

Characterization of the 2,3-Oxidosqualene Cyclase Gene from *Antrodia cinnamomea* and Enhancement of Cytotoxic Triterpenoid Compound Production

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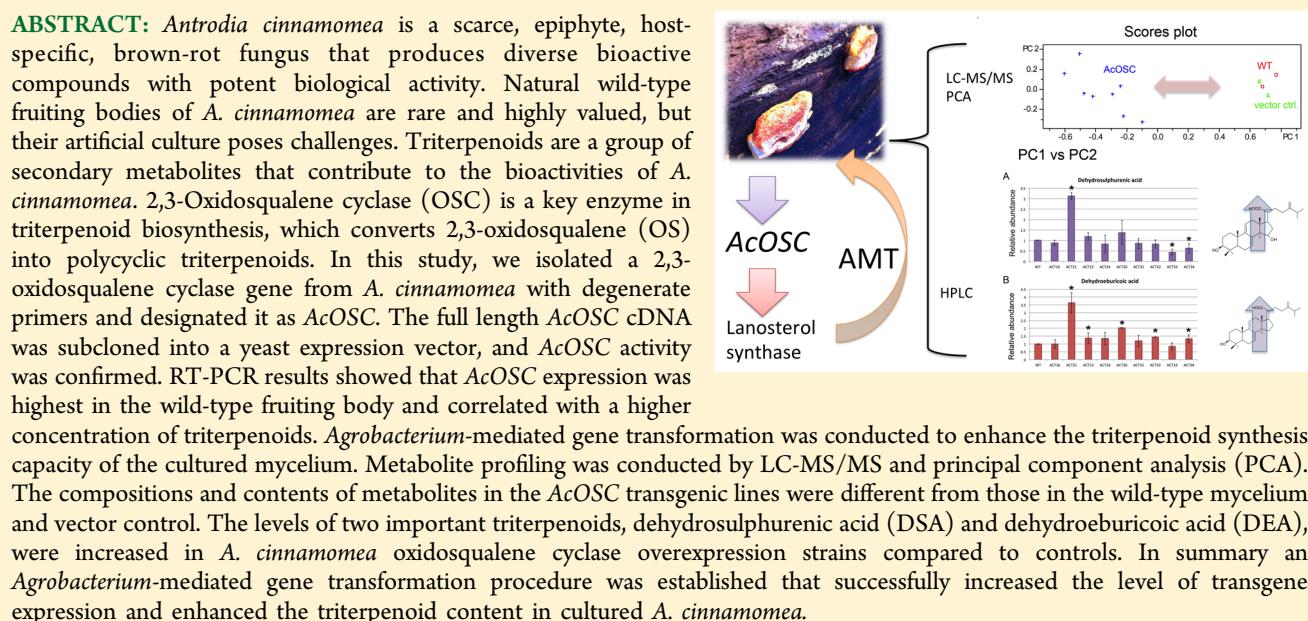
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Supporting Information



Many mushrooms are used as folk medicines and often contain a variety of bioactive compounds including polysaccharides and triterpenoids.^{1,2} *Antrodia cinnamomea* (syn. *Antrodia camphorata* and *Taiwanofungus camphoratus*) is a scarce and unique edible fungus that is endemic to the forests of Taiwan. It is a precious epiphyte brown-rot saprophytic fungus specific to the inner cavity of *Cinnamomum kanehirae* Hayata that is native to Taiwan and is an endangered species.³ The wild-type *A. cinnamomea* fruiting body is traditionally used as a folk medicine for diarrhea, alcohol poisoning, drug intoxication, abdominal pain, hypertension, and liver cancer and is colloquially named “the ruby of the forest” by the Taiwanese.⁴ Several *in vivo* and *in vitro* studies have indicated that *A. cinnamomea* possesses a diverse range of biological activities, such as antihepatitis,⁵ anti-inflammatory,^{6,7} antiox-

idative,^{8,9} cytotoxic,¹⁰ hepatoprotection,^{11,12} and vasorelaxation.¹³ Many studies have shown that triterpenoids contribute to the bioactivities of *A. cinnamomea*.^{14,15}

Triterpenoids are a large group of natural products, some of which participate in the major physiology of fungal growth, such as sterol synthesis, and many of which have extensive pharmaceutical bioactivities.^{16–18} More than 100 different triterpenoid skeletons have been described, all of which are generated by biosynthesis through the mevalonate and terpene backbone synthesis pathway to generate the 30-carbon isoprenoid squalene, which is then oxidized into (3S)-2,3-oxidosqualene through squalene monooxygenase (Figure 1).

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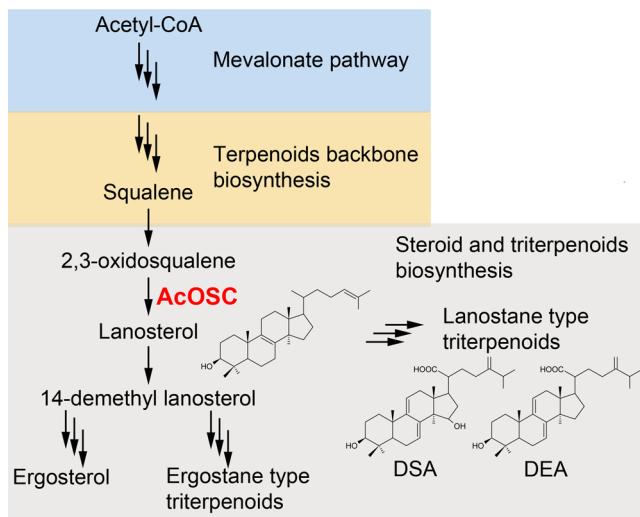


Figure 1. AcOSC involved in the terpenoid biosynthesis pathway. DSA: dehydrosulphurenic acid, DEA: dehydroeburicoic acid.

Oxidosqualene cyclase catalyzes a cyclization step that produces multiple closed-ring systems in just one single transformation via the “chair–boat–chair” conformation.^{19–21} With different downstream modification enzymes numerous natural products can be synthesized.^{22,23} To date over 40 triterpenoid metabolites have been isolated from *A. cinnamomea*, and these can be classified into lanostane- and ergostane-type triterpenoids.²⁴

Because of its potential pharmacological applications, the demand for wild-type *A. cinnamomea* fruiting bodies is increasing. However, the wild-type fruiting body can grow in only one specific host, grows extremely slowly, and is causing a severe illegal logging issue in Taiwan. Although the artificial culture of mycelia is feasible, the artificially cultivated basidomes (fruiting bodies) have been found deficient in certain medically effective compounds.⁶ Concentrations of triterpenoids are reported to be 10- to 30-fold higher in the wild-type fruiting body compared to the submerged cultured mycelium.²⁵ The natural wild-type fruiting bodies of *A. cinnamomea* are rare and highly valued, and there are no cultivated alternatives. Numerous studies have focused on enhancing compound production by the artificial culture system, increasing the biomass of mycelium, and inducing fruiting body formation.^{8,26,27}

In the present study, an AcOSC gene was cloned from *A. cinnamomea*. The heterologous protein expression with a lanosterol synthase-deficient yeast expression system was used for functional characterization of the AcOSC gene. Agrobacterium-mediated gene transformation increased the AcOSC gene expression and enhanced triterpenoid production.

RESULTS AND DISCUSSION

Molecular Cloning and Sequence Analysis of AcOSC.

The complete full-length AcOSC cDNA contains 2552 base pairs (bp) with a 128 bp 5' untranslated region (UTR), a 219 bp 3' UTR, and a 2205 bp coding region, which can be translated into a peptide with 734 amino acids and a 84.4 kDa predicted molecular weight (GenBank accession no. KJ094413).

Amino acid sequence alignment with oxidosqualene cyclase genes from several other basidiomycetes is shown in Figure 2,

and the protein sequence similarity is listed in Table S1 (Supporting Information). AcOSC protein sequence similarity with other enzymes identified from several basidiomycetes was between 61.94% and 72.18%. The most similar enzyme was a lanosterol synthase (LSS) from *Ganoderma lucidum* (72.18%), an important basidiomycete with diverse triterpenoids called ganoderic acids.²⁸ Although AcOSC and LSS shared about a 70% similarity in amino acid sequence, the enzymatic function was the same. The substrate binding activity center that is conserved in the oxidosqualene cyclase family [(VSDC(T/V)(G/A)E)] is marked in Figure 2. There were six highly conserved repetitive QW motifs (amino acid glutamine and tryptophan) found in the alignment that may play important roles in structural catalysis stabilization in oxidosqualene cyclase and squalene cyclase across various species.^{29–31}

The three-dimensional protein structure constructed with SWISS-MODEL is shown in Figure S1 (Supporting Information). The catalytic reaction center is enlarged in the inset of the figure. Asp454 was predicted as the polar cap in the hydrophobic activation cavity that protonates the epoxide group of the prefolded (3S)-2,3-oxidosqualene, resulting in the initiation of the polycyclization reaction. Cys455 and Cys532, also shown in the inset, were assigned to act as hydrogen-bonding partners with Asp454.

Cloning and Analysis of AcOSC Activity in Yeast. For the further functional analysis, the full length of AcOSC coding sequence was inserted into the yeast protein expression vector pYES2/CT and named pYES-AcOSC. After transformation and protein induction in lanosterol synthase-deficient yeast (the workflow and the results of colony polymerase chain reaction (PCR), reverse transcriptase (RT)-PCR, and Western blot are shown in Figure S2, Supporting Information), the liquid broth was then extracted with hexane and silylation derivatives prepared with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). The authentic standards (ergosterol and lanosterol) were directly dissolved in hexane and silylated with MSTFA. The GC/MS analysis result is shown in Figure 3. Compared to the authentic standard, mass fragmentation, and the vector control, the pYES-AcOSC could produce lanosterol by the AcOSC enzyme complement *erg7* mutant yeast, which is lanosterol synthase deficient.

Identification of Transformants. AcOSC expression patterns under different culture conditions are shown in Figure 4A. AcOSC in the wild-type fruiting body possessed the highest transcript level among the culture conditions tested. This result corresponds with the higher concentration of triterpenoid in the wild-type fruiting body.²⁵ To improve the capacity of the artificially cultured *A. cinnamomea* mycelium to produce triterpenoids, Agrobacterium-mediated transformation was used to generate AcOSC overexpression isolates. Single colonies of transformed *A. cinnamomea* were chosen by hygromycin antibiotic selection. Successfully transformed *A. cinnamomea* were separately cultured in MEA medium with hygromycin antibiotic. The genomic DNA of different transformed lines was purified and checked using PCR with primer pairs specific for the sequences of the CaMV 35S promoter and the hygromycin resistance gene. The PCR result is shown in Figure S3 (Supporting Information). PCR of genomic DNA confirmed successful integration of AcOSC T-DNA in selected transformants, while no PCR products were observed for the wild-type control. Successfully transformed cell lines were prefixed ACT. ACT 16 to 20 were transformed with pCAMBIA1301 as the vector control, ACT 21 to 30 were AcOSC constructed in

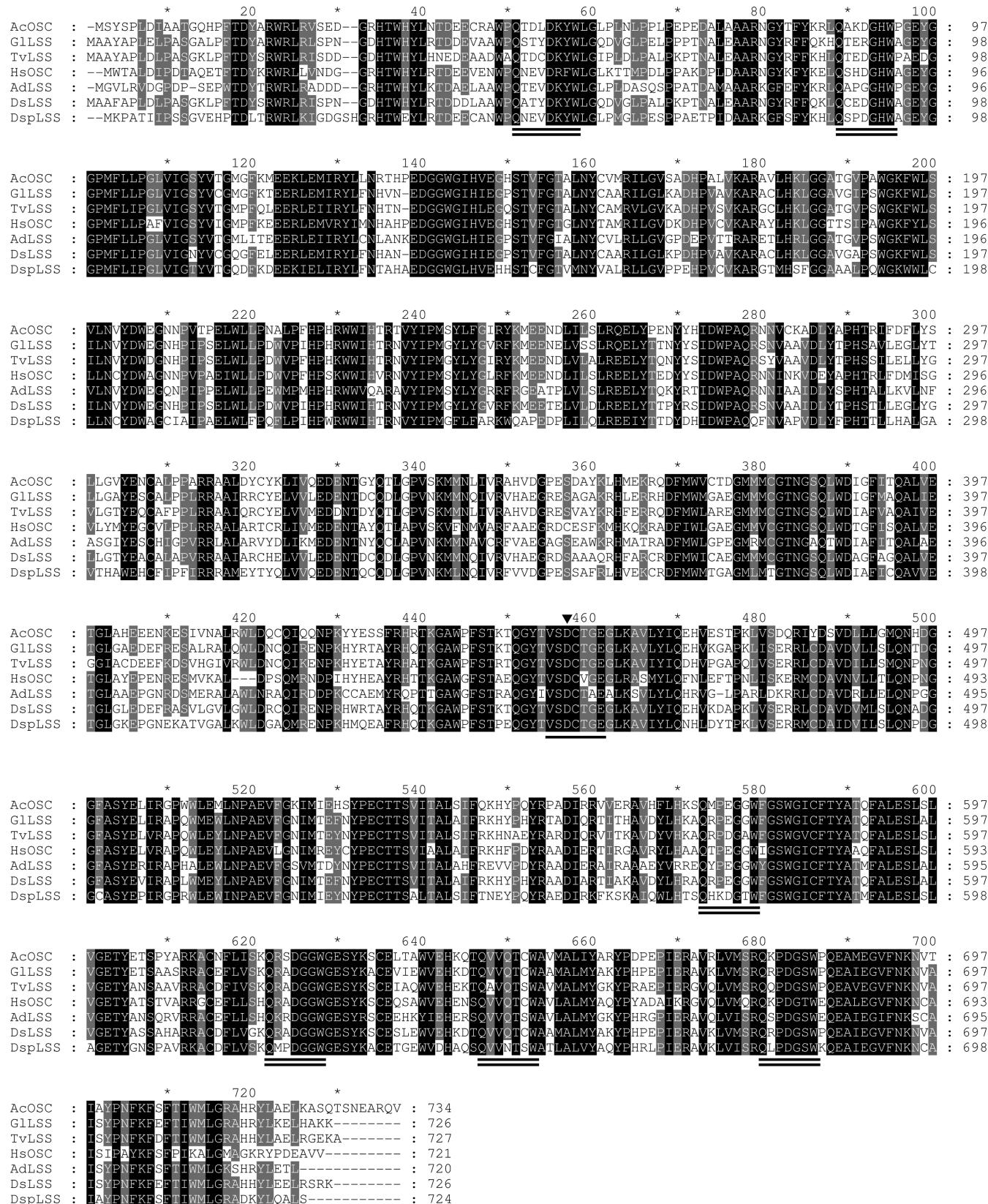


Figure 2. Amino acid sequence alignment of AcOSC and oxidosqualene cyclases from several representative basidiomycetes. GenBank sequence ID: G1LSS (ADD60470: *Ganoderma lucidum*), TvLSS (EIWS9845: *Trametes versicolor*), HsOSC (ACF70484: *Hypholoma sublateritium*), AdLSS (EJD47041: *Auricularia delicata*), DsLSS (EJF64326: *Dichomitus squalens*), DspLSS (EJU03611: *Dacryopinax* sp.). The catalytic reaction center is underlined. The repetitive conserved QW motifs are double underlined.

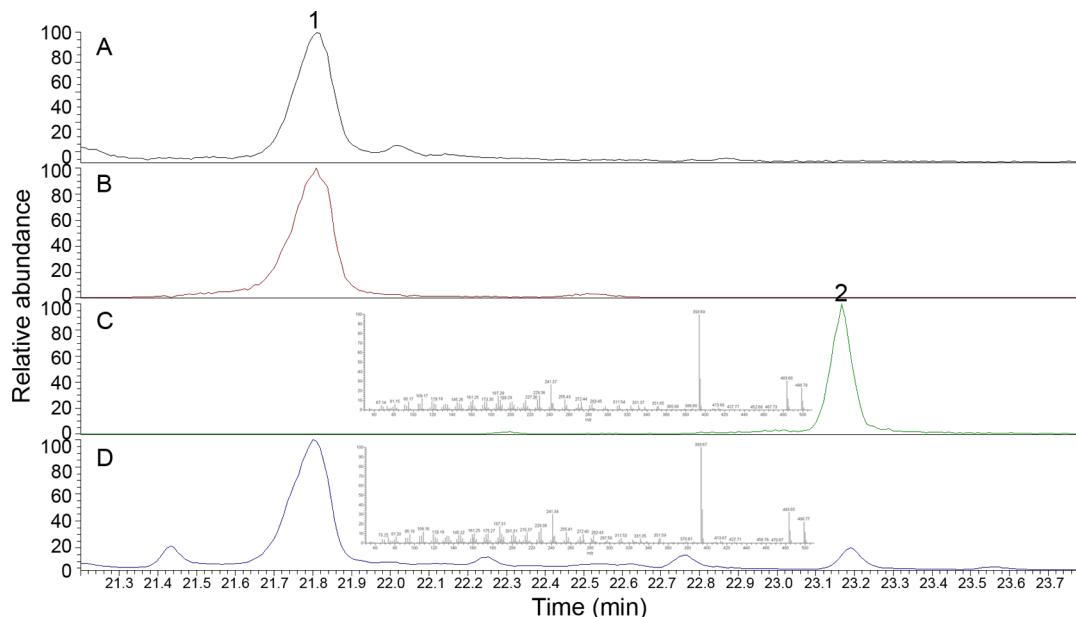


Figure 3. Chromatogram of the hexane extract from vector control and pYES-AcOSC after silylation with MSTFA analyzed using GC/MS. (A) 1: ergosterol standard, (B) vector control, (C) 2: lanosterol standard, (D) pYES-AcOSC.

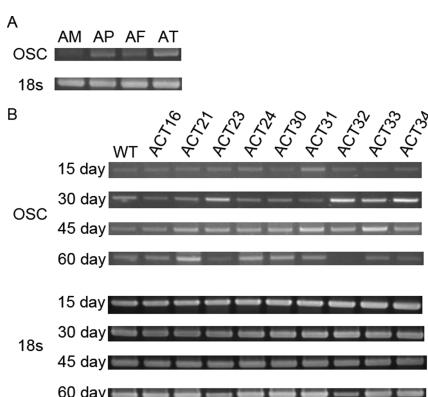


Figure 4. AcOSC gene expression patterns in different culture conditions and transgenic lines as shown by RT-PCR. (A) Different culture conditions (AM: liquid culture mycelium; AL: solid culture mycelium; AF: solid culture fruiting body; AT: natural fruiting body) (B) WT: liquid culture mycelium strain WSY-01; ACT for different transgenic lines.

pCAMBIA1301 under the CaMV 35S promoter, ACT 31 and 32 were AcOSC constructed in pCAMBIA under the 0.5 kbp endoglucanase promoter, and ACT 33 and 34 were AcOSC constructed in pCAMBIA under 1.0 kbp endoglucanase promoter.

A. cinnamomea wild-type mycelium, vector control (ACT 16), and several transgenic lines (ACT 21, 23, 24, 30, 31, 32, 33, 34) were chosen for further metabolite analysis. First the RT-PCR result for different culture periods is shown in Figure 4B. Except for ACT21 having the highest expression at 60 days, no matter the transformed cell lines driven by the 35S CaMV or endoglucanase promoter, the AcOSC gene expression was generally increased after 15 days and was highest at 45 days.

Bioactive Compound Analysis. The crude extracts from *A. cinnamomea* transgenic lines were collected for further HPLC and liquid chromatography tandem mass detector (LC-MS/MS) analysis. The LC-MS/MS analysis data from different transgenic lines were used for principal component analysis

(PCA), and the result is shown in Figure S4 (Supporting Information). In the scores plot, the AcOSC transgenic lines are in a different cluster from the nontransgenic wild-type mycelium and vector control. This shows that the AcOSC transgenic lines undergo different metabolite synthesis activity as a result of the transgenic process. The monitoring variables in the bucket are shown in the loadings plot. Two major triterpenoids had a significant effect on the group pattern. These two samples were also separated with HPLC and collected for NMR analysis (Figure SSB and C, Supporting Information), and triterpenoids were identified as dehydro-sulphurenic acid (DSA) and dehydroeburicoic acid (DEA), separated with retention times of 73.8 and 104.6 min (Figure SSA, Supporting Information), respectively.

These two lanostane-type triterpenoids had different concentrations among the transformants. DSA and DEA are the dominant triterpenoids found in the mycelium of *A. cinnamomea* and can serve as an index of triterpenoid levels. We measured these two compounds from the crude extract of untransformed mycelium and the transgenic lines. The results are shown in Figure 5. The transgenic line ACT 21 showed a significant increase in DSA and DEA of more than 3 times that of the nontransgenic line. ACT 30 showed a 2-fold increase in DEA. ACT 23, ACT 32, and ACT 34 showed about a 50% increase in DEA content.

Triterpenoids are a large class of natural products distributed among almost all living organisms, and they have many bioactivities.^{16,24} To date, more than 40 triterpenoids have been isolated from *A. cinnamomea*, and these can be classified as either lanostane- or ergostane-type. Many of these terpenoids have been demonstrated to have diverse bioactivities such as anti-inflammatory, cytotoxic, hepatoprotective, and analgesic.²⁴ Unfortunately, a lower efficiency of triterpenoid production has been found in the artificial culture system of *A. cinnamomea* mycelia.^{14,24} Improvements of the culture system were developed, including regulating the carbon, nitrogen source, and pH,³² or adding *in vitro* induction such as by wounding, air exchange,²⁶ or plant extracts.³³ This is the first report that

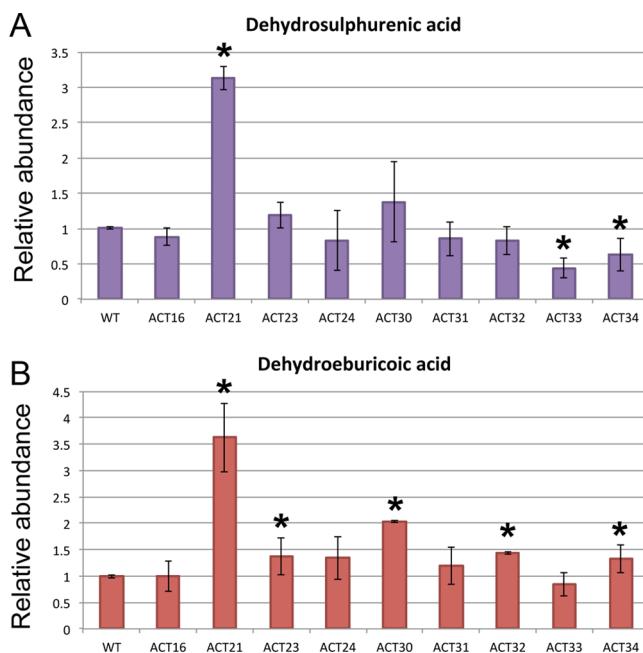


Figure 5. Metabolic analysis of *A. cinnamomea* transgenic lines with HPLC. The contents of two triterpenoids, (A) dehydrosulphurenic acid and (B) dehydroeburicoic acid, were measured. Asterisks denote a significant difference from the untransgenic line WT ($p < 0.05$). WT: wild-type mycelium strain WSY-01, ACT 16: pCAMBIA1301 vector control transgenic line, ACT 21-34: AcOSC transgenic line.

focuses directly on the triterpenoid biosynthesis pathway, and we found that AcOSC overexpression in *A. cinnamomea* can be an effective strategy to enhance triterpenoid synthesis in the artificial culture system. ACT21 is one of the most efficient transgenic lines in producing DSA and DEA, as we can develop the industrial-scale production of ACT21. At the same time, several unidentified modifying enzymes are involved in the transformation of lanosterol to DSA or DEA and other ergostane-type triterpenoids (Figure 1). Analysis of the transcriptome of ACT21 compared to wild-type mycelium with next-generation sequencing techniques may help us identify other important enzymes or transcription factors that are coexpressed with AcOSC.

DSA and DEA are the two major triterpenoids found in *A. cinnamomea* and were first identified by Yang *et al.* in 1996.³⁴ Several bioactivity studies have verified that DSA improves antioxidative capacity³⁵ and possesses cytotoxic activity against human leukemia cell line U397, pancreatic cancer BxPC3 cells,³⁶ and *Spodoptera frugiperda* Sf9 insect cells.³⁷ DEA has cytotoxic activity against several cancer cell lines including glioblastoma cell line U87MG,³⁸ colon cell lines HT-29 and HCT116, breast cancer cell line MDAMB231, and lung cancer cell lines A549 and CL1-0, without effecting normal epithelial cells.³⁹ DEA also demonstrated anti-inflammatory activity in the inhibition on fMLP-induced superoxide generation in human neutrophils⁴⁰ and mice.⁴¹ Since DSA and DEA contribute much of the bioactivities of *A. cinnamomea*, increasing the contents of these bioactive triterpenoids in artificial culture mycelium is of utmost interest.

In this study, AcOSC gene expression was increased by heterologous and homologous promoters (Figure 4B). The CaMV 35S promoter has been extensively used in many transformation experiments, but the efficiency of this promoter in fungal species was uncertain. According to our RT-PCR

result (Figure 4B), using the CaMV 35S promoter in *A. cinnamomea* was feasible. Godio *et al.*⁴² applied different gene promoters from different fungi species to upregulate the antitumor compound clavaric acid from the basidiomycete *Hypholoma sublateritium*. Several promoters were tested, but only the promoter from the basidiomycete *Agaricus bisporus* resulted in good transformation efficiency. It may also be possible to obtain high levels of gene expression using different promoters from other basidiomycete species. In a previous study,⁴³ endoglucanase from *A. cinnamomea* was found to have a high level of expression in an artificial culture medium. Therefore, the endoglucanase promoter was selected for use in this study for upregulation of AcOSC gene expression in the artificially cultured mycelium. The RT-PCR result also showed high levels of expression in ACT 31 to 34 and also at an earlier time-point than other transformants (Figure 4B). The level of expression decreased after a long culture period. This might be because, in this study, the carbon source in the medium may be exhausted after a long culture period, and this may cause the downregulation of the related genes.

Although we successfully obtained transgenic lines with enhanced triterpenoid synthesis, most of the transgenic lines showed upregulated gene expression but a lower abundance of downstream DSA and DEA contents. This may be because there were over 40 triterpenoids isolated from *A. cinnamomea*, and it is possible that the AcOSC gene induces some minor triterpenoid production in the transgenic lines. The triterpenoid synthesis pathway shares several key enzymes with the ergosterol synthesis pathway (Figure 1), and increasing the level of AcOSC expression might lead to an increase in steroid accumulation. As shown in Figure 1, several enzymes might be involved in the pathway from lanosterol to lanostane- or ergostane-type triterpenoids. The metabolite synthesis routes are complicated and tightly regulated, and downregulation of DSA and DEA in some transgenic lines may contribute to the accumulation of other unidentified metabolites.

EXPERIMENTAL SECTION

General Experimental Procedures. Gas chromatography (GC/MS) was performed using a Thermo Finnigan Trace GC Ultra equipped with a DB-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) and coupled with an ITQ 900TM mass instrument (Thermo Fisher Scientific, Waltham, MA, USA). Liquid chromatography (LC-MS/MS) was performed by a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific) equipped with a Phenomenex Luna 5 μm C₁₈ (2) column (250 × 4.60 mm, Phenomenex, Torrance, CA, USA). High-performance liquid chromatography (HPLC) was performed using an Agilent quaternary HPLC 1200 series (Agilent, Santa Clara, CA, USA) equipped with Phenomenex Luna 5 μm C₁₈ (2) column (250 × 4.60 mm). The NMR spectra were obtained using a Bruker ASCEND-400 in CDCl₃ with TMS as internal control. The chemicals and culture medium used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Fungal Strain and Culture Media. *Antrodia cinnamomea* isolate WSY-01 was provided by Prof. Sheng-Yang Wang, National Chung-Hsing University, Taiwan. The mycelium was maintained in MEA medium (2% malt extract, 2% D-glucose, and 2% phytoagar if cultured in agar plates) in the dark at 24 °C without shaking.

RNA, Genomic DNA Preparation, and Molecular Cloning. The total RNA and genomic DNA preparation was as described by Chu *et al.*⁴³ and Lin *et al.*⁴⁴ Single-strand cDNAs were generated with oligo-dT primer by Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The degenerate primers designed from 2,3-oxidosqualene cyclases found from the National Center for

Biotechnology Information (NCBI) database were used to clone the conserved region of the gene. The sequences above were aligned by the ClustalW2 online server (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) from European Bioinformatics Institutes, and the primer sequences are listed in Table S2 (Supporting Information). The 5' and 3' Rapid Amplification of cDNA ends system version 2.0 (Invitrogen) