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Understanding the Essential Metabolic Nodes in the Synthesis of 4-Acetylantroquinol B (4-AAQB) by *Antrodia cinnamomea* Using Transcriptomic Analysis

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Abstract: 4-Acetylantroquinol B (4-AAQB) is a ubiquinone that has been shown to have multiple anticancer activities and is only found in the rare medicinal fungus *A. cinnamomea* in Taiwan. The large-scale production and application of 4-AAQB is thus limited due to the high host specificity, long production cycle, and low 4-AAQB content of *A. cinnamomea*. Additionally, the lack of molecular genetic studies on *A. cinnamomea* has hindered the study of the synthetic pathway of 4-AAQB. In this work, transcriptomic analysis was conducted to understand the essential metabolic nodes in the synthesis of 4-AAQB by *A. cinnamomea* based on the differences using glucose and fructose as carbon sources, respectively. The results showed that the glyoxylate and TCA cycle, terpenoid synthesis pathway, and the quinone ring modification pathway were clarified as the most significant factors associated with 4-AAQB synthesis. The enzymes ACS, ACU7, ACUE, GPS, PPT, P450, GEDA, YAT1, CAT2, and METXA in these pathways were the essential metabolic nodes in the synthesis of 4-AAQB. When fructose was used as the substrate, the expressions of these enzymes were upregulated, and the synthesis of some important intermediate metabolites was enhanced, thus promoting the accumulation of 4-AAQB. Our work understood the mechanism of fructose promoting the synthesis of 4-AAQB and identified the essential metabolic nodes which could provide the theoretical basis for the development of fermentation strategies to produce 4-AAQB by *A. cinnamomea*.

Keywords: 4-Acetylantroquinonol B; *Antrodia cinnamomea*; transcriptomic analysis; fermentation; metabolic nodes; fructose

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1. Introduction

4-AAQB is one of the important active substances unique to *A. cinnamomea*, a new ubiquinone compound isolated from the mycelia of *A. cinnamomea* in 2009 [1]. Subsequently, Lin et al. targeted the antiproliferative activity of HepG2 cells, and the final isolated extract of *A. cinnamomea* was also identified as 4-AAQB, whose IC50 value was only 0.1 μg/mL against liver cancer cells HepG2 [2]. Further exploration demonstrated that 4-AAQB was able to prevent osteoporosis [3], improve sepsis [4], and ameliorate nonalcoholic fatty liver disease in mice [5]. In addition, 4-AAQB also exhibited a variety of anticancer activities, and it showed significant inhibitory effects on breast cancer cells, rectal cancer cells, and ovarian cancer cells [6–8]. In 2019, Li et al. found that 4 AAQB was able to effectively activate the immune effects of cytokines by stimulating the expression of receptors indicated by hepatocellular carcinoma cells, confirming the potential of 4-AAQB to prevent hepatocellular carcinoma, treat hepatocellular carcinoma, and inhibit metastasis [9,10]. Therefore, the production and application of 4-AAQB has received increasing attention in recent years due to its outstanding physiological activity and anticancer potential.

4-AAQB has only been identified in the fruit body and mycelium of *A. cinnamomea*, which is a valuable medicinal fungus unique in Taiwan and has been used as medicine for hepatitis for many years [11]. Due to the slow growth rate and the high host specificity, the natural *A. cinnamomea* fruit body is difficult to obtain. The submerged cultivation method, with the advantages of simple operation and short production cycle, was thus developed for *A. cinnamomea*, and it has been utilized to produce triterpenes by its mycelium. Therefore, the submerged cultivation of *A. cinnamomea* is considered the most promising method to obtain 4-AAQB. Furthermore, Lin et al. found that the content of 4-AAQB in the mycelium of *A. cinnamomea* was higher than that in the fruit body of *A. cinnamomea*.

To better regulate the synthesis of 4-AAQB, some studies have attempted to resolve the synthesis pathway of 4-AAQB. Yang et al. found that the addition of 4-hydroxybenzoic acid (4-HBA) significantly increased the yield of 4-AAQB, and they suspected that that the synthesis of 4-AAQB was similar to the synthesis of CoQ via the condensation of the benzene ring and farnesene chain precursors, while 4-HBA was the benzene ring precursor [12]. But Chou et al. perceived that the ring precursor of 4-AAQB was derived from orsellinic acid (OA) instead of 4-HBA [13,14]. Yu et al. further proved that the ring precursor of 4-AAQB was solely derived from OA though knockout of the pathway [15,16]. The farnesene chain precursor of 4-AAQB was suspected to be derived from the intermediate metabolite farnesyl pyrophosphate (FPP) in the MVA pathway, which was lactonized to form farnesyl pyrophosphate with ends lactonization (FPPB) [12,14]. Additionally, polyisoprene transferase was considered to be the enzyme catalyzing the condensation of the ring precursor OA and farnesene chain precursor FPPB. These works made contributions to the resolution of the 4-AAQB synthesis pathway.

Based the speculated pathway, some reports attempted to add potential precursors to enhance the synthesis of 4-AAQB. Yang et al. added CoQ0 and 4-hydroxybenzoic acid (4-HBA) during the submerged cultivation of *A. cinnamomea*, which led to the yield of 4-AAQB increasing to 27.80 mg/g, about 16 times greater than the yield without these compounds [12]. Liu et al. investigated several benzene derivates in the promotion of 4-AAQB production by *A. cinnamomea*, and 4-methylbenzoic acid was proven to be the optimum. Coupled with the application of the in situ extractive fermentation strategy, the yield of 4-AAQB reached 17.27 mg/g [17]. Chiang et al. added volatile compounds during the cultivation of *A. cinnamomea*, which improved the yield of 4-AAQB about twofold [18]. These works indicated that it was important to understand the synthesis pathway of 4-AAQB, which was helpful for the development of efficient fermentation strategy.

