Antrodia camphorata* during liquid-state fermentation

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Abstract: The solid-state fermentation of *Antrodia camphorata* could produce a variety of ubiquinone compounds, such as antroquinonol (AQ). However, AQ is hardly synthesized during liquid-state fermentation (LSF). To investigates the mechanism of AQ synthesis, three precursors (ubiquinone 0 UQ0, farnesol and farnesyl diphosphate FPP) were added in LSF. The results showed that UQ0 successfully induced AQ production; however, farnesol and FPP could not induce AQ synthesis. The precursor that restricts the synthesis of AQ is the quinone ring, not the isoprene side chain. Then, the *Agrobacterium*-mediated transformation system of *A. camphorata* was established and the genes for quinone ring modification (*coq*2-6) 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 *coq*2 and *coq*5 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 camphorate*, Antroquinonol, *Agrobacterium*-mediated transformation system, Liquid-state fermentation, Biosynthesis

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, AQB, 4-acetyl-AQB, 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 antrodin compounds (Xia et al., 2014). However, the mycelia of *A. camphorata* mainly contain antrodin 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 *in situ* extractive fermentation system (Liu et al., 2020). UQ0 upregulates the expression of S-adenosylmethionine synthetase and provides a methyl group for the biosynthesis of AQ (Hu et al., 2016). Moreover, UQ0 induces the biosynthesis of 4-acetyl-AQ (Chiang et al., 2013). The quinone ring of AQ and 4-acetyl-AQ are synthesized via the polyketide pathway (Chou et al., 2017).

Although previous studies have shown that the biosynthetic pathway of AQ is related to UQ synthesis, and the 4-hydroxybenzoate polyprenyltransferase is important to AQ biosynthesis (Liu et al., 2021), the effect of other key enzymes of related to UQ synthesis on AQ biosynthesis remains unclear. *Agrobacterium tumefaciens* could integrate the T-DNA region of the Ti plasmid into the host genome (Bourras et al., 2015). Utilizing this feature, *A. tumefaciens* is widely used in the genetic transformation of plants and eukaryotic microorganisms. In this study, we established an *Agrobacterium*-mediated transformation (ATMT) method for *A. camphorata*. The effect of several key genes related to quinone ring modification and isoprene side chain synthesis in *A. camphorata* on AQ production was studied. These data will increase our understanding of the mechanism of AQ synthesis during LSF.

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Table 1 Primers Used in This Experiment

Primers	Sequences
coq2-F	tggagagaacacgggggactcttgaCCATGGATGTCTCCGAGTCGTTCTACTG
coq2-R	gtttgaacgatcggggaaattcgagctGGTAACTCATGCAAGCTGCGGGTGCT
coq3-F	ggagagaacacgggggactcttgaCCATGGATGAACCCAGTAAGGATGCAA
coq3-R	gtttgaacgatcggggaaattcgagctGGTAACTCATTCCATCGGCCTTCTTA
coq5-F	ggagagaacacgggggactcttgaCCATGGATGTCTCAGGAGAAGAAGGG
coq5-R	tgtttgaacgatcggggaaattcgagctGGTAACTTAGACGCGGCAAAGGATTT
coq6-F	ggagagaacacgggggactcttgaCCATGGATGTTGTATTGGCGTGCGAA
coq6-R	gtttgaacgatcggggaaattcgagctGGTAACTCATCTCGAGCCCAATACCT
fps-F	ggagagaacacgggggactcttgaCCATGGATGACCACGAAGGATGAACT
fps-R	gtttgaacgatcggggaaattcgagctGGTAACCTACTCGGTAACTCACTTCGT
HMGR-F	ggagagaacacgggggactcttgaCCATGGATGGTGCGACAGTCGTTCCAC
HMGR-R	gtttgaacgatcggggaaattcgagctGGTAACTCATGTTCATGCCCATGGCGT
hyg-F	CTATTTCTTTGCCCTCGGAC
hyg-R	CCTGACCTATTGCATCTCCC

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 were 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 1×10^8 spores/mL) was used to inoculate 100 mL of seed culture medium (SCM) in a 500 mL Erlenmeyer flask. The SCM contained glucose 20 g/L, soy bean meal 4 g/L, corn steep liquor 20 mL/L, citric acid 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L and Na_2HPO_4 0.5 g/L, pH 5.5.

To assess the ability of AQ production, the transformants of *A. camphorata* S-29 was cultured in LSF with the precursor of UQ0. The culture medium contains glucose 60 g/L, soy bean meal 4 g/L, corn steep liquor 8 mL/L, citric acid 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L and Na_2HPO_4 0.5 g/L, pH 5.0. The 0.3 g/L UQ0 was added into culture medium at 72 hr of fermentation.

