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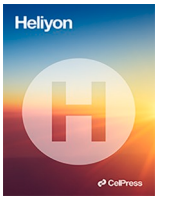

Improvement of triterpenoid production in mycelia of *Antrodia camphorata* through mutagenesis breeding and amelioration of CCl_4-induced liver injury in mice

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ABSTRACT

Due to the scarcity of wild fruiting bodies, submerged fermentation of the medicinal fungus *Antrodia camphorata* is attracting much attention, but the production of bioactive triterpenoids is low. Therefore, there is an urgent need to improve the triterpenoid yield of submerged fermentation. Here, the *A. camphorata* mutant E3-64 was generated from strain AC16101 through random mutagenesis breeding, producing 172.8 mg triterpenoid per gram of dry mycelia. Further optimization of culture parameters resulted in a yield of 255.5 mg/g dry mycelia (i.e., an additional >1.4-fold increase), which is the highest reported yield thus far. Notably, mutant E3-64 produced 94% and 178% more of the triterpenoid components antcin A and antcamphin A, respectively, while it produced 52% and 15% less antcin B and G, respectively. Mutant E3-64 showed increased expression of key genes involved in triterpenoid biosynthesis, as well as different genome-wide single-nucleotide polymorphisms as compared with AC16101. Triterpenoids of the E3-64 mycelia exhibited remarkably protective activity against acute CCl_4-induced liver injury in mice. This study shows the potential of *A. camphorata* for scientific research and commercial application.

1. Introduction

The medicinal mushroom *Antrodia camphorata* is known to produce a diverse array of bioactive compounds, specifically triterpenoids. It is popularly used as a traditional Chinese medicine to treat various human illnesses, such as alcohol intoxication, cancer,

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and inflammation [1]. *A. camphorata* grows naturally on the bull camphor tree (*Cinnamomum kanehira* Hayata) in Taiwan. Owning to its unique features such as an extremely strict host range and requirements for specific environmental conditions, wild fruiting bodies are very limited and expensive, and market demands are not met. Therefore, the cultivation of *A. camphorata* has attracted much attention. Submerged fermentation (SmF) seems to be a potential approach due to the short cultivation period and easy operation of large-scale production [2]. However, the amounts of bioactive compounds produced by cultured *A. camphorata* mycelia are much lower than those produced by wild fruiting bodies [2].

Terpenoids, including triterpenoids, which have medicinal potential, are the main bioactive compounds of *A. camphorata* [3,4]. Current studies mainly focus on (i) optimizing culture constituents and fermentation conditions to increase biomass accumulation and (ii) screening efficiently of specific additives, including chemical elicitors such as chitosan and metabolite precursors such as squalene, as well as environmental factors, to enhance terpenoid production [2]. However, terpenoid production is still low, seriously hampering possible clinical application.

Notably, compared with optimization of culture constituents and parameters, breeding of *A. camphorata* for high terpenoid biosynthesis could be more effective. Mutagen-mediated genetic breeding of fungal strains is an efficient approach to improve the production of secondary metabolites. Common mutagens include ultraviolet radiation, radioactive ^{60}Co, ethyl methane sulfonate (EMS), and the recently developed atmospheric and room temperature plasma (ARTP) [5,6]. EMS causes transition mutations through alkylating nucleotides at random positions [7], while ARTP leads to DNA damage by changing the structure and permeability of the cell wall and plasma membrane [8]. However, to the best of our knowledge, no studies using random mutagenesis to improve terpenoid production in *A. camphorata* have been reported.

To obtain high terpenoid-producing *A. camphorata* strains, in the present study, we carried out random mutagenesis breeding with a combination of EMS and ARTP. Furthermore, the culture conditions of the resulting *A. camphorata* mutant were optimized. The triterpenoid composition, single-nucleotide polymorphisms, and transcript levels of major genes involved in triterpenoid biosynthesis were compared between the mutant and start strain AC16101 through liquid chromatography–tandem mass spectrometry (LC-MS/MS), genome re-sequencing, and real-time reverse transcription quantitative PCR (RT-qPCR) assays, respectively. Finally, the hepatoprotective effects of the extracted triterpenoids from *A. camphorata* mutant mycelia were evaluated in mice.

2. Materials and methods

2.1. A. camphorata strains and their culture conditions

A. camphorata wild-type strain AC16101 and mutant strains were cultured on potato dextrin agar (PDA) plates for 14 days at 28 °C. Spores were collected with sterile water. Strains AC16101 and E3-64 were deposited in the China Center for Type Culture Collection with accession numbers M20211322 and M2021756, respectively. Fresh spores (5.0×10^7) were inoculated into liquid medium (0.1 g/L KH_2PO_4, 0.05 g/L $MgSO_4{\cdot}7H_2O$, 10.5 g/L glucose, 10 g/L yeast extract 10) and then incubated in a shaker at 150 rpm for 28 days at 25 °C. The mycelia were separated by filtering with filter paper and used for determination of triterpenoid content.

2.2. Mutagenesis assay

EMS- and ARTP-induced mutagenesis of *A. camphorata* was performed based on a previously described method [9]. In brief, for EMS-mediated mutagenesis, fresh *A. camphorata* spores were suspended in phosphate-buffered saline and adjusted to a concentration of 9×10^7 spores/mL. Subsequently, EMS (1.2%, w/v) was mixed with the fresh spore solution and placed in a shaker at 180 rpm for 2–10 h at 28 °C. EMS treatment was stopped by adding an equal volume of sodium thiosulfate. The treated spores were washed several times using sterile water to remove the remaining EMS, until no white flocculent precipitate was observed anymore. The spores were collected by centrifugation for 10 min at 11,300×*g* and 4 °C and resuspended in sterile water.

In addition to EMS mutagenesis, ARTP-induced mutagenesis was carried out in an ARTP mutagenesis breeding machine (Wuxi Tmaxtree Biotechnology Co., Ltd., Wuxi, China). Fresh *A. camphorata* spores were suspended in 5% (w/v) glycerol to a final concentration of 9×10^7 spores/mL. The spores were seeded into carrier plates and treated with ARTP for mutagenesis. The mutagenic parameters were set as follows: flow rate, 10 L/min; radiofrequency power input, 130 W; and treatment time, 0–240 s. The treated spores were washed with sterile water.

The treated spores were diluted by gradient dilution (commonly 10^{-1}–10^{-5}), seeded on PDA plates, and then incubated for 6 days at 28 °C. The colonies were counted and lethality curves were drawn based on the following equation:

$$\text{fatality rate } (\%) = (N_0 - N_t)/N_0 \times 100\%$$

where N_0 is the colony number on plates inoculated with untreated spores and N_t is the colony number on plates inoculated with treated spores.

Colonies on plates inoculated with EMS/ARTP-treated spores were picked, re-spread on PDA plates, and incubated for 14 days. The fresh spores were collected with sterile water and cultivated in liquid medium for 28 days, and finally the triterpenoid content was determined.

2.3. Determination of triterpenoid content in A. camphorata mycelia

Triterpenoid contents were measured by spectrophotometry with vanillin-perchloric acid [10,11]. In brief, *A. camphorata* mycelia were harvested and dried for 24 h at 50 °C. The dry mycelia were ground into powder with liquid nitrogen. Approximately 0.1 g of mycelial powder was added into 5 mL of 95% (v/v) ethanol, mixed, and placed for 3 h at 60 °C. After all ethanol had evaporated, the dried matter was resuspended in a freshly prepared mixture of glacial acetic acid containing vanillin (5% [v/v]; 300 μL) and perchloric acid (800 μL), and the reaction was allowed to take place for 30 min at 60 °C. Then, the reaction solution was immersed in ice water and cooled to room temperature, and 5 mL glacial acetic acid was added. The absorption value at 550 nm (A_{550}) was measured using a spectrophotometer. The triterpenoid content in the mycelia was calculated according to the standard curve of oleanolic acid ($y = 3.611x - 0.0033$, $R^2 = 0.9996$, where y is A_{550} and x is the concentration of oleanolic acid).

2.4. Optimization of culture conditions for A. camphorata E3-64

In order to further improve the triterpenoid content, culture conditions of *A. camphorata* were optimized, including initial pH of the medium, temperature, carbon source, and nitrogen source. The effects of initial pH (3.0, 4.0, 5.0, and 6.0) on the triterpenoid content of the mycelia of *A. camphorata* mutant E3-64, when cultivated for 15 days, were observed. Moreover, the influence of temperature (22, 25, 28, and 31 °C) was investigated at the optimum pH.

In addition, the effects of several carbon sources (xylose, glucose, maltose, sucrose, corn starch, and cassava starch) on the triterpenoid content of E3-64 mycelia cultivated for 15 days were assessed at the optimal pH and incubation temperature, as well as the optimal concentration of the selected source carbon. Similarly, the effects of several nitrogen sources (peptone, beef extract, yeast extract, NH_4Cl, and $(NH_4)_2SO_4$) and the optimal concentration were determined.

2.5. Phenotypic analysis of A. camphorata

Fresh spore solution (5×10^7 spores) of mutant E3-64 and start strain AC16101 was seeded onto solid plates containing potato medium, carrot medium, wheat bran medium, or PDA and cultivated for 14 days at 28 °C. Colonies were imaged using a Canon camera (EOS 6D; Canon Inc., Tokyo, Japan). Similarly, the morphology of hypha cultivated in liquid medium for 7, 14, and 28 days was observed and images were captured using the same Canon camera.

2.6. Investigation of mycelial growth of A. camphorata

Fresh spores (5×10^7) of mutant E3-64 and start strain AC16101 were inoculated into 100 mL of the optimal liquid medium and subsequently cultured for 8–28 days under the optimal conditions. Samples were taken every 4 days. The harvested mycelia were separated by filtering with filter paper, dried at 50 °C, and weighted.

2.7. Genome re-sequencing

Genomic DNA was extracted from the mycelia of mutant E3-64 and start strain AC16101 and randomly fragmented by ultrasonication. DNA fragments of 300–400 bp were collected and used for construction of the read library. DNA sequencing was performed on a BGISEQ-500 platform at the Beijing Genomics Institute (BGI, Shenzhen, China). To guarantee data accuracy and reliability, low-quality reads and adaptors were removed to generate clean reads for subsequent bioinformatics analysis. The genome of *A. camphorata* strain S27 [12] was used as a reference. Detection of single-nucleotide polymorphisms and insertions/deletions (InDels) was carried out by BWA v0.7.10 and GATK v3.4.0 [13–16].

2.8. RT-qPCR

Transcript levels of genes encoding major enzymes involved in triterpenoid biosynthesis were assessed using RT-qPCR as previously described [17]. Total RNA of *A. camphorata* mycelia cultivated for 28 days was extracted using the TRIzol™ Plus RNA Purification Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the instructions. The extracted RNA was used for first-strand cDNA synthesis. SYBR® qPCR Master Mix (Vazyme, Nanning, China) was used for further PCR amplification. Relative expression of each tested gene was determined by normalizing transcript levels to the levels of the internal control (tyrosine kinase gene) in strain AC16101. Fold change values were calculated using the $2^{-\Delta\Delta Ct}$ method [18].

2.9. LC-MS/MS analysis

LC-MS/MS analysis was performed on a Thermo Scientific Q-Exactive platform (Thermo Fisher Scientific). LC analysis was carried out using a Hypersil GOLD C18 column (50 mm × 2.1 mm, 1.9 μm) with 0.1% formic acid in water (A) and acetonitrile (B) as the mobile phase. The injection volume was 1 μL and the flow rate was 0.3 mL/min. MS parameters were set as follows: ion source, ESI; ion detection mode, positive; sheath gas (N_2) pressure, 40 psi; auxiliary gas (N_2) pressure, 10 psi; spray voltage, 3.5 kV; ion transfer tube temperature, 320 °C; auxiliary gas temperature, 350 °C. The MS scanning range was 200–2000 *m/z* with full MS/dd-MS2 model.

2.10. Identification of individual triterpenoids

Individual triterpenoids in ethanol-extracted *A. camphorata* triterpenoids (ACET) were identified by LC-MS/MS analysis as described above, based on the molecular formulas of triterpenoids in positive ion mode. Compound Discoverer 3.2 was employed with the mzCloud database (Thermo Fisher Scientific).

2.11. Hepatoprotective effects of ACET in CCl_4-treated rat liver cells

The effects of ACET on the viability of CCl_4-treated rat liver cells were investigated with the methyl thiazolyl tetrazolium (MTT) method [19]. Normal liver cells (5×10^5) of neonatal Sprague-Dawley rats were inoculated into RPMI1640 medium containing fetal bovine serum at a final concentration of 10% (v/v) and incubated in a 5% CO_2 incubator for 5 days at 37 °C. The medium was replaced by fresh RPMI1640 medium and CCl_4 was added at a final concentration of 8 mM. After 6 h, ACET containing different amounts (2.5–20 μg) of triterpenoids was added and then cells were cultivated for 24–72 h. Experiments were performed in triplicate. Subsequently, MTT was added to all wells at a final concentration of 0.5 mg/mL, followed by incubation for 4 h at 37 °C. The generated formazan was dissolved by dimethyl sulfoxide, and the absorbance value at 570 nm was measured in a spectrophotometer. The cell survival rate was calculated according to the generated absorbance values.

2.12. Animals and treatments

Healthy SPF grade Institute of Cancer Research mice with a body weight of 18–22 g were provided by the Laboratory Animal Center of Guangxi Medical University (Laboratory Animal Production License No.: SCXK (Gui) 2020–0003). Animals were reared in separate cages with free access to food and water at a temperature of 23 ± 2 °C and a relative humidity of 60% ± 5%, with a natural light/dark cycle. All animal procedures were approved by the Animal Ethics Committee of Guangxi Botanical Garden of Medicinal Plants (SYXK (Gui) 2020–0004).

The mice were randomly divided into the following six groups ($n = 10$ mice per group): (i) the blank control group, (ii) the CCl_4 group, (iii) the Bifendatatum group (8 mg/kg), (iv) the low-dose ACET group (12.5 mg triterpenoids/kg), (v) the middle-dose ACET group (25.0 mg/kg), and (vi) the high-dose ACET group (50.0 mg/kg). Mice in the blank control group and the CCl_4 group were given 0.08% sodium carboxymethyl cellulose aqueous solution, and mice in the other groups were fed the corresponding drugs. After continuous administration for 14 days, mice in the blank control group received an intraperitoneal injection of pure peanut oil, and mice in all other groups were injected intraperitoneally with peanut oil containing 0.15% CCl_4. Blood was collected 16 h after injection and centrifuged at 1000×*g* for 10 min. The upper serum was collected, and alanine aminotransferase (ALT) activity, aspartate aminotransferase (AST) activity, the total bile acid (TBA) content, and the total bilirubin (TBIL) content were determined using the Alanine aminotransferase Assay Kit (#C009-2-1), the Aspartate aminotransferase Assay Kit (#C010-2-1), the Total bile acid kit (#E003-2-1), and the Total bilirubin kit (#C019-1-1), respectively (all purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.13. Histological analyses

Mouse liver tissue was fixed with 10% formaldehyde solution, followed by dehydration, transparentization, wax dipping, embedding, sectioning, and staining with hematoxylin–eosin. Finally, the degree of liver tissue damage was observed under a light microscope (OLYMPUS DP480; Olympus, Tokyo, Japan).

2.14. Statistical analysis

All experimental data generated were analyzed with Microsoft Excel (Microsoft, Redmond, WA, USA). Significance of differences was evaluated by Student's *t*-test and/or one-way ANOVA.

2.15. Accession number

Genome re-sequencing data have been deposited in the Sequence Read Archive with accession number PRJNA737152.

3. Results

3.1. Screening for triterpenoid hyperproducers of A. camphorata through chemical and physical mutagenesis

To choose the appropriate EMS treatment duration, *A. camphorata* AC16101 lethality was determined after treatment for 2–10 h. The results showed that lethality increased over time, reaching 94.6% at 8 h (Fig. S1A). Moreover, the lethality of mutant E1-1-5, which was isolated after EMS treatment, upon ARTP treatment was investigated, and treatment with a radiofrequency power input of 130 W and a flow rate of 10 L/min for 200 s resulted in 96.5% lethality (Fig. S1B). These treatment conditions were used for EMS- and ARTP-mediated mutagenesis.

Multiple rounds of EMS and ARTP treatments were performed according to the steps described in Fig. 1A. Fresh spores of the start

strain were spread on PDA plates and then cultivated for 7 days at 28 °C. A total of 275 colonies were selected, including 245 from EMS mutagenesis and 30 from ARTP mutagenesis. We further chose 201 colonies for direct inoculation into liquid medium for cultivation for 28 days and measured the triterpenoid content in mycelia. Comparative analysis revealed that mycelia of mutants E, E1-1-5, E3-64, and AE-5, which were obtained after each round of treatment, contained 105.8–172.8 mg triterpenoids per gram dry weight, which was 30.0%–88.6% more than the triterpenoid content of strain AC16101, except for strain E, whose triterpenoid content was similar to that of strain AC16101 (Fig. 1B). The mycelia of mutant E3-64 showed the highest triterpenoid content (172.8 mg/g dry weight). These results suggested that both EMS and ARTP could promote triterpenoid biosynthesis in *A. camphorata*.

To assess the stability of mutant E3-64 regarding triterpenoid biosynthesis, triterpenoid content of mycelia was determined after each of six successive rounds of sub-culture. No significant differences were observed (Fig. 1C), indicating that mutant E3-64 is genetically stable.

3.2. Optimization of cultivation conditions for mutant E3-64

Culture parameters considerably affect *A. camphorata* growth and triterpenoid biosynthesis [2]. To further improve the triterpenoid contents of mutant E3-64 mycelia, liquid culture conditions were optimized, including the initial pH of the medium, temperature, carbon source, and nitrogen source. The triterpenoid content of mutant E3-64 mycelia increased with the increase in pH, reaching a peak at pH 5.0, and then dropped (Fig. 2A). Temperature slightly affects the triterpenoid content of mycelia; we found that 28 °C was the most suitable temperature for triterpenoid biosynthesis (Fig. 2B). Moreover, 85 g/L glucose and 10 g/L yeast extract improved triterpenoid biosynthesis compared with the other tested carbon and nitrogen sources (Fig. 2C–F). Altogether, the optimal cultivation conditions for triterpenoid biosynthesis of *A. camphorata* mutant E3-64 were 85 g/L glucose, 10 g/L yeast extract, pH 5.0, and 28 °C. Under these conditions, E3-64 synthesized 255.5 mg triterpenoids per gram dry mycelia at 28 days, i.e., an increase of 47.9% compared with the yield under non-optimal conditions.

3.3. Phenotypic and growth analyses of mutant E3-64

To investigate the effects of EMS treatment on fungal growth and phenotypes, *A. camphorata* mutant E3-64 and start strain AC16101 were cultivated on solid plates containing PDA, potato medium, carrot medium, and wheat bran medium for 14 days. Colony

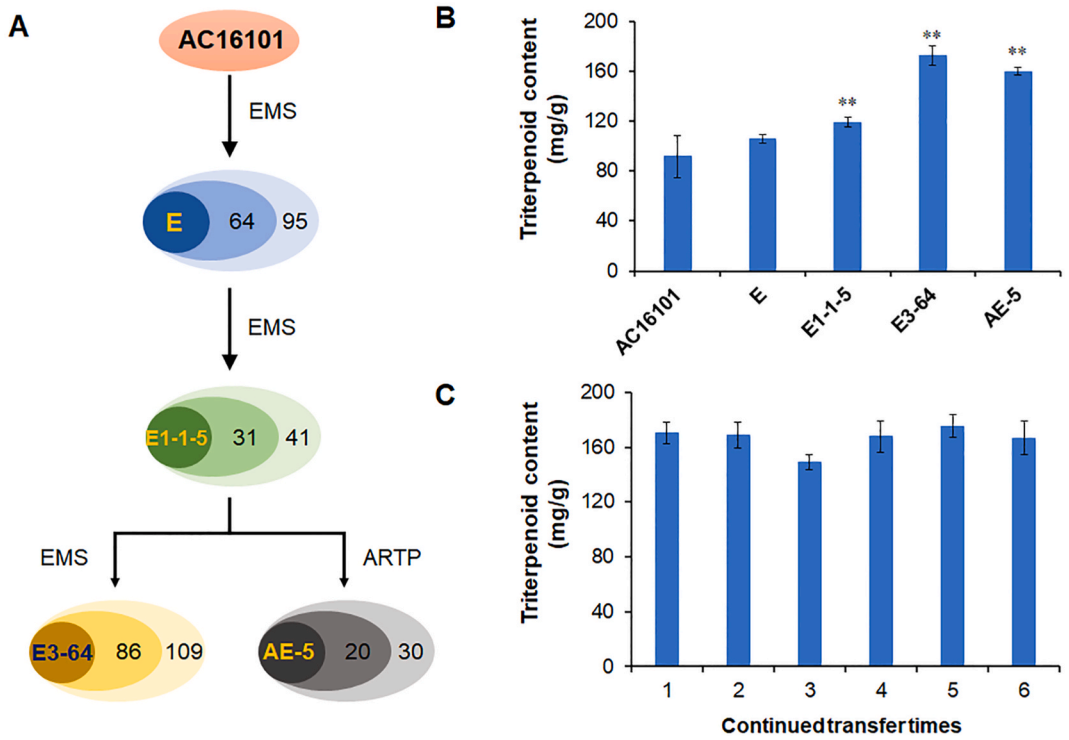

Fig. 1. EMS/ARTP-mediated mutagenesis and screening for triterpenoid hypersynthesizers of *A. camphorata*. (A) Schematic illustration of the mutagenesis and screening process. (B) Investigation of triterpenoid biosynthesis by potential *A. camphorata* mutants selected from each round of mutagenesis. (C) Triterpenoid contents of mutant E3-64 sub-cultured consecutively six times. All fungal strains were cultivated for 28 days. $^{**}P < 0.01$ between the isolates and the start strain AC16101, as determined by Student's *t*-test. EMS: ethyl methane sulfonate; ARTP: atmospheric and room temperature plasma.

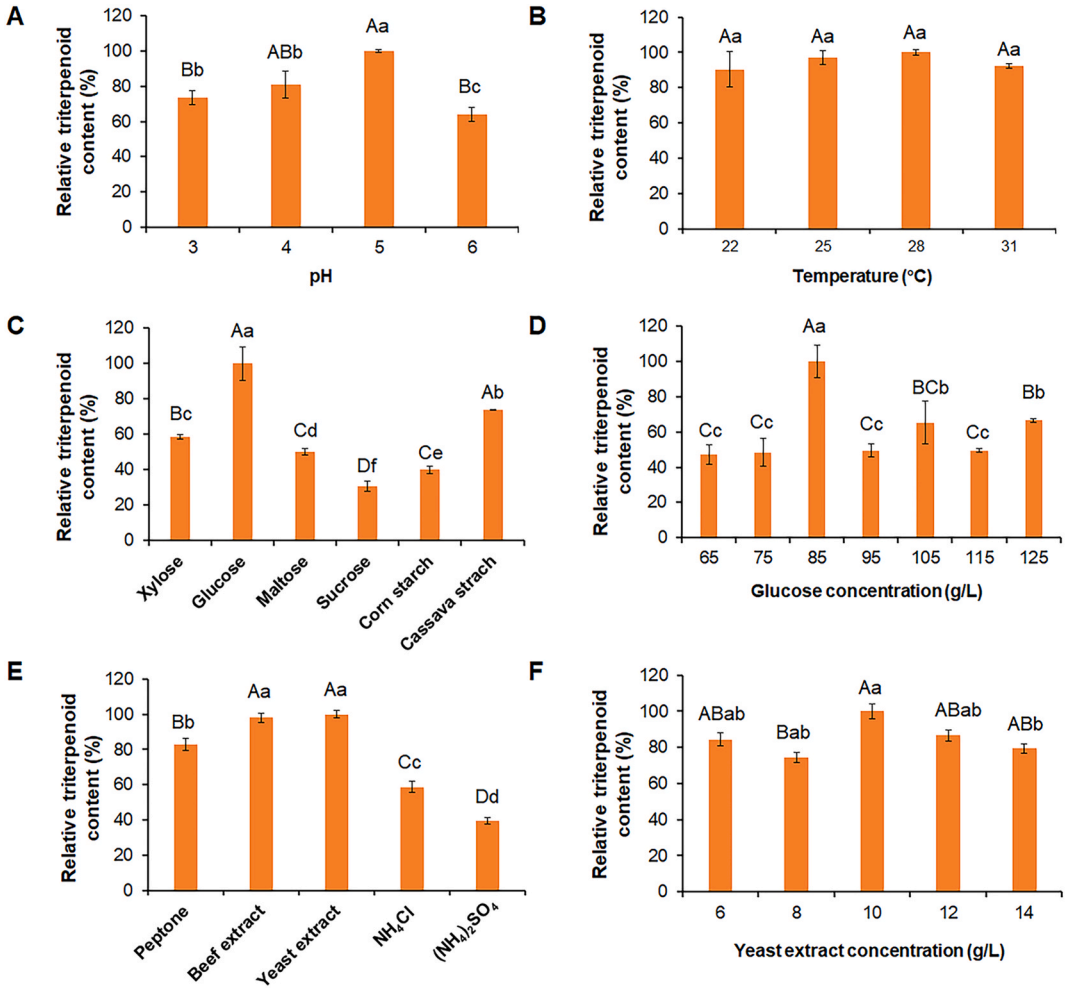

Fig. 2. Effects of culture conditions and medium components on triterpenoid biosynthesis in *A. camphorata* mutant E3-64. (A) Initial medium pH. (B) Incubation temperature. (C) Carbon source. (D) Glucose concentration. (E) Nitrogen source. (F) Yeast extract concentration. Different uppercase and lowercase letters indicate $P < 0.01$ and $P < 0.05$, respectively, as determined by Student's *t*-test.

sizes of both E3-64 and AC16101 looked similar, whereas the colony color of E3-64 was slightly darker than that of AC16101 (Fig. 3A). Mycelial colors of both E3-64 and AC16101, as well as liquid cultures, gradually became from yellow to orange yellow during cultivation. Interestingly, the mycelial color of E3-64 is darker than that of AC16101 at the same cultivation time (Fig. 3B). These results hinted that pigment production of *A. camphorata* might be associated with triterpenoid biosynthesis. Moreover, growth curve analysis revealed that mycelial accumulation of E3-64 was similar to that of AC16101 (Fig. 3C) in the optimized liquid medium, suggesting that improvement of triterpenoid content in mutant E3-64 is attributed to changes of the regulatory network of triterpenoid biosynthesis.

3.4. Analysis of triterpenoid compounds in the mycelia of mutant E3-64

Furthermore, the composition of triterpenoids produced in the mycelia of *A. camphorata* mutant E3-64 and strain AC16101 was compared. Four ergostane triterpenoids (antcin A, antcin B, antcin G, and antcamphin A) with changes of contents between mutant E3-64 and start strain AC16101 were detected through LC-MS/MS analysis in positive ion mode and comparison of the molecular formulas of triterpenoids [3]. The contents of antcin A and antcamphin A increased by 94% and 178% in E3-64 mycelia, respectively (Fig. 4A and B), while antcin B and antcin G contents decreased by 52% and 15%, respectively, in comparison with AC16101 (Fig. 4C and D).

3.5. Analysis of transcript levels of major genes involved in triterpenoid biosynthesis in mutant E3-64

Five key enzymes are involved in triterpenoid biosynthesis in *A. camphorata*, including pyrophosphomevalonate decarboxylase (MVD; EMD33647.1), lanosterol 14α-demethylase (CYP; ABV66226.1), squalene synthase (SQS; AFR13032.1), squalene epoxidase

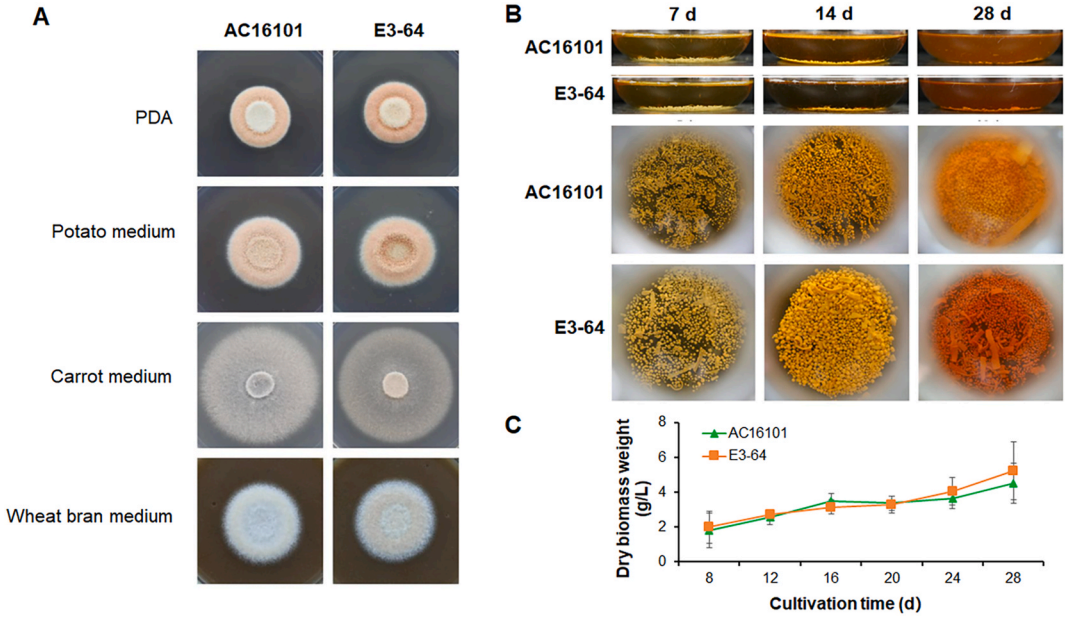

Fig. 3. Phenotypic (A, B) and growth (C) analysis of *A. camphorata* mutant E3-64 and start strain AC16101. In panel (A), all fungal strains were cultivated for 7 days.

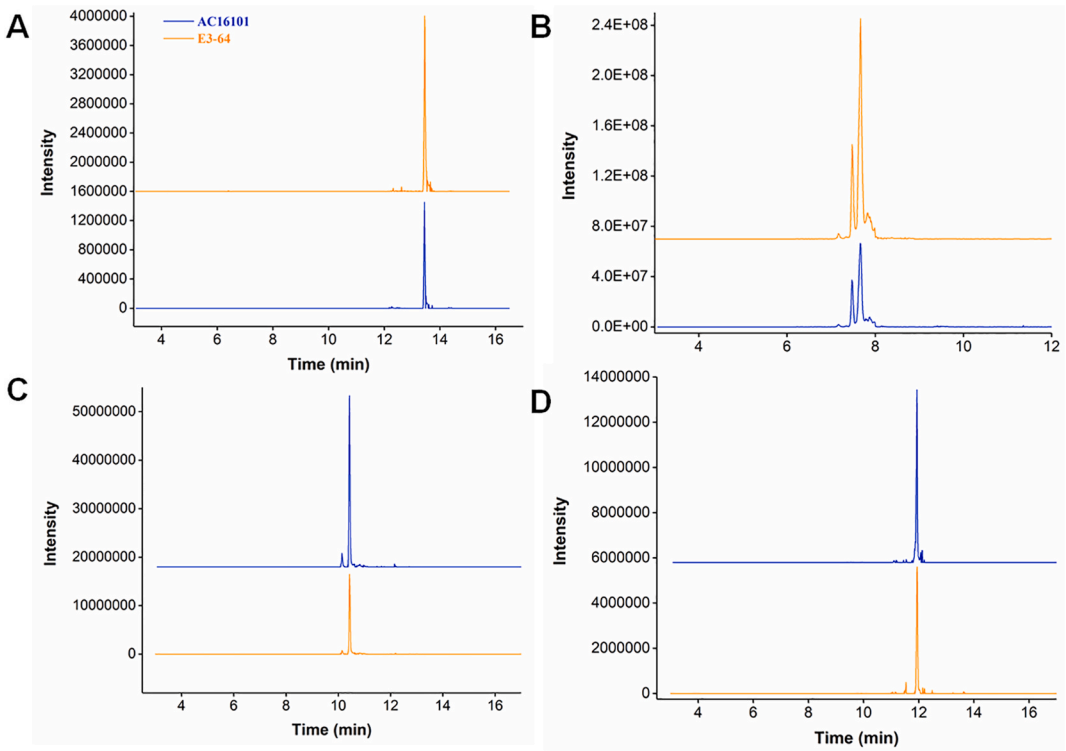

Fig. 4. Analysis of triterpenoid compounds from *A. camphorata* by LC-MS/MS. (A) Antcin A. (B) Antcin B. (C) Antcin G. (D) Antcamphin A.

(SE; CCM03056.1), and 2,3-oxidosqualene cyclase/lanosterol synthase (OSC/LS; D7NJ68) [1,12]. Remarkably, RT-qPCR analysis revealed that the transcript levels of all five genes encoding these key enzymes were considerably enhanced in mutant E3-64 (by 59.7%–206.1%) compared with strain AC16101 (Fig. 5). High expression of these key genes contributed to enhanced triterpenoid production of mutant E3-64.

3.6. Genome re-sequencing of mutant E3-64

In order to understand the reasons behind the observed differences in phenotypes and triterpenoid contents, genome re-sequencing of both mutant E3-64 and strain AC16101 was performed, followed by comparative analysis. A total of 3130 and 2876 Mb of clean reads was obtained for AC16101 and E3-64, respectively, with an average sequencing depth of more than 80 × . Comparative analysis showed that coverage of the *A. camphorata* strain S27 genome was over 95% [12].

In total, 2952 InDels and 24,662 single-nucleotide variations (SNVs) were detected in mutant E3-64 compared with AC16101. Of these, 1840 InDels (984 deletions and 856 insertions) were distributed in coding sequences (CDSs) (Table S1) and 1112 InDels (566 deletions and 546 insertions) were distributed in intergenic regions (Table S2). Among the 1112 InDels in intergenic regions, 384 InDels were located <1000 bp upstream of an ATG start codon and 223 InDels were located <300 bp downstream of a termination codon (TAG, TGA, or TAA) (Table S2). Moreover, 14,932 SNVs were located in intergenic regions, including 5719 SNVs located <1000 bp upstream of an ATG start codon, 2668 SNVs located <300 bp downstream of a termination codon (TAG, TGA, or TAA), and 6545 SNVs located in other regions (Table S3). The remaining SNVs were located in CDSs (Fig. 6A; Table S4). In total, 5886 SNVs and 1567 InDels were distributed in genes in mutant E3-64. Interestingly, among SNVs in CDSs, 78 SNVs resulted in a premature stop codon and 5132 led to a non-synonymous mutation, in 74 and 2673 genes. Moreover, 70 SNVs resulted in non-synonymous changes of stop codons (Fig. 6A; Table S4). Kyoto Encyclopedia of Genes and Genomes annotation revealed that the genes with SNVs and InDels were mainly involved in metabolism, followed by global and overview maps (Fig. 6B).

Screening of these genes revealed that 156 terpenoid biosynthesis genes (TBGs) were detected in mutant E3-64, which contained 525 SNVs and 69 InDels, which accounted for 64.2% of total TBGs annotated in the genome of *A. camphorata* strain S27 [12]. Changes of promoter and terminator regions may affect gene transcription, while non-synonymous mutations of CDSs may disturb protein structure and function. There were 24 and 55 TBGs containing InDels and non-synonymous SNVs in CDSs, respectively, and there were 12 and 100 TBGs containing InDels and non-synonymous SNVs in promoter/terminator regions, respectively (Fig. 6C). We found 13 genes involved in terpenoid backbone biosynthesis (map00900 at Kyoto Encyclopedia of Genes and Genomes), whose encoded proteins included 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS; GME3566), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR; GME2824), phosphomevalonate kinase (MPK; GME5956), pyrophosphomevalonate decarboxylase (MVD; GME8756), isopentenyl-diphosphate isomerase (IDI; GME2391), geranyl diphosphate synthase (GPP; GME2121), farnesyl diphosphate synthase (FPP; GME9130), undecaprenyl diphosphate synthase (UPPs; GME6314), and five protein-S-isoprenylcysteine O-methyltransferases (ICMTs; GME6924, GME6424, GME593, GME7381, and GME1794) (Fig. 6D).

In addition, many genes encoding monoterpene synthases (13), sesquiterpene synthases (11), cytochrome P450s (45), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferases (11), 4-hydroxy-3-methylbut-2-enyl diphosphate reductases (8), and polyketide beta-ketoacyl synthases (4), as well as genes encoding polyketide beta-ketoacyl-synthases (4), lipases (3), 3-ketoacyl-CoA thiolases (2), and enoyl-CoA reductase (1), were also found to contain SNVs and InDels.

Notably, changes in all four regions described above were observed in *GME7184*, including T−166A, T−399G, and I7 (CGTCCGT) at −410 in the promoter region, T51C in the terminator region, and A206G at 823 leading to N206S and I1 (T) at 294 in the CDS. *GME7184*, a homolog of *ACg008754* in the S27 genome, was predicted to encode a monoterpene synthase. GME8756, which harbors an R84K mutation in mutant E3-64, is annotated as the key MVD in triterpenoid biosynthesis. Moreover, the putative promoter region of *GME6984* was found to harbor a T−397C mutation, which might result in enhanced expression in mutant E3-64.

Gene expression is strictly regulated by specific transcription factors (TFs). We found 17 TFs in which 41 non-synonymous SNVs and SNVs in the putative promoter and terminator regions and five InDels in the CDSs and putative promoter and terminator regions were detected. Interestingly, in three genes (*GME3788*, *GME3338*, and *GME3964*) that were annotated to encode Gti1/Pac2 family TFs, 13 non-synonymous SNVs and one deletion in the putative promoter and terminator regions were found in both *GME3788* and

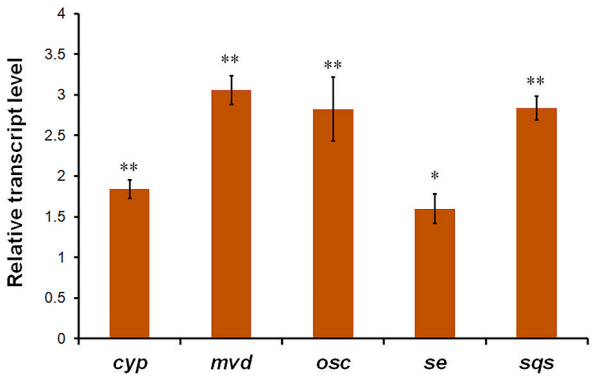

Fig. 5. Transcript levels of genes involved in triterpenoid biosynthesis in *A. camphorata*. Expression levels of the tested genes in mutant E3-64 were normalized against those in the start strain AC16101. The tyrosinase gene was used as a reference. *$P < 0.05$, **$P < 0.01$ between mutant E3-64 and start strain AC16101, as determined by Student's *t*-test. Each experiment included three biological replicates. Each data point represents mean ± SD. *cyp*: lanosterol 14α-demethylase gene; *mvd*: pyrophosphomevalonate decarboxylase gene; *osc*: 2,3-oxidosqualene cyclase/lanosterol synthase gene; *se*: squalene epoxidase gene; *sqs*: squalene synthase gene.

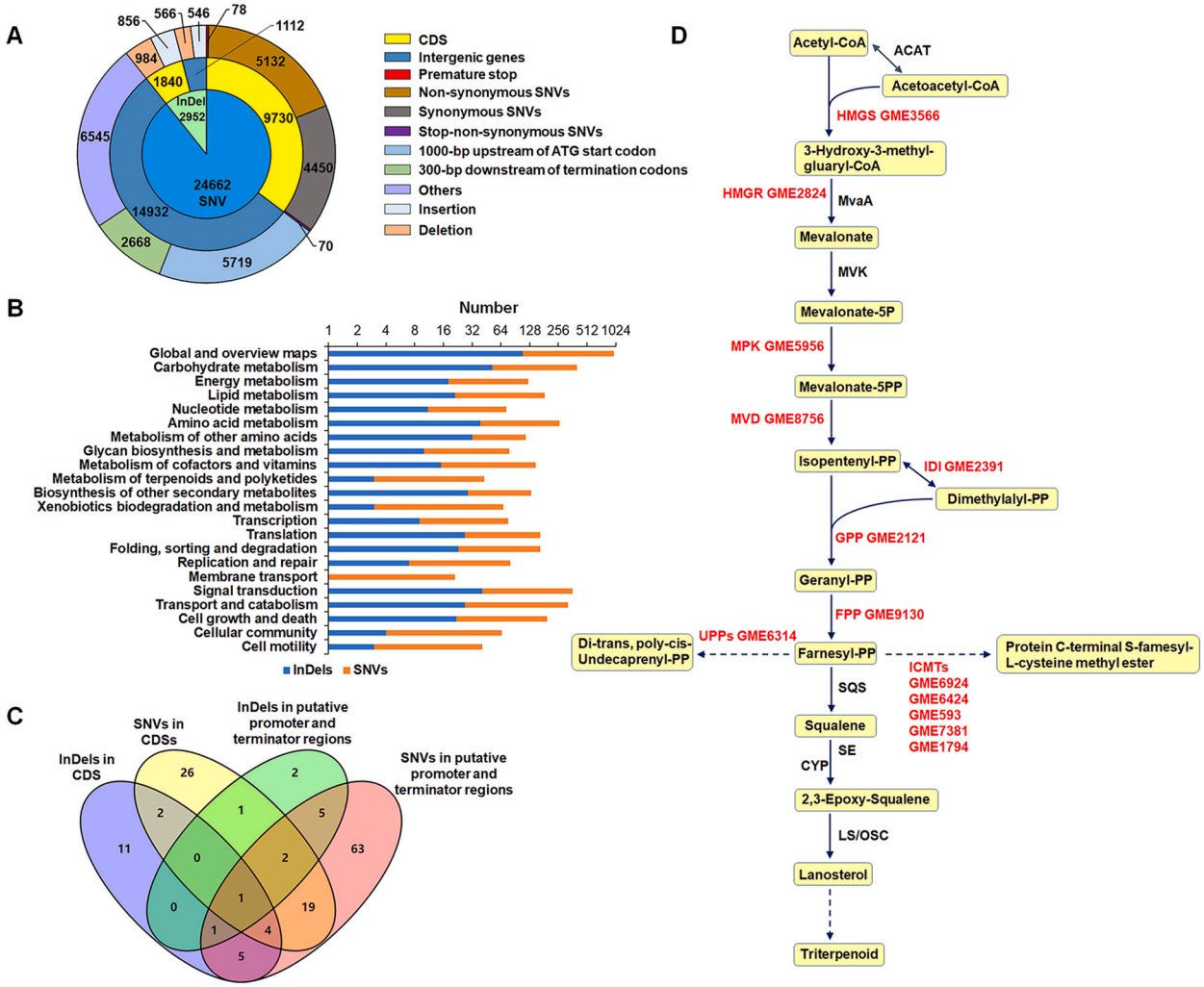

Fig. 6. Genome re-sequencing comparative analysis of *A. camphorata* mutant E3-64 and the parental strain AC16101. (A) Distribution of single-nucleotide variations (SNVs) and Insertion/deletions (InDels) in mutant E3-64. CDS: coding DNA sequence. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of genes containing SNVs and InDels. (C) Venn diagram showing unique and shared genes containing non-synonymous SNVs and InDels. (D) Putative pathway of triterpenoid biosynthesis in *A. camphorata*. Red color indicates the appearance of non-synonymous SNVs and/or InDels. The putative promotor is defined as the 1000-bp region upstream of the ATG start codon; the putative terminator is the 300-bp region downstream of the termination codon (TAG, TGA, or TAA).

GME3338, as well as one deletion in the putative promoter region of *GME3964*. *GME1342* encodes a homolog of *Saccharomyces cerevisiae* OPI1, which mediates repression of phospholipid biosynthesis [20]. *GME1970*, which has three non-synonymous SNVs and one insertion, encodes a homolog of the *S. cerevisiae* oxidative stress sensing biosensor YAP1 [21]. In addition, many SNVs and InDels were found in genes encoding pre-initiation complexes, such as the transcription initiation factor TFII subunits TFIID and TFIIF and mediators MED13 and MED4.

3.7. Hepatoprotective activity of triterpenoids extracted from mutant A. camphorata mycelia against CCl_4-induced liver injury

To investigate the hepatoprotective effects of ACET from mutant E3-64 mycelia, rat liver cells were treated with CCl_4. In the CCl_4 group (no triterpenoid added), survival rates of the liver cells were 64.3%–67.5% when cultivated for 24–72 h after 6 h of CCl_4 treatment compared with the untreated control. ACET containing 5–20 μg triterpenoids considerably improved survival rates of the CCl_4-treated liver cells; when ACET containing over 10 μg triterpenoids was added, survival rates could reach the same levels as in the Bifendatatum group (positive control). However, ACET containing 2.5 μg triterpenoids did not significantly influence the survival rate of liver cells (Table 1).

Moreover, the effects of ACET on CCl_4-induced liver injury in mice were investigated. In general, CCl_4 treatment results in the destruction of the cell membrane of liver cells and hence leakage of ALT and AST into the blood circulation. Indeed, intraperitoneal

injection of CCl_4 could remarkably increase the activities of ALT and AST. However, pretreatment with a high dose of triterpenoids clearly decreased the activities of ALT and AST, reaching levels that were comparable to those of the Bifendatatum group (positive control) (Fig. 7A and B).

Similarly, the serum concentrations of TBA and TBIL in mice treated with middle- or high-dose triterpenoids were markedly lower than in the CCl_4 group, again reaching levels that were comparable to those in the Bifendatatum group (Fig. 7C and D).

Furthermore, microscopic analysis was conducted. In untreated mice, the hepatocytes were intact and well ordered, with round and central nuclei and a well-preserved cytoplasm (Fig. 8A). Comparatively, CCl_4 treatment resulted in serious hepatocyte necrosis, including nuclear shrinkage, membrane loss, and nucleolar fragmentation (Fig. 8B). In comparison with those of Bifendatatum (Fig. 8C), low- or middle-dose ACET slightly mitigated hepatocyte necrosis (Fig. 8D and E). High-dose ACET exerted similar hepatoprotective effects as Bifendatatum (Fig. 8C and F). These results indicated that triterpenoid-containing ACET exhibits hepatoprotective activity in a dose-dependent manner.

4. Discussion

In this present study, we firstly employed mutagenesis breeding with EMS and/or ARTP and optimized culture conditions to enhance the triterpenoid yield of the medicinal fungus *A. camphorata*. Mycelia of the resulting mutant E3-64 contained a triterpenoid content of 255.5 mg/g dry weight, i.e., an increase of 137.7% in comparison with strain AC16101. To the best of our knowledge, this is the highest triterpenoid content reported to date in *A. camphorata* flask cultures, even higher than that in its fruiting body. The mycelia of most *A. camphorata* strains contain 23.3–117.2 mg triterpenoids/g dry weight, and fruiting bodies typically contain 164–175 mg/g dry weight (Table 2). For example, Ma et al. [23] reported that the triterpenoid content of mycelia reached 117.2 mg/g dry weight in flask cultures by adding 4% tangerine peel extract. Mutant E3-64 can achieve triterpenoid levels of 1640.1 mg/L, and is therefore only outperformed by the fruiting bodies reported by Huang et al. [30].

Interestingly, when cultivated in optimized glucose medium, the culture color of mutant E3-64 exhibited marked changes (darker orange) compared to the start strain AC16101, suggesting that more orange pigment was produced during triterpenoid biosynthesis. It is not surprising that biosynthesis of several secondary metabolites is generally co-regulated. For example, in *Fusarium graminearum*, ergosterol biosynthesis and pigment production are regulated by the TF FgSR [31]. However, how pigment and triterpenoid biosynthesis are co-regulated in *A. camphorata* requires further study.

It should be noted that mycelial accumulation is vital to triterpenoid biosynthesis in *A. camphorata* [2]. However, biomass of both mutant E3-64 and AC16101 is quite low compared with that of most *A. camphorata* strains; for example, Li et al. [4] reported a biomass of 7.8 g and Yang et al. [24] even reported a biomass of 22.0 g. These results indicate that the liquid medium used in the present study benefits the production of secondary metabolites such as triterpenoids, as well as orange pigments, but not mycelial growth. Therefore, it is necessary to improve the biomass of mutant E3-64 in the future.

Moreover, we found that mycelial accumulation of mutant E3-64 was similar to that of AC16101, suggesting that the improvement of triterpenoid content in mutant E3-64 was attributed to changes of the regulatory network of triterpenoid biosynthesis. For example, five major genes (*cyp*, *mvd*, *osc*, *se*, and *sqs*) involved in triterpenoid biosynthesis in *A. camphorata* were considerably upregulated in mutant E3-64 at the transcriptional level. MVD catalyzes the conversion of mevalonate into isopentenyl-PP (IPP), serving as the fundamental building block for downstream terpenoid biosynthesis. CYP converts lanosterol to 4,4-dimethyl-zymosterol through promoting 14α-demethylation, which is the rate-limiting step in ergosterol biosynthesis. CYP51 plays an important role in ergostane biosynthesis and the formation of fruiting bodies. *CYP51* (*ACg002141*) in *A. camphorata* strain S27 shows high expression in the binucleated mycelium and the fruiting body [12]. The intermediate squalene is derived from farnesyl-PP via SQS and then converted to 2,3-oxidosqualene by SE [1,12]. OSC/LS promotes cyclization of the generated 2,3-oxidosqualene to lanosterol. Overexpression of *osc/ls* efficiently enhances the triterpenoid content of *A. camphorata* [32].

Furthermore, various SNVs and InDels were found in mutant E3-64 compared with AC16101. Many of them were detected in genes associated with terpenoid backbone biosynthesis and genes encoding TFs. However, how these alterations influence the expression of genes participating in triterpenoid synthesis has been unclear thus far. Especially, *GME3788*, *GME3338*, and *GME3964* were annotated to encode Gti1/Pac2 family TFs. In total, 13 non-synonymous SNVs and one deletion were found in the putative promoter and

Table 1
Hepatoprotective effects of ACET against CCl_4-treated rat liver cells.

Group	Triterpenoid concentration (μg)	Survival rate (%)		
		24 h	48 h	72 h
ACET_0	0	67.52 ± 4.35	64.32 ± 3.91	66.34 ± 4.25
ACET_2.5	2.5	64.12 ± 3.29	67.08 ± 7.02	65.45 ± 6.56
ACET_5	5	71.13 ± 3.71*	80.17 ± 4.32**	85.15 ± 4.43**
ACET_10	10	83.22 ± 3.53**	87.43 ± 4.63**	89.24 ± 3.12**
ACET_20	20	87.41 ± 5.31**	90.42 ± 5.23**	91.33 ± 3.87**
DDB	Bifendate	79.61 ± 2.12**	86.23 ± 3.65**	89.76 ± 2.43**
NC	Negative control	100.00 ± 0.43**	100.00 ± 1.41**	100.00 ± 2.23**

Note: ACET, alcoholic extracted triterpenoid of *A. camphorata* mutant E3-64. ACET_0: CCl_4-treated control group (no triterpenoid added); NC: negative control (CCl_4-untreated control). * and ** represent $p < 0.05$ and $p < 0.01$ between ACET_0 group and other groups, respectively, accessed by Student's *t*-test.

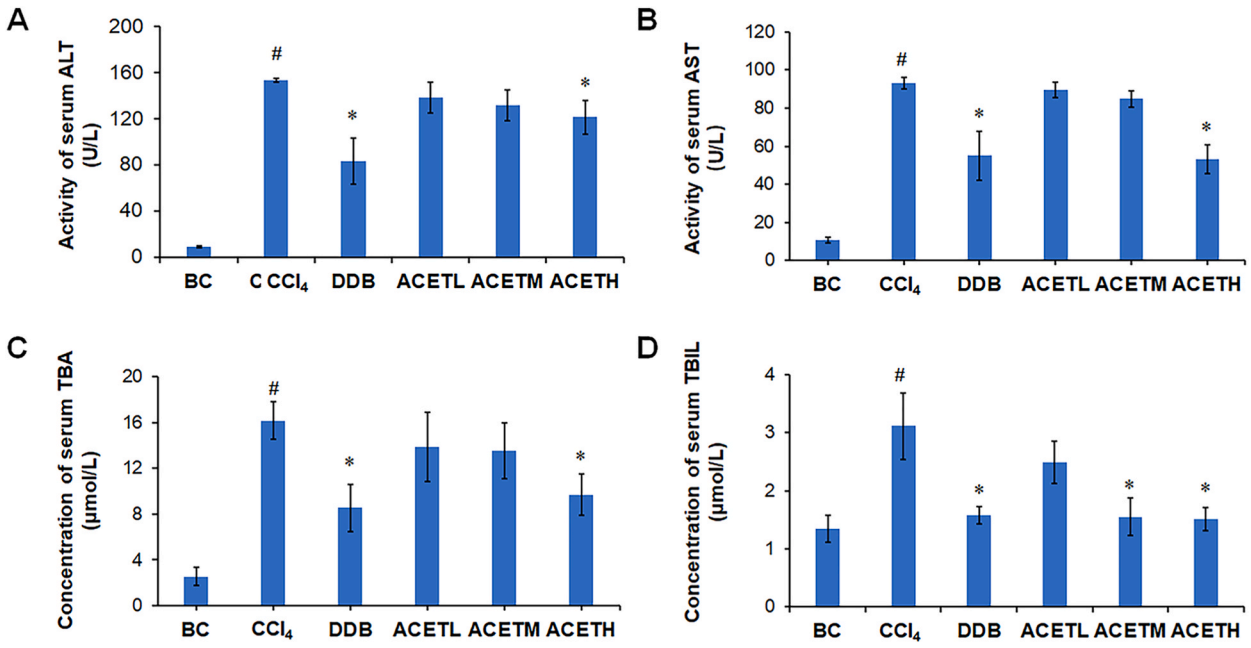

Fig. 7. Effects of ethanol-extracted triterpenoids from *A. camphorata* mutant E3-64 (ACET) on serum biomarkers in CCl_4-treated mice. (A) Alanine aminotransferase (ALT) activity. (B) Aspartate aminotransferase (AST) activity. (C) Total bile acid (TBA) content. (D) Total bilirubin (TBIL) content. Data are presented as mean ± SEM ($n = 10$). $^{*}P < 0.05$ between the CCl_4 group and the ACETL, ACETM, or ACETH group. $^{\#}P < 0.01$ between the CCl_4 group and the blank control. Statistical analysis was conducted by one-way ANOVA. BC: blank control; DDB: Bifendatatum; ACETL: low-dose ACET; ACETM: medium-dose ACET; ACETH: high-dose ACET.

terminator regions of both *GME3788* and *GME3338* happened, and one deletion was found in the putative promoter region of *GME3964*. Wor1, a member of the Gti1/Pac2 family, is widely present in fungi; it regulates multiple morphological changes such as fungal growth, mating, conidiation, and virulence [33,34]. *GME1342* encodes a homolog of *S. cerevisiae* OPI1, which mediates repression of phospholipid biosynthesis [20]. *GME1970*, which encodes a homolog of the *S. cerevisiae* oxidative stress biosensor YAP1 [21], was found to have three non-synonymous SNVs and one insertion. In addition, many SNVs and InDels were found in genes encoding pre-initiation complexes, such as the transcription initiation factor TFII subunits TFIID and TFIIF and mediators MED13 and MED4.

Finally, as expected, ACET from the mutant E3-64 showed excellent hepatoprotective activity against CCl_4-induced liver damage, suggesting that ACET when produced under the optimal cultivation conditions contained triterpenoids, and exerted promising hepatoprotective effects, comparable to the previously reported effects of the fruit body and spore powder [1,35–37]. The hepatoprotective activity of E3-64 mycelia is attributed to antcins A, B, and G and antcamphin A, which were detected by LC-MS/MS. Li et al. reported that 16 of 29 triterpenoids from the crude extract of *A. camphorata* exerted significant protective effects against CCl_4 toxicity in HepG2 cells in vitro, and further confirmed that antcin B and K ameliorate CCl_4-induced liver injury in mice [36].

Altogether, the *A. camphorata* mutant E3-64 was obtained. This mutant shows improved triterpenoid production compared with the parental strain AC16101; under optimized cultivation conditions, its mycelia contained 255.5 mg triterpenoid per gram dry weight. LC-MS/MS analysis revealed that the antcin A and antcamphin A contents increased by 94% and 178%, respectively, in E3-64 mycelia, which might result from increased expression of major genes involved in triterpenoid biosynthesis. Notably, the antcin B and G contents decreased by 52% and 15%, respectively. ACET from E3-64 mycelia exerted excellent hepatoprotective activity against CCl_4-induced liver damage in mice. These results are promising for the commercial application of *A. camphorata*.

5. Limitations of the study

Cultivation conditions were optimized in culture flasks, and it is not known how high the production of triterpenoids will be in large-scale fermentation systems. In addition, ACET is not pure. Therefore, we cannot exclude the possibility that other unknown factors contribute to its hepatoprotective activity.

Author contribution statement

Huan-Ju Wang; Ce Cui: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Xiao-Mei Gong; Cheng-Xi Li; Hao Guo; Ya-Ling Wang; Yu-Dan Huang; Jian-Lin Jiang; Xue-Mei Luo; Tian-Qi Liu: Analyzed and interpreted the data. Jian-Hua Miao: Contributed reagents, materials, analysis tools; Shuo Wang; Shuai Zhao; Jia-Xun Feng: Conceived and designed the

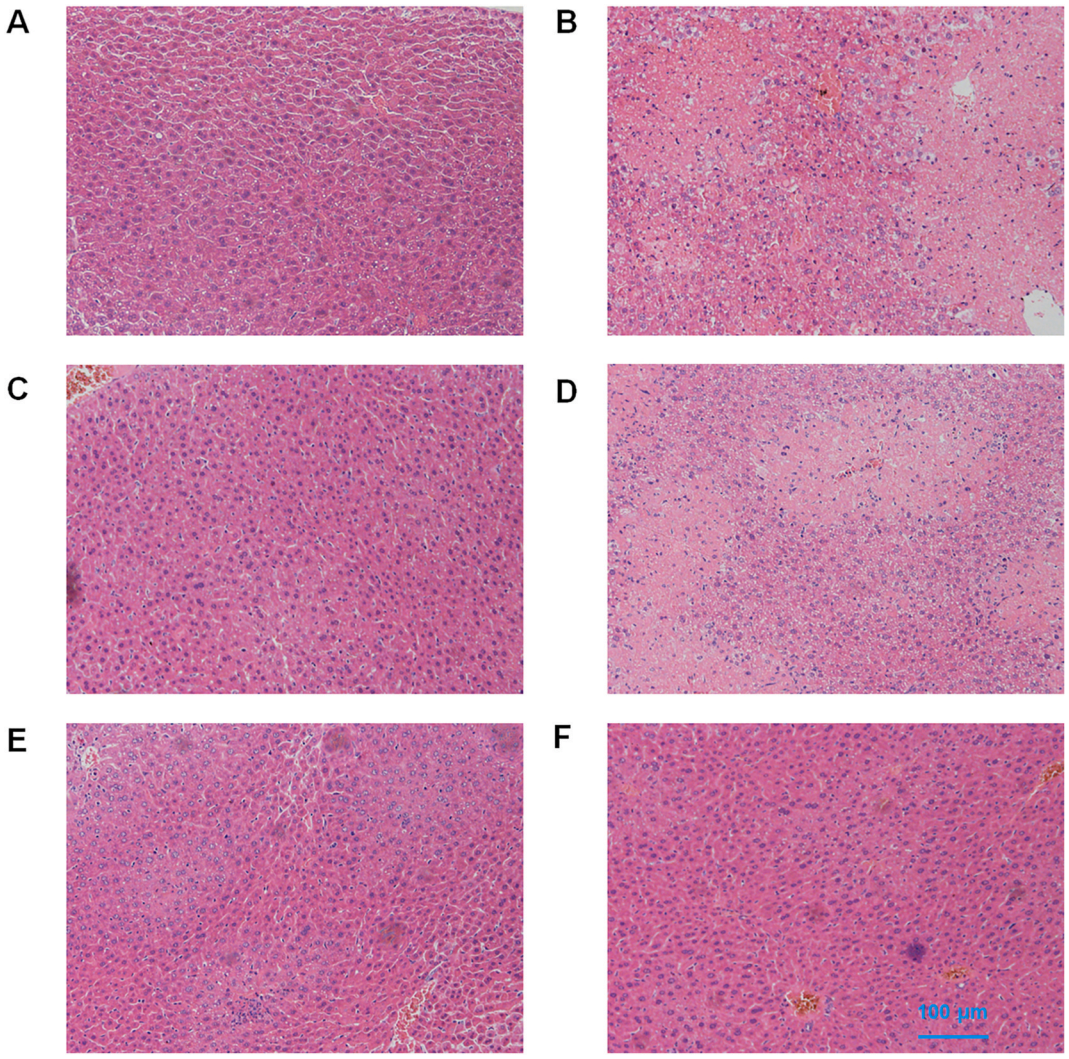

Fig. 8. Effect of ethanol-extracted triterpenoids from *A. camphorata* mutant E3-64 (ACET) on liver pathology in CCl_4-treated mice as determined by HE staining. (A) Blank control; (B) CCl_4; (C) Bifendatatum; (D) Low-dose ACET; (E) Medium-dose ACET; (F) High-dose ACET.

Table 2
Comparison of the production of triterpenoid among *A. camphorata* mutant E3-64 and other known strains.

A. camphorata strains	Sample	Cultivation time (d)	Elicitor	Content of triterpenoid (mg/g DW)	Total triterpenoid production (mg/L)	References
E3-64	Mycelia under	28	–	255.5	1640.1	This study
AC16101	SmF	28	–	107.5	738.2	This study
ATCC 200183		12	α-terpineol	23.3	91.3	[22]
CCRC35396		28	Tangerine peel extract	117.2	1417.8	[23]
CCRC35396		28	Citrus peel extract	NA	283	[24]
AC0623		7	–	31.8	NA	[25]
ATCC 200183		10	–	NA	64.2	[26]
BCRC35716		21	Chitosan	40	NA	[27]
		7	–	10.2	70	[4]
–	Mycelia under SSF	30	α-terpineol	32.5	NA	[28]
	Fruiting body	–	–	175	NA	[29]
		–	–	164.2	NA	[30]

Note: SmF, Submerged fermentation; SSF: Solid-state fermentation; NA: Not tested.

experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data associated with this study has been deposited at Sequence Read Archive (SRA) under the accession number PRJNA737152. Declaration of interests.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19621.

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