Nevertheless, due to the incomplete resolution of the synthesis pathway, some essential metabolic nodes affecting the production of 4-AAQB are unclear, which limits the exploitation of strategies for its efficient production. In this work, fructose was proven to be more beneficial for the production of 4-AAQB than glucose as the carbon source, which led to the yield of 4-AAQB increasing about 4.8-fold. It was suspected that the utilization of fructose might affect some essential metabolic nodes and promote the synthesis of 4-AAQB. Transcriptomic analysis was thus applied to analyze the expression of the pathways related to the synthesis of 4-AAQB and to understand the mechanism of fructose promoting the production of 4-AAQB. Some essential metabolic nodes in the synthesis process were clarified (Figure 1), which could provide a theoretical basis for the resolution of the complete 4-AAQB synthesis pathway and the development of novel fermentation strategies for 4-AAQB production.

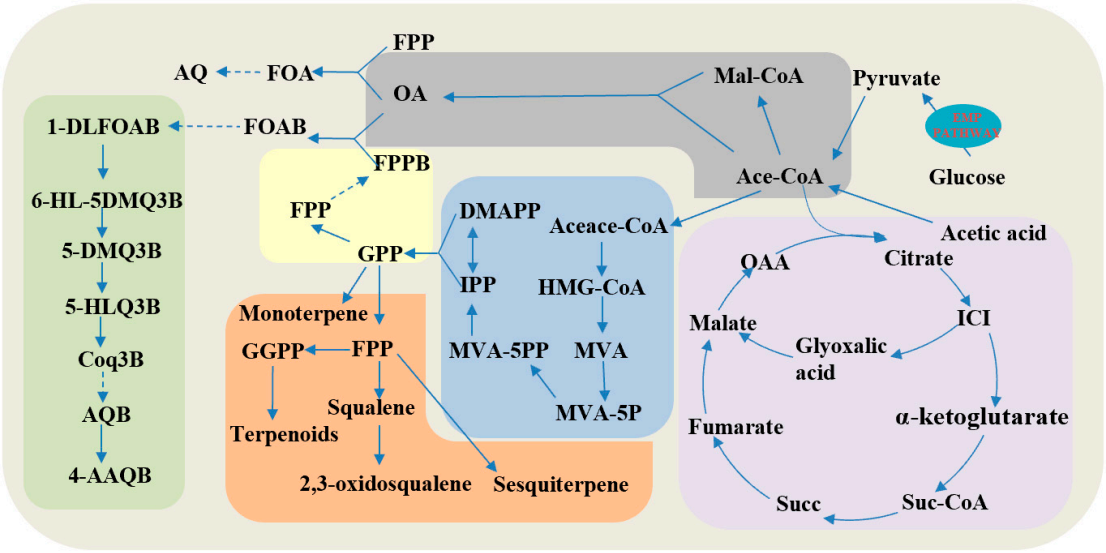

Figure 1. The putative synthetic pathways of 4-AAQB in *A. cinnamomea*. The purple box is the TCA cycle and glyoxylate pathway; the blue box is the MVA pathway; the orange box is the terpene synthesis pathway; the yellow box is the FPPB synthesis pathway; the green box is the quinone ring modification pathway; and the gray box is the ring precursor OA synthesis pathway. Ace-CoA, acetyl CoA; Mal-CoA, malonyl CoA; Aceace-CoA, acetylacetyl CoA; HMG-CoA, hydroxymethylglutaryl-CoA; MVA, mevalonate; MVA-5P, mevalonate-5-phosphate; MVA-5PP, mevalonate diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl pyrophosphate; FPPB, farnesyl pyrophosphate with ends lactonization; ICI, isocitric acid; Succ, succinic acid; Suc-CoA, succinic acid CoA; OAA, oxaloacetic acid; OA, orsellinic acid; FOA, 3-farnesyl pyrophosphate-orsellinic acid; AQ, antroquinonol; 1-DLFOAB, 1-decarboxylation-3-farnesyl pyrophosphate B-orsellinic acid; 6-HL-5-DMQ3B, 6-Hydroxyl-5-demethoxylation-Coenzyme Q3 B; 5-DMQ3B, 5-Demethoxylation-coenzyme Q3 B; 5-HLQ3B, 5-Hydroxyl-coenzyme Q3 B; CoQ3B, Coenzyme Q3 with ends lactonization; AQB, antroquinonol B; 4-AAQB, 4-Acetylantroquinonol B.

2. Materials and Methods

2.1. Strain and Medium

A. cinnamomea (YMT 1002) was obtained from Chaoyang University of Science and Technology (Taizhong, Taiwan). The strain was maintained on agar slant culture medium by periodical subculture following incubation at 30 °C for 20 days and was stored at 4 °C. The compositions of the seed medium were glucose 20 g/L, peptone 10 g/L, KH_2PO_4 0.75 g/L, and Mg_2SO_4 1.5 g/L. The compositions of the glucose/fructose fermentation medium were glucose/fructose 40 g/L, peptone 10 g/L, K_2HPO_4 0.5 g/L, and Mg_2SO_4 0.5 g/L. The compositions of the agar slant culture medium were glucose 20 g/L, peptone 2 g/L, malt extract 10 g/L, and ager 25 g/L.

2.2. Culture Methods

The mycelium was washed with 10–15 mL sterile water to obtain a spore suspension, inoculated at 0.6% into 250 mL triangular flasks containing 100 mL of seed medium and incubated at 28 °C, 150 rpm, for 5 days in a constant temperature shaker (HZC-280, Peiying, China) to obtain seed medium. Then, 20% of the seeds were inoculated into 250 mL triangular flasks containing 80 mL of glucose medium and fructose medium, respectively, and incubated at 28 °C, 150 rpm, for 20 days in a constant temperature shaker. Farnesol and geraniol were added on day 10 to the fermentation of *A. cinnamomea* in glucose medium, and the total fermentation time was 20 days.

2.3. Analysis Methods

2.3.1. Dry Cell Weight of *A. cinnamomea* and 4-AAQB Detection Method

The mycelium of *A. cinnamomea* was dried at 60 °C for 24 h to a constant weight to determine the dry cell weight. Then, 0.1 g dried mycelium was mixed with 2 mL 90% ethanol to extract 4-AAQB in *A. cinnamomea* cells at 30 °C, 150 rpm, for 2 h using constant temperature oscillation.