A. tumefaciens strains LBA4404, GV3101 and EHA105 were a kind of gift from Doctor Jianhua Li (CAS Center for Excellence in Molecular Plant Sciences), grown in YEP medium (yeast extract 5 g/L, tryptone 10 g/L, sucrose 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L pH 7.0). *Escherichia coli* Top 10 was manipulated (Sambrook & Russell, 2001).

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 *Nco* I and *BstE* II restriction sites was 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 *Nco* I and *Bst* EII. Subsequently, the backbone of pCAMBIA1301 and the DNA fragments of target genes were assembled to produce the plasmids using ClonExpress 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 OD_{600} 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

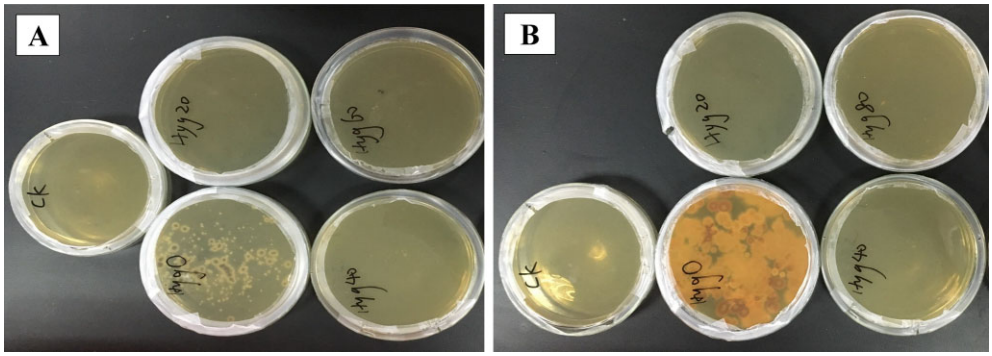

Fig. 1. Effect of different concentrations of hygromycin B on the growth of *A. camphorata* S-29. A: The cell age of *A. camphorata* S-29 was 8 days; B: The cell age of *A. camphorata* S-29 was 31 days. Cells growth at 20, 40, 60 and 80 mg/L hygromycin B and the plate without antibiotic was set as a control.

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, 0.2, 0.5 and 1.5 g/mL. 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, 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 (0.1,0.5,1,10 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_2O (pH 3 with TFA) and B: CH_3CN. 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,

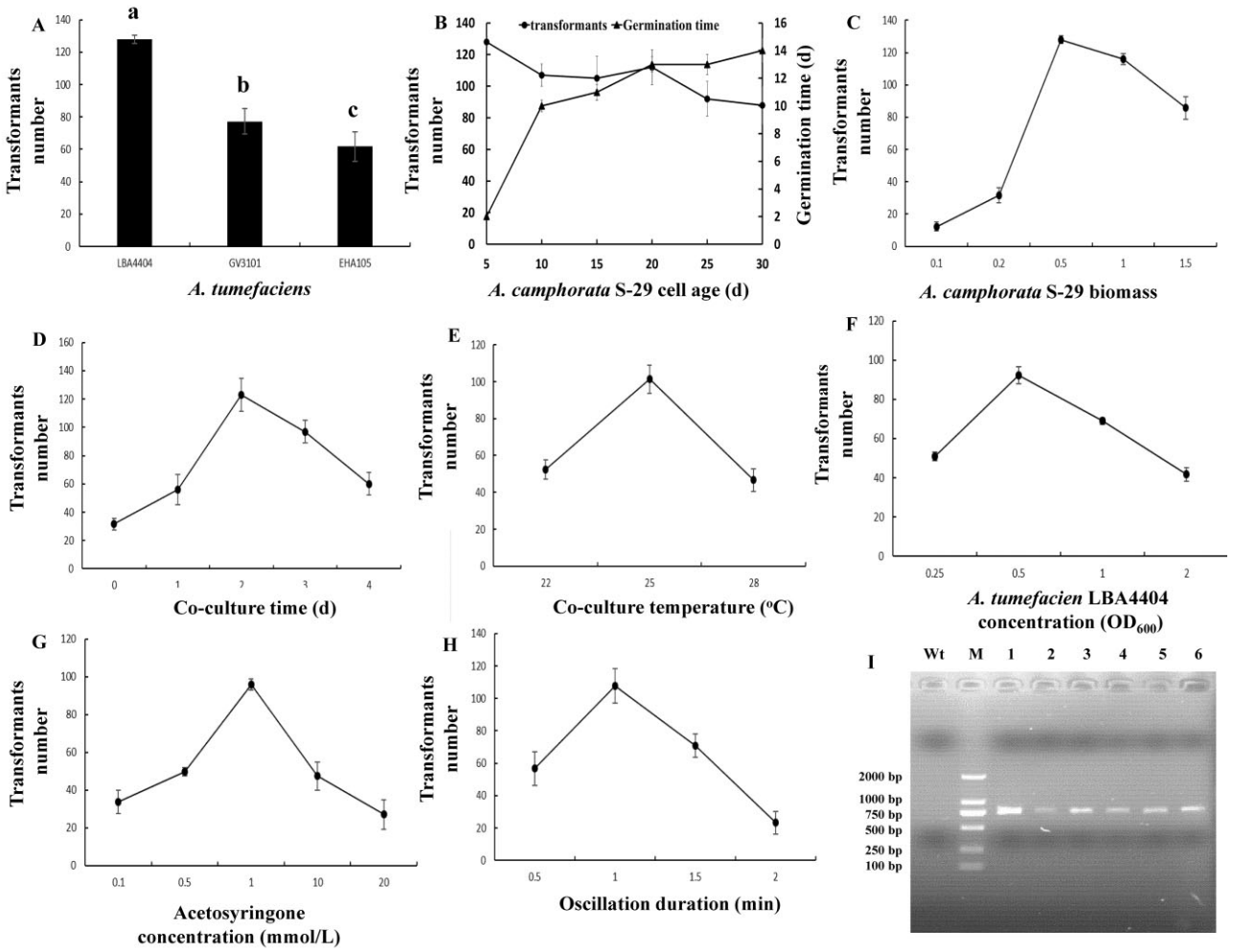

Fig. 2. Agrobacterium-mediated transformation system of the *A. camphorata* S-29. A: Selection of *A. tumefaciens*; B: *A. camphorata* S-29 cell age; C: *A. camphorata* S-29 biomass; D: Co-culture time of *A. tumefacien* LBA4404 and *A. camphorata* S-29; E: Co-culture temperature; F: *A. tumefacien* LBA4404 concentration; G: Acetosyringone concentration; H: Oscillation duration; I: PCR detection of *hyg* gene in the transformants of *A. camphorata* S-29 (wt: wild-type; M: Maker of DL2000; 1-6: Transformants of *A. camphorata* S-29). Results 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 $p < 0.05$.

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. tumefacien* 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 OD_{600} 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 thatUQ0 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

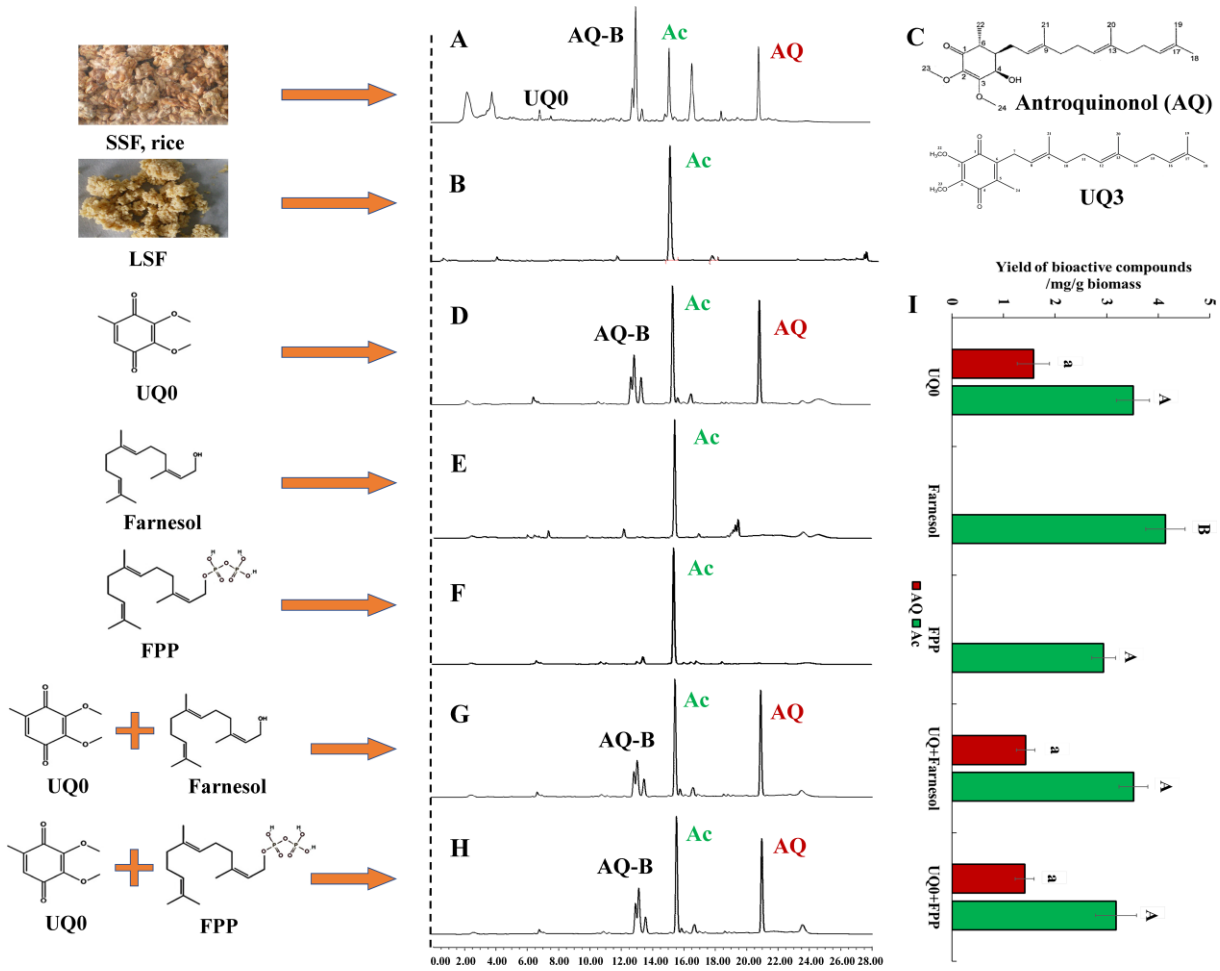

Fig. 3. Evaluation the role of exogenous addition precursors on AQ biosynthesis. A: Chromatogram of *A. camphorata* in SSF; B: Chromatogram of *A. camphorata* in LSF; C: AQ was a structural analog of UQ3; D: Chromatogram of *A. camphorata* in LSF with addition of UQ0; E: Chromatogram of *A. camphorata* in LSF with addition of farnesol; F: Chromatogram of *A. camphorata* in LSF with addition of FPP; G: Chromatogram of *A. camphorata* in LSF with addition of UQ0 and farnesol; H: Chromatogram of *A. camphorata* in LSF with addition of UQ0 and FPP; I: Yield of AQ and Ac in different groups.

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*, *coq6*, *HMGR* 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-*HMGR* 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 in PDA plate contain hygromycin B 20 mg/L (Fig. 5B).

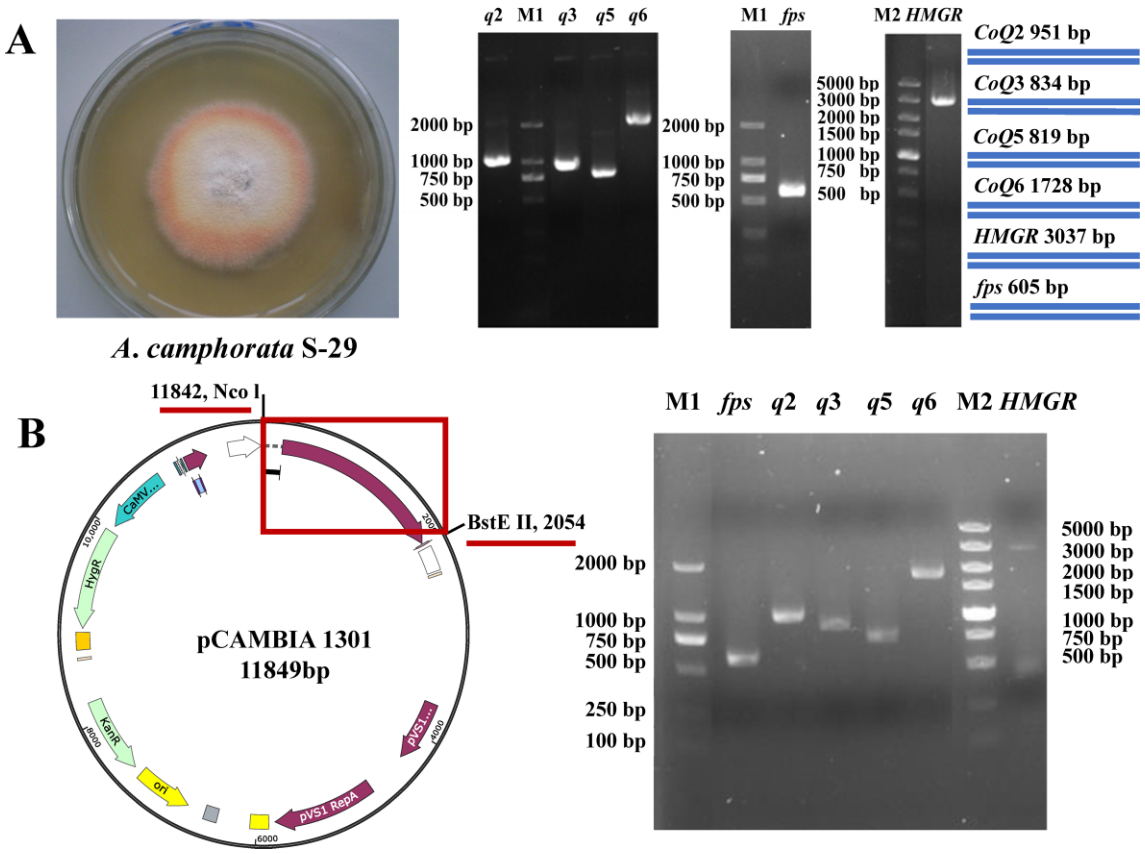

Fig. 4. Construction of the recombinant plasmid based on pCAMBIA 1301. A: *A. camphorata* S-29 and the target gene (*coq2*, *coq3*, *coq5*, *coq6*, *fps*, *HMGR*); B: Construction of plasmids based on pCAMBIA 1301 and verified in *A. tumefaciens* LBA4404. M, M1 and M2 = markers of DNA with different molecular weight. Target gene: *q2* = *coq2*, *q3* = *coq3*, *q5* = *coq5*, *q6* = *coq6*, *HMGR* = HMG-CoA reductase, *fps* = FPP synthase. wt = wild-type.

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

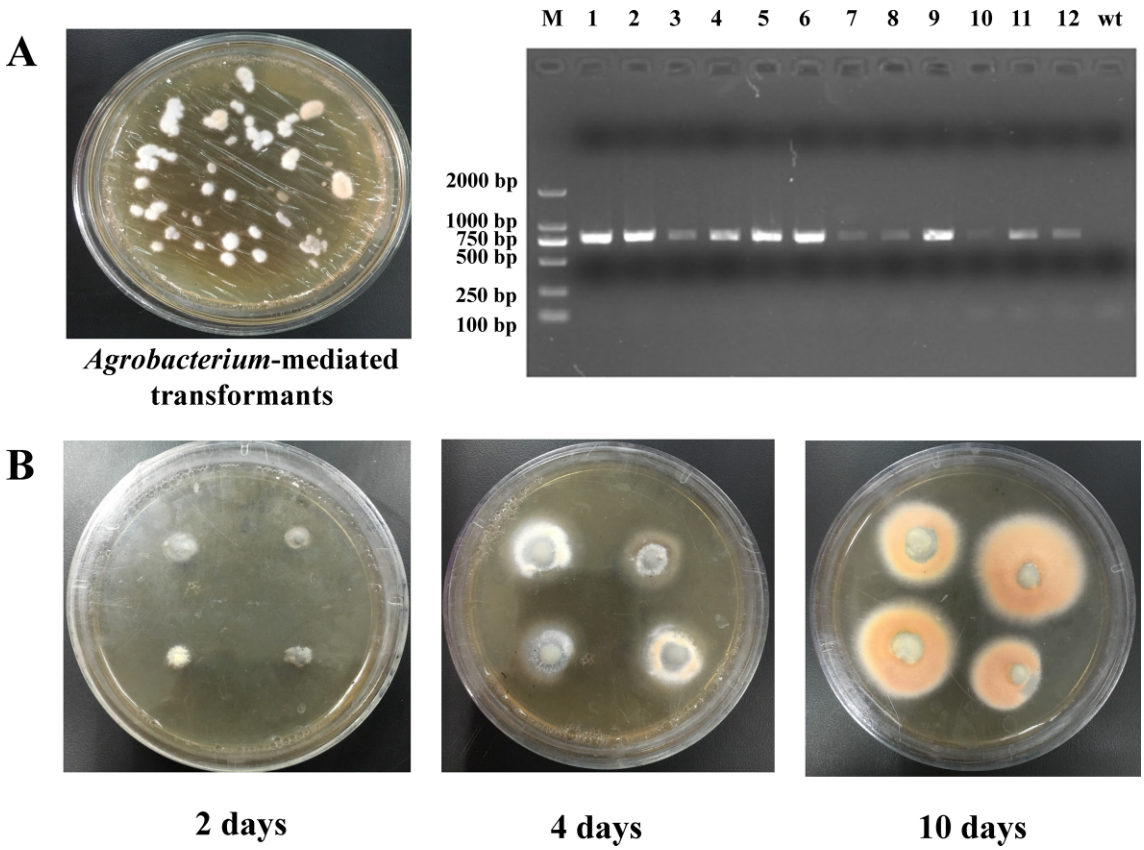

Fig. 5. Transformation of *A. camphorata* S-29. A: *Agrobacterium*-mediated transformants and detection of *hyg* gene in transformants of *A. camphorata* S-29. B: Growth of transformants of *A. camphorata* S-29 in PDA plate with hygromycin B. M = marker of DNA. wt = wild-type.

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

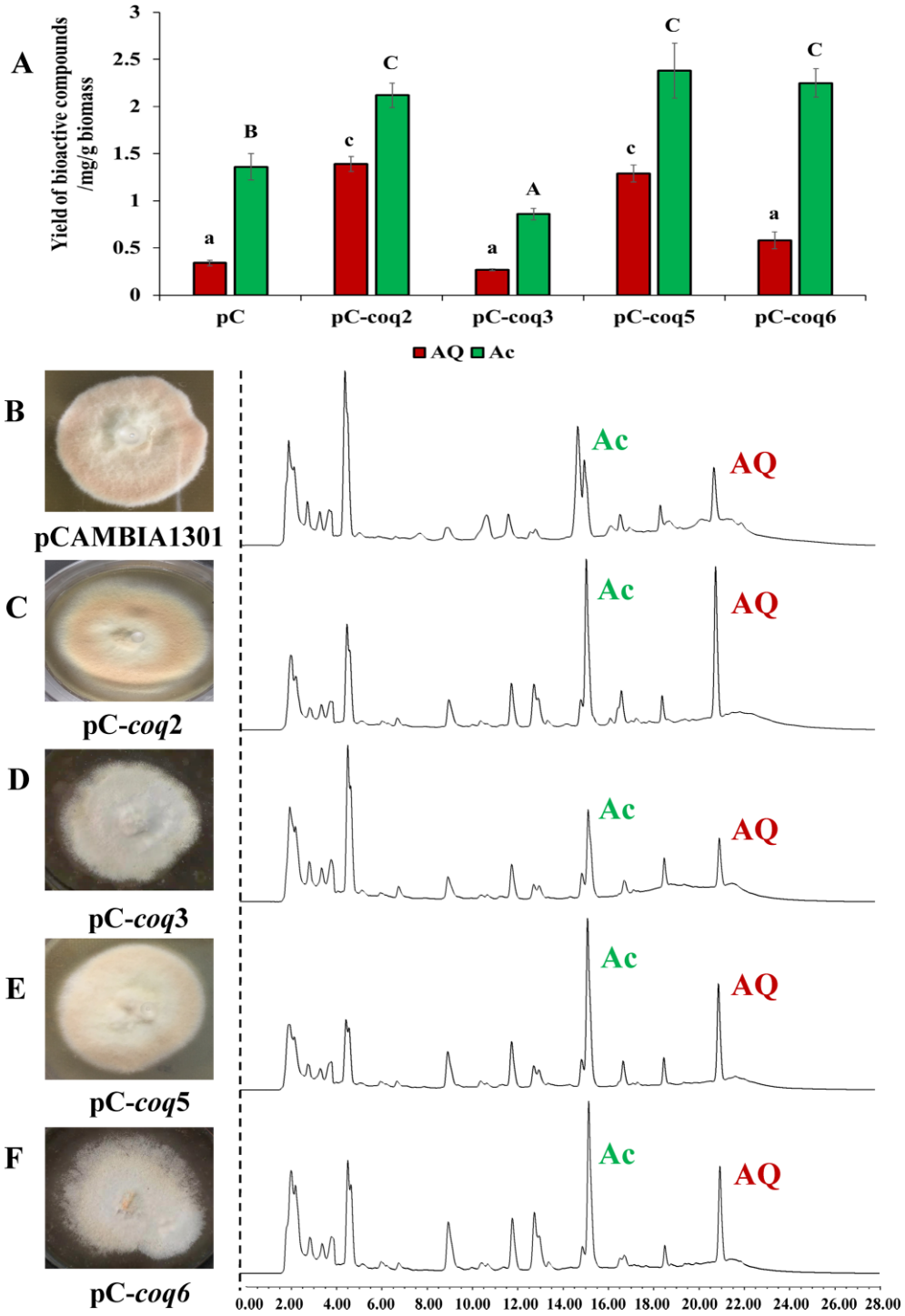

Fig. 6. Effect of overexpression of quinone ring modification genes from *A. camphorata* S-29 on AQ biosynthesis. A: Yield of AQ and Ac in different transformants. B: The colony morphology and chromatogram of transformant with pCAMBIA1301. C: The colony morphology and chromatogram of transformant with pCAMBIA1301-*coq*2. D: The colony morphology and chromatogram of transformant with pCAMBIA1301-*coq*3. E: The colony morphology and chromatogram of transformant with pCAMBIA1301-*coq*5. F: The colony morphology and chromatogram of transformant with pCAMBIA1301-*coq*6.

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 ± 0.72^{bc}	8.11 ± 0.52^{ab}	9.83 ± 0.83^{c}	8.65 ± 0.55^{abc}	7.47 ± 0.83^{a}	7.87 ± 0.80^{bc}	7.07 ± 0.69^{a}
Aq (mg/L)	3.10 ± 0.26^{a}	11.25 ± 0.56^{c}	2.62 ± 0.17^{a}	11.17 ± 0.83^{c}	4.36 ± 0.34^{b}	2.25 ± 0.12^{a}	2.48 ± 0.13^{a}
Ac (mg/L)	12.36 ± 0.49^{b}	17.15 ± 0.64^{c}	8.42 ± 0.44^{a}	20.50 ± 1.71^{d}	16.74 ± 0.79^{c}	12.59 ± 1.25^{a}	11.97 ± 0.63^{a}

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 $P < 0.05$.

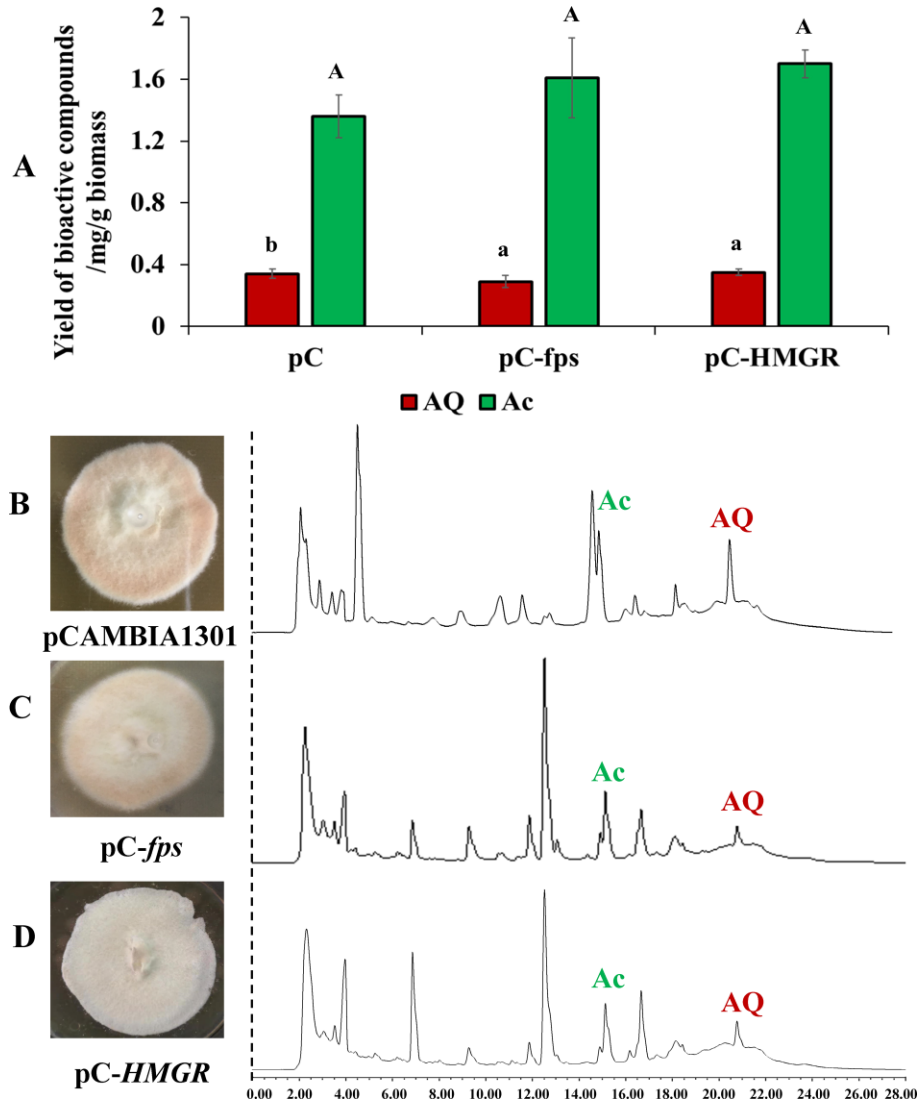

Fig. 7. Effect of overexpression of side chain genes from *A. camphorata* S-29 on AQ biosynthesis. A: Yield of AQ and Ac in different transformants. B: The colony morphology and chromatogram of transformant with pCAMBIA1301. C: The colony morphology and chromatogram of transformant with pCAMBIA1301-*fps*.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

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.

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 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

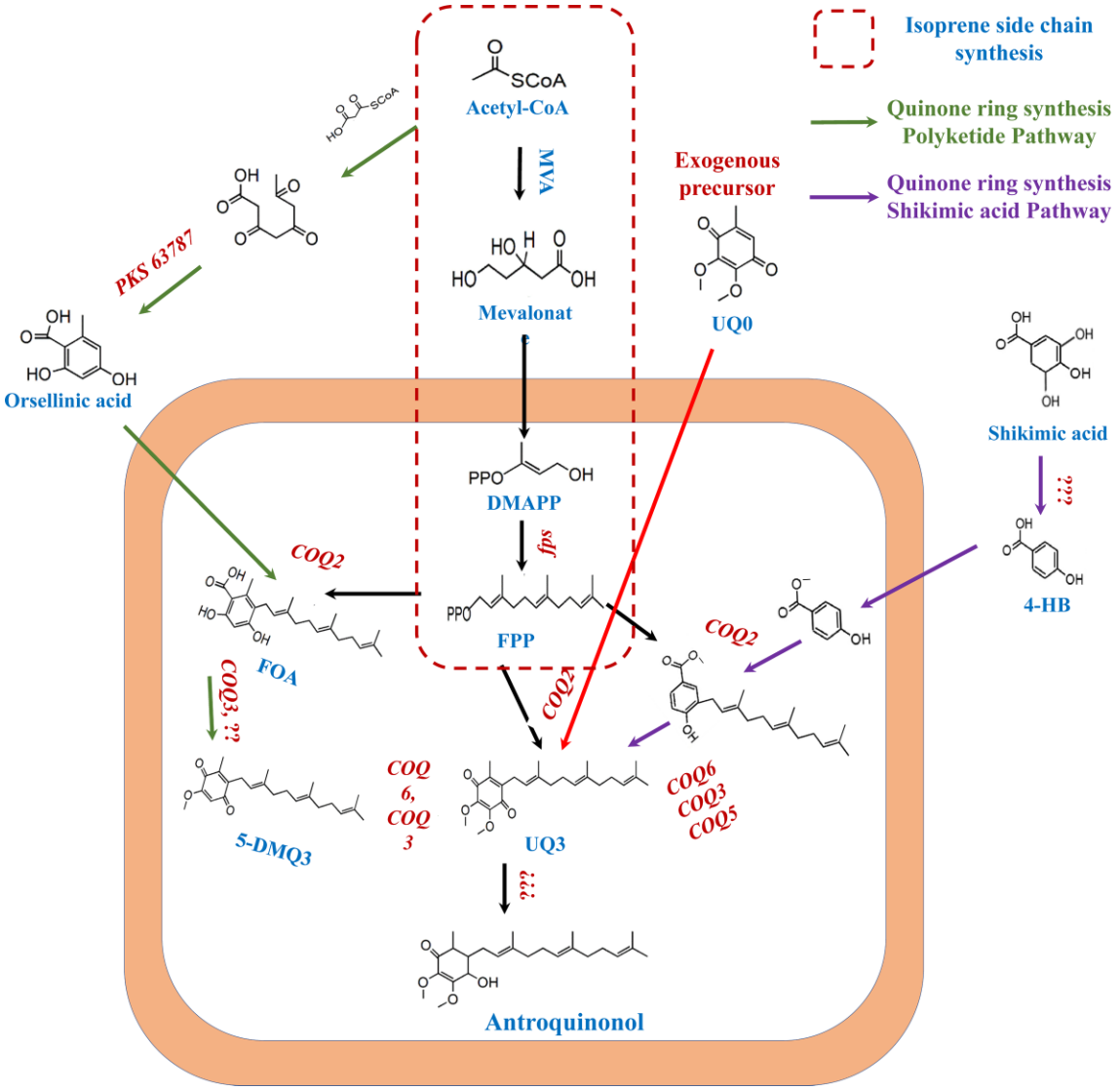

Fig. 8. The global pathway of AQ synthesis in *A. camphorata*. As a precursor, UQ0 could significantly reduce the synthesis steps of AQ.

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 Ac 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 pyrophsphate 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 *coq5* 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.

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Data Availability

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

Conflict of Interest

The authors declare no conflict of interest.

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