4-AAQB was detected using the HPLC system equipped with a UV detector (1100, Agilent, Santa Clara, CA, USA) and ODS Hypersil C18 column (250 × 4.6 mm, 5 μm, Thermo Scientific, Waltham, MA, USA). Elution was performed at a flow rate of 0.8 mL/min with a column temperature of 30 °C and UV wavelength of 248 nm with acetonitrile (A) and 0.2% acetic acid solution (B) as the mobile phase. The mobile-phase composition was changed linearly from 60% A to 75% A in 30 min. All experiments were duplicated three times, and average data are reported.

2.3.2. Transcriptome Analysis

After the mycelium of *A. cinnamomea* was cultured in different media for 15 days, it was frozen with liquid nitrogen. The mRNA extraction, cDNA library construction, and transcriptome sequencing were completed using Novogene (Novogene, Beijing, China). The Gene Ontology database (GO, http://geneontology.org/, accessed on 10 November 2022) classified the differentially expressed genes to biological processes, cellular components, and molecular functions. The Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/, accessed on16 November 2022) classified differentially expressed genes to different pathways.

2.3.3. Quantitative PCR (qPCR) Analysis

The mycelium of *A. cinnamomea* cultured for 15 days in different media was used to prepare samples for qPCR analysis. Total RNA was extracted from *A. cinnamomea* using the Vazyme Plant Total RNA Extraction Kit (Vazyme, Nanjing, China). Then, the RNA was reverse transcribed into cDNA using the NovoScript® Plus All-in-one 1st Strand cDNA Synthesis SuperMix (Novoprotein, Shanghai, China). The qPCR analysis was performed using NovoStart®SYBR qPCR SuperMix Plus Kit (Novoprotein, Shanghai, China). The *A. cinnamomea* 18S rRNA gene was used as the housekeeping gene for qPCR. The expression levels of the key genes in the synthesis of 4-AAQB were detected by qPCR instrument RealPlex2 (Eppendorf, Hamburg, Germany). The primers sequences for qPCR analysis are listed in Supplementary Data.

3. Results and Discussion

3.1. Comparison of 4-AAQB Production from Fructose and Glucose

The different kinds of carbon sources have significant impacts on the growth cells and the synthesis of products. In our previous work, the effects of carbon sources on the cultivation of *A. cinnamomea* were explored and fructose showed an excellent promotion in the synthesis of 4-AAQB compared to other substrates. Herein, we compared the cell growth of *A. cinnamomea* and 4-AAQB production in detail using glucose and fructose as the substrates, respectively. As shown in Figure 2, the cell growth of *A. cinnamomea* reached the logarithmic stage on the fifth day and stabilized on the twelfth day in both the glucose and the fructose medium. Glucose was a preferable substrate for *A. cinnamomea* and led to faster cell growth. However, fructose was more beneficial for producing 4-AAQB, as it increased the content of 4-AAQB up to 1.52 mg/g, about 4.8-fold more than the content of 4-AAQB in glucose medium. In particular, the significant difference in the production of 4-AAQB was gradually exhibited in the two media after 15 days of incubation. Therefore, it was suspected that the utilization of fructose might affect some essential metabolic nodes in the synthesis of 4-AAQB. Transcriptomic analysis was subsequently conducted to analyze the expression of the pathways related to the synthesis of 4-AAQB and to understand the mechanism of fructose promoting the production of 4-AAQB.

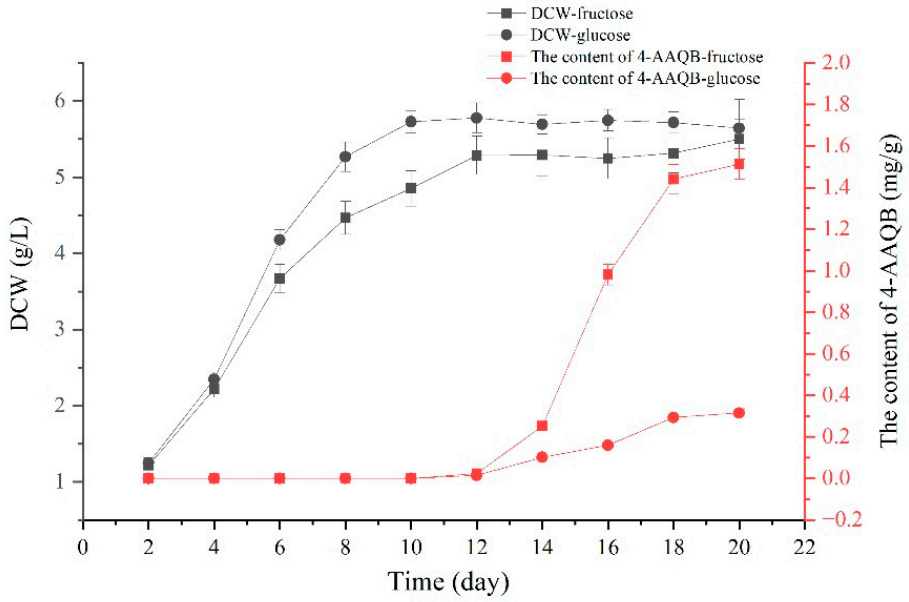

Figure 2. Growth curves of fermentation of *A. cinnamomea* to produce 4-AAQB in different kinds of media. DCW, the dry cell weight of *A. cinnamomea*.

3.2. Transcriptomic Analysis of 4-AAQB Synthesis Using Glucose and Fructose

According to the results of transcriptomic analysis, 3845 genes showed significant expression differences, including 1821 upregulated genes and 2024 downregulated genes. As shown in Figure 3, these differentially expressed genes were classified into three functional classifications using GO terms: biological processes, cellular components, and molecular functions. In the biological processes category, a large number of differentially expressed genes were involved in redox and carbohydrate metabolism. In the cellular components category, many differentially expressed genes were related to the formation of membranes and membrane components. In the molecular functions category, many differentially expressed genes were enriched to oxidoreductase and the processes of methylation. It could be inferred that multiple reduction, carbon methylation, and oxygen methylation are involved in the synthesis process of 4-AAQB.

Combined with the analysis using KEGG terms, the differentially expressed genes were annotated into different metabolic pathways. As shown in Figure 4, the differentially expressed genes were enriched in a total of 20 metabolic pathways, among which the secondary metabolite biosynthesis pathway contained the most differentially expressed genes. The reason might be that 4-AAQB is a secondary metabolite and some of the pathways are involved in its biosynthesis process. At the same time, a large number of differentially expressed genes were enriched in the carbon metabolism pathway. This was due to the different central carbon flux metabolism pathways using glucose and fructose as the carbon sources, respectively.

To further clarify the expression of different genes associated with 4-AAQB synthesis, the differentially expressed genes were classified into several modules according to the metabolic pathways in which they were involved (Table 1).

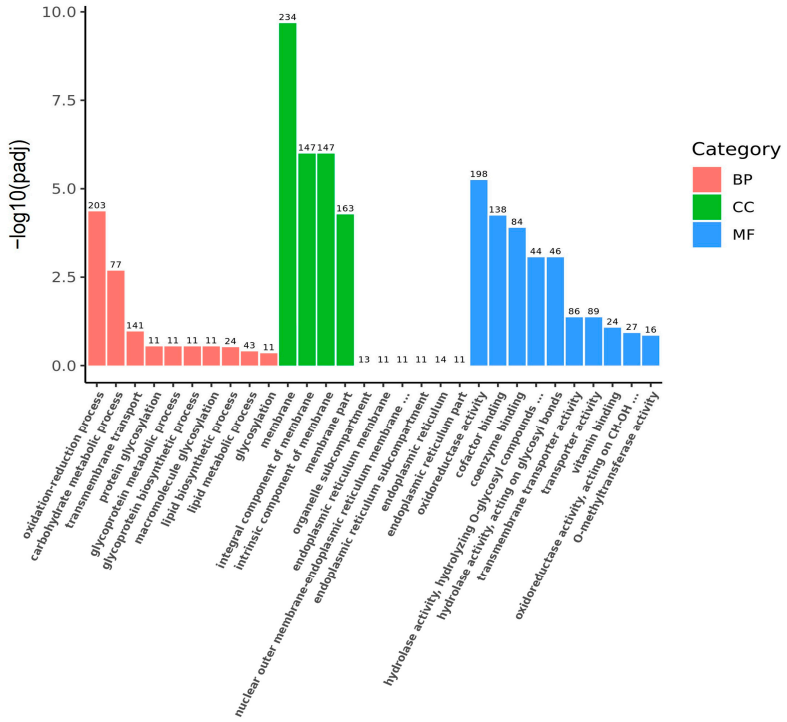

Figure 3. Functional classifications of differentially expressed genes of *A. cinnamomea* in different kinds of media based on GO terms. BP, biological processes; CC, cellular components; MF, molecular functions.

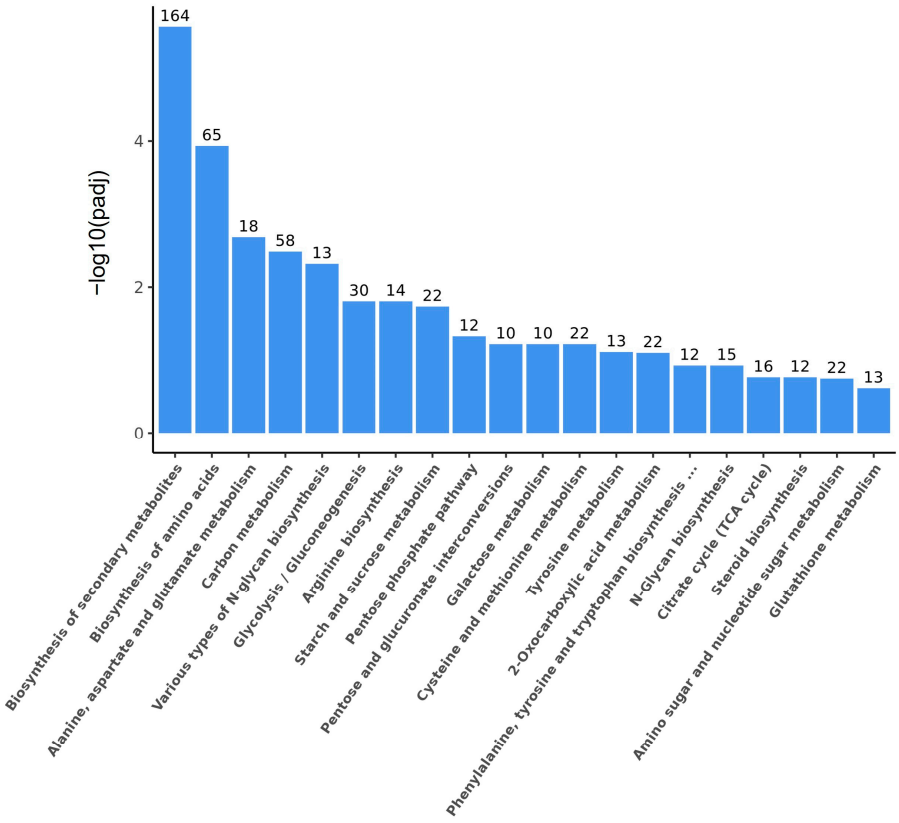

Figure 4. KEGG pathway analysis of differentially expressed genes of *A. cinnamomea* in different kinds of media based on GO terms.

Table 1. Classification of differentially expressed genes in *A. cinnamomea* in different kinds of media.

Models	Enzymes	Function
Glyoxylate and citrate cycle	ACS	Acetyl CoA synthase
	CS	Citrate synthase
	ACUE	Malate synthase
	ACU7	Isocitrate lyase
	IDH	Isocitrate dehydrogenase
MVA pathway and terpenoid biosynthesis	SE	Squalene epoxidase
	SQS	Squalene synthase
	HMGS	Hydroxymethylglutaryl-CoA
	HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
	AACT	Acetyl CoA acetyltransferase A
	MVD	Diphosphomevalonate decarboxylase
	MPK	Phosphomevalonate kinase
	FPS	Farnesyl pyrophosphate synthase
	GPS	Geranyl pyrophosphate synthase
Ubiquinone biosynthesis	PPT1/2	4-hydroxybenzoate polyprenyltransferase
	P4501/2/3	Monooxygenase
	GEDA1/2/3	Oxygen methyltransferase
	COQ6	Phenol hydroxylase
	NAHG	Salicylate hydroxylase
	YAT1	Oxygen acetyltransferase
	CAT2	Oxygen acetyltransferase
	METXA	Oxygen acetyltransferase

3.2.1. Analysis of the TCA Cycle and Glyoxylate Pathway Module

The tricarboxylic acid cycle (TCA cycle) was the most important metabolic pathway in microbes, in which acetyl CoA was oxidized to produce CO_2 and ATP to provide energy for cell growth. It was the most efficient way to convert carbohydrates into energy as the metabolism link of sugars, lipids, and proteins [19,20]. Moreover, the TCA cycle was also capable of generating amino acid precursors and the cofactor NAD(P)H for a variety of chemical reactions [21]. The glyoxylate pathway was a bypass of the tricarboxylic acid cycle (TCA cycle), in which two molecules of acetyl CoA were converted into succinate instead of being oxidized to carbon dioxide [22]. The glyoxylate pathway was important for microbes since it was able to produce succinate in the absence of oxaloacetate, which could maintain the integrity of the TCA cycle. Moreover, it also provided the basis for the biological use of two-carbon substrates such as acetate and increased the efficiency of acetyl CoA utilization. Due to the importance of the TCA cycle and glyoxylate pathway, they were first focused according to the transcriptomic analysis results. As shown in Figure 5, several genes in the TCA cycle and glyoxylate pathway showed significant expression differences. The relative expression level of related enzymes was obtained by transcriptome analysis.

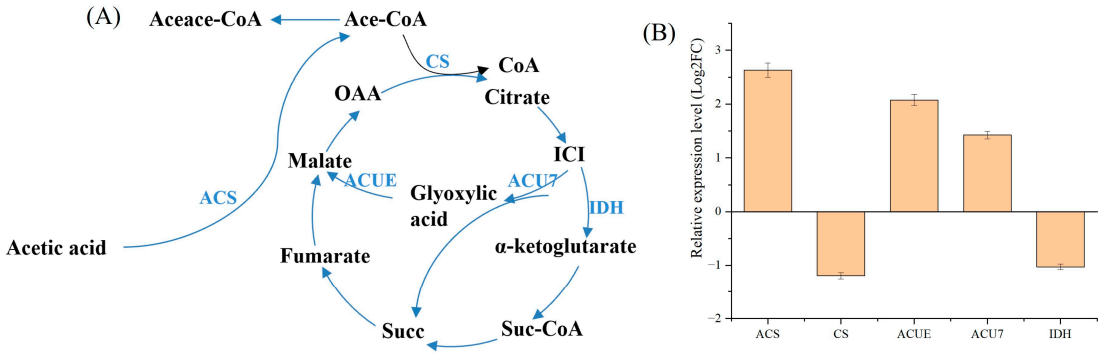

Figure 5. (**A**) Schematic diagram of TCA cycle and glyoxylate pathway module; (**B**) the relative expression level of related enzymes. ACS, acetyl-CoA synthase; CS, citrate synthase; ACU7, isocitrate lyase; ACUE, malate synthase; IDH, isocitrate dehydrogenase; Ace-CoA, acetyl CoA; ICI, isocitric acid; Succ, succinic acid; Suc-CoA, succinic acid CoA; OAA, oxaloacetic acid; Aceace-CoA, acetoacetyl CoA.

ACS was the acetyl CoA synthase, which catalyzed the formation of acetyl-CoA from acetate. Acetate was a common byproduct due to the overflow of carbon metabolism. Many studies have reported that the expression of ACS could facilitate the reconversion of the byproduct acetate to acetyl-CoA to improve the efficiency of central carbon flow utilization [23]. When fructose was used to cultivate *A. cinnamomea*, the expression level of ACS was upregulated about 6.21-fold. The improvement in ACS expression promoted the formation of acetyl CoA and enhanced the utilization of central carbon flux. However, the expression of the CS and IDH, important enzymes in the TCA cycle, was downregulated. CS is citrate synthase, which was able to catalyze the condensation of acetyl-CoA and oxaloacetate to generate citrate. The downregulated expression of CS led to the reduction in carbon flux toward the TCA cycle and the substantial accumulation of acetyl-CoA. IDH is isocitrate dehydrogenase, which functioned to convert isocitrate to 2-oxoglutarate. Due to the limitation of carbon flux in the TCA cycle, the expression of IDH was downregulated and the glyoxalate pathway was activated to avoid the loss of carbon atoms in the form of carbon dioxide and to improve the utilization efficiency of carbon flux. As shown in Figure 5, ACU7 and ACUE, isocitrate lyase and malate synthase, were involved in the glyoxylate pathway; while ACU7 catalyzed the formation of glyoxylic acid from isocitric acid and ACUE catalyzed the formation of malic acid from glyoxylic acid. The expression levels of ACU7 and ACUE enzymes were upregulated 2.68- and 4.21-fold, respectively. It was suggested that the glyoxylate pathway was significantly activated in *A. cinnamomea* to maintain the limited TCA cycle when fructose was used as the substrate. This led to the accumulation of acetyl CoA, which is the foundation for the synthesis of the OA and FPPB (two potential precursors of 4-AAQB), and also an adequate donor for the acetyl groups in 4-AAQB. Therefore, compared to glucose, the utilization of fructose could promote the accumulation of acetyl CoA and thus provide more available precursors for the synthesis of 4-AAQB.

3.2.2. Analysis of Terpene Synthesis Module

The terpene synthesis pathway in *A. cinnamomea* was the same as it in other eukaryotes. Acetyl CoA was the precursor to generating the isoprene units IPP and DMAPP via the MVA pathway, and then to forming active compounds such as monoterpenes, sesquiterpenes, diterpenes, and triterpenes via multiple condensation modifications [24]. The chain precursor FPPB for the synthesis of 4-AAQB was considered to be produced in the terpene synthesis pathway. Therefore, the expression of the enzymes in the pathway was analyzed as shown in Figure 6.

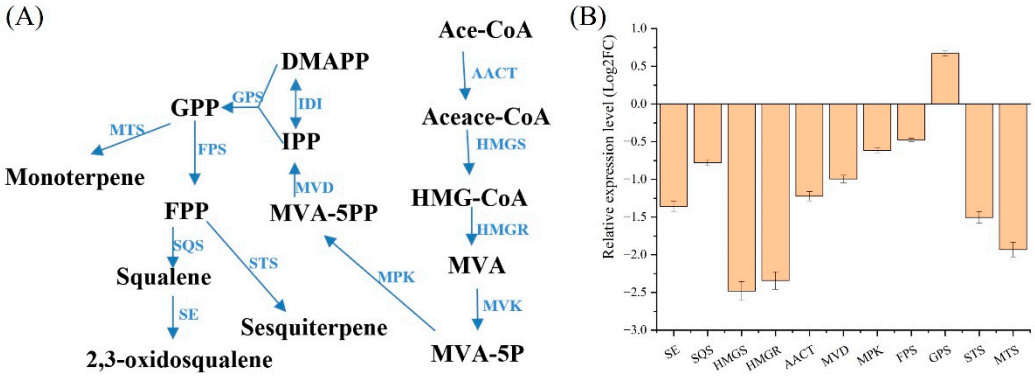

Figure 6. (**A**) Schematic diagram of terpene synthesis module; (**B**) the relative expression level of related enzymes. AACT, acetyl-CoA acetyltransferase; HMGS, hydroxymethylglutaryl-CoA synthase; HMGR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; MVK, mevalonate kinase; MPK, phosphomevalonate kinase; MVD, mevalonate diphosphate decarboxylase; IDI, isopentenyl diphosphate isomerase; GPS, geranyl pyrophosphate synthase; MTS, monoterpene synthase; FPS, farnesyl pyrophosphate synthase; STS: sesquiterpene synthase; SQS, squalene synthase; SE, squalene epoxide; Ace-CoA, acetyl CoA; Mal-CoA, malonyl CoA; Aceace-CoA, acetylacetyl CoA; HMG-CoA, hydroxymethylglutaryl-CoA; MVA, mevalonate; MVA-5P, mevalonate-5-phosphate; MVA-5PP, mevalonate diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate.

It could be seen that the expression levels of almost all the enzymes in the MVA pathway were downregulated when fructose was utilized as a carbon source by *A. cinnamomea*, including AACT, HMGS, HMGR, MPK, and MVD. The downstream products of the MVA pathway include squalene and ergosterol, which were associated with cell membrane synthesis [25]. This might be related to the fact that the cell growth of *A. cinnamomea* had basically stabilized after 15 days of cultivation. However, the expression of geranyl pyrophosphate synthase (GPS) appeared to be significantly upregulated, while farnesyl pyrophosphate synthase (FPS) and monoterpene synthase (MTS), the enzymes in GPP consumption pathways, were downregulated, which might mean that more GPP was accumulated. Meanwhile, the expression of squalene synthase (SQS), squalene epoxide (SE), and sesquiterpene synthase (STS) participating in the consumption of FPP was also significantly downregulated. This also led to the accumulation of FPP, thus suppressing the expression of FPS and promoting the accumulation of GPP. In the previous reports, the chain precursor FPPB was suspected to be formed by the spontaneous lactonization of FPP. According to our work, fructose was more beneficial for the production of 4-AAQB, while the accumulation of GPP, instead of that of FPP, was enhanced in the fructose-based medium. We thus concluded that GPP might be the potential precursor for the formation of FPPB.

3.2.3. Analysis of the Quinone Ring Modification Module

OA was regarded as the ring precursor of 4-AAQB, which was ligated to the chain precursor FPPB to generate 3-farnesyl pyrophosphate B-orsellinic acid (FOAB) by the function of aromatic prenyltransferase. FOAB underwent a series of modifications to form 4-AAQB, as shown in Figure 7. PPT is 4-hydroxybenzoate polyprenyltransferase, which catalyzed the ligation of 4-hydroxybenzoate or other analogues with the polyisoprene chain. It was also found to play an important role in the synthesis of antroquinonol, another kind of ubiquinone produced by *A. cinnamomea*. In the synthesis of antroquinonol [26], CoQ2, which is functionally identical to PPT, catalyzes the ligation process of FPP to 4-HBA. The expression levels of PPT1/2 were significantly upregulated in the cultivation of *A. cinnamomea* in the fructose-based medium, which meant that fructose could promote the ligation of OA to FPPB to improve the synthesis of FOAB, as shown in Figure 7. P450

enzymes are the most common oxidase family in the fungal metabolism, which generally catalyzes the oxidation process in metabolite synthesis, and has been found to be closely related to the oxidation synthesis of antroquinonol [27]. In 4-AAQB synthesis, P450 enzymes catalyzed the oxidation of 1-decarboxylation-3-farnesyl pyrophosphate B-orsellinic acid to 6-hydroxyl-5-demethoxylation-coenzyme Q3 B, as shown in Figure 7. The expression of three kinds of P450 enzymes was found to be upregulated in *A. cinnamomea* in the fructose medium, namely, P450-1/2/3. Notably, the expression of P450-1/2 increased about 14.61- and 13.98-fold, respectively. Oxygen methyltransferase was indispensable in Coq-like compound synthesis [28]. In the synthesis of 4-AAQB, the transformation of hydroxyl groups to form oxymethyl groups in the modification of ring structures was similar to the process in synthesis of Coq6, which catalyzes 6-hydroxyl-5-demethoxylation-coenzyme Q3 B and 5-hydroxyl-coenzyme Q3 B to 5-demethoxylation-coenzyme Q3 B and Coenzyme Q3 B (Figure 7), respectively. The expression of three kinds of oxygen methyltransferase was found to be upregulated, namely, GEDA-1/2/3, the isoenzyme of CoQ5 in yeast. The expression levels of GEDA1/2/3 increased about 3.78-, 3.03-, and 1.48-fold, respectively, when the carbon source changed from glucose to fructose. In addition, hydroxylases played important roles in the synthesis of secondary metabolites such as ubiquinone compounds. COQ6 and NAHG are the phenol hydroxylase and salicylate hydroxylase. In the synthesis of Coq6 in yeast, COQ6 catalyzed the hydroxylation of the quinone ring [28]. The expression levels of COQ6 and NAHG were upregulated 1.47- and 1.86-fold, respectively, in the synthesis of 4-AAQB in the fructose-based medium. It was suggested that COQ6 and NAHG might be involved in the hydroxylation modification of the ring structure from 5-Demethoxylation-coenzyme Q3 B to 5-Hydroxyl-coenzyme Q3 B (Figure 7). 4-AAQB was finally synthesized via the oxygen acetylation of Antroquinonol B. Three putative oxygen acetyltransferases, YAT1, CAT2, and METXA, were identified according to the transcriptional analysis, and were significantly upregulated in *A. cinnamomea* in the fructose-based medium. They were assumed to be key enzymes for the catalysis of oxygen acetylation in the synthesis of 4-AAQB. The results demonstrated that these enzymes are the essential enzymes involved in the quinone ring modification of FOAB, while fructose could improve their expression levels and thus improve the accumulation of 4-AAQB.

In summary, the mechanism of fructose promoting the production of 4-AAQB was revealed via the transcriptomic analysis. Firstly, when fructose was used as a carbon source, the TCA cycle was weakened and the glyoxylate pathway was activated, which led to the accumulation of Acetyl-CoA, providing sufficient precursors for the synthesis of OA and FPPB. Secondly, the synthesis of GPP, instead of FPP, was enhanced in the *A. cinnamomea* cultivated in the fructose-based medium, which was different from the previous reports. Additionally, we suspected that GPP might be the potential precursor for the formation of FPPB. Thirdly, the expression of aromatic prenyltransferase as well as the enzymes involved in the modification of FOAB was enhanced using fructose as the substrate, which was also one of the reasons for the promotion of 4-AAQB production. Furthermore, the expressions of the enzymes in these modules were further validated using qPCR and the same results were obtained. It was demonstrated that the glyoxylate and TCA cycle, the terpenoid synthesis pathway, and the quinone ring modification pathway were the essential modules, while the enzymes ACS, ACU7, ACUE, GPS, PPT, P450, GEDA, YAT1, CAT2, and METXA in these pathways were the essential metabolic nodes in the synthesis of 4-AAQB.

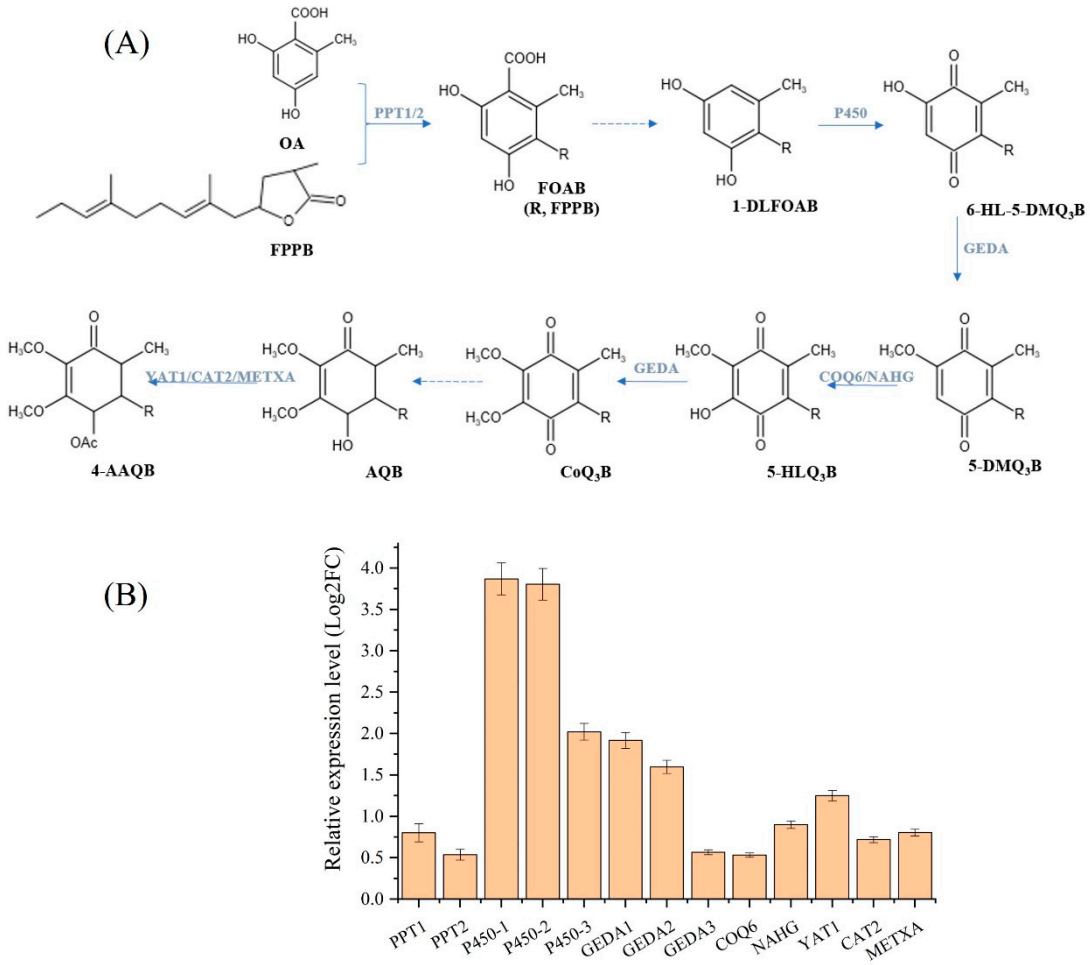

Figure 7. (**A**) Schematic diagram of quinone ring modification module; (**B**) the relative expression level of related enzymes. PPT, 4-hydroxybenzoate polyprenyltransferase; P450, monooxygenase; GEDA, oxygen methyltransferase;COQ6, Phenol hydroxylase; NAHG, Salicylate hydroxylase; RDS, reductase; YAT1, CAT2, METXA, Oxygen acetyltransferase; FPPB, farnesyl pyrophosphate with ends lactonization; OA, orsellinic acid; FOA, 3-farnesyl pyrophosphate-orsellinic acid; AQ, antroquinono; 1-DLFOAB, 1-decarboxylation-3-farnesyl pyrophosphate B-orsellinic acid; 6-HL-5-DMQ3B, 6-Hydroxyl-5-demethoxylation-Coenzyme Q3 B; 5-DMQ3B, 5-Demethoxylation-coenzyme Q3 B; 5-HLQ3B, 5-Hydroxyl-coenzyme Q3 B; CoQ3B, Coenzyme Q3 with ends lactonization; AQB, antroquinonol B; 4-AAQB, 4-Acetylantroquinonol B.

3.3. Effects of Geraniol and Farnesol on the Production of 4-AAQB

Based on the transcriptomic analysis results, GPP was suspected to be the potential precursor for the formation of FPPB, which was contradictory to the previous speculation that FPP was the precursor. It has been proven that cells were able to efficiently convert geraniol and farnesol to GPP and FPP, respectively [29]. To verify our suspicions, geraniol and farnesol were added to the glucose-based medium to investigate their effects on the production of 4-AAQB. Geraniol and farnesol, with different concentrations (0.01–0.2%, v/v), were added to the medium on the 10th day, respectively. In order to increase the conversion efficiency of farnesol and geraniol, 0.01% (v/v) phosphoric acid was added simultaneously. As shown in Figure 8, the addition of both geraniol and farnesol promoted the production of 4-AAQB. The highest yield of 4-AAQB was about 1.16 mg/g with a 0.1% addition of geraniol, which was about 4.06 times higher than that of the control. However, the highest yield of 4-AAQB was only 0.39 mg/g with a 0.2% addition of farnesol. It was demonstrated that the promotion effect of geraniol was more significant than that of farnesol.

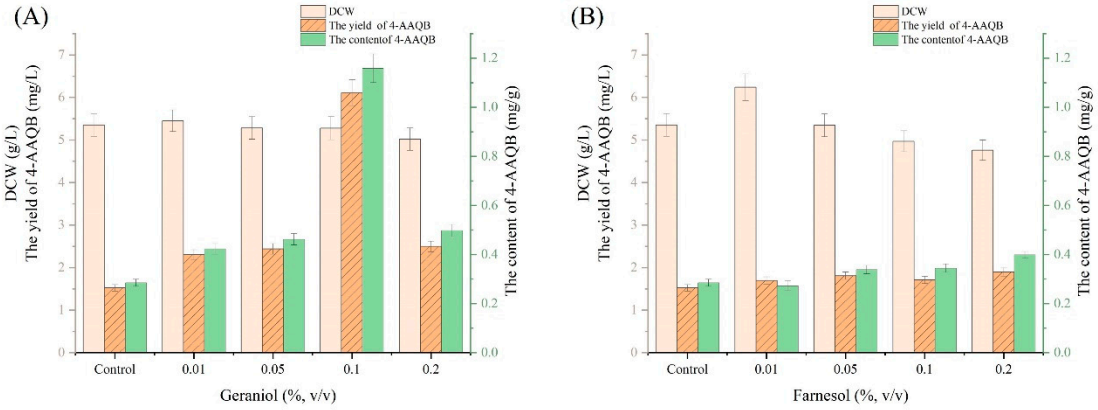

Figure 8. (**A**) Effects of different concentrations of geraniol on production of 4-AAQB by *A. cinnamomea*; (**B**) effects of different concentrations of farnesol on production of 4-AAQB by *A. cinnamomea*.

The addition of geraniol improved the content of GPP and provided a sufficient precursor for the synthesis FPPB, thus promoting the accumulation of 4-AAQB. This reinforced our suspicions that GPP was the most likely precursor for the synthesis of FPPB. It also laid the foundation for the further development of suitable fermentation strategies to enhance the synthesis of 4-AAQB.

4. Conclusions

In this work, fructose was found to be more beneficial than glucose for the production of 4-AAQB in the cultivation of *A. cinnamomea*. Transcriptomic analysis was conducted to analyze the expression of the pathways related to the synthesis of 4-AAQB and to understand the mechanism of fructose promoting the production of 4-AAQB. According to the transcriptomic analysis result, the TCA cycle and the glyoxylate pathway were proven to affect the accumulation of acetyl CoA as the foundation of ring precursor OA, while the GPP generated in the terpene synthesis pathway was considered the potential foundation of the chain precursor FPPB. The aromatic prenyltransferase catalyzing the ligation of OA to FPPB to form FOAB and the enzymes ACS, ACU7, ACUE, GPS, PPT, P4501/2/3, GEDA1/2/3, YAT1, CAT2, and METXA, which are involved in the modification of FOAB, played important roles in the synthesis of 4-AAQB. On this basis, geraniol and farnesol were added to the glucose-based medium to investigate their effects on the production of 4-AAQB, which further reinforced our suspicions that GPP was the most likely precursor for the synthesis of FPPB. Our work clarified some essential metabolic nodes in the synthesis of 4-AAQB which could provide a theoretical basis for the resolution of the complete 4-AAQB synthesis pathway and promote the development of novel fermentation strategies for 4-AAQB production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9060542/s1, Table S1: Primers used for q-PCR.

Author Contributions: H.L. and L.D.: Conception; Y.J. and H.L.: Writing—original draft; Y.J., H.L. and Y.N.: Investigation and Data Curation; Y.-M.T.: Resources; L.D. and F.W.: Writing—review and editing, Supervision, Funding acquisition, Project administration. All authors have read and agreed to the published version of the manuscript.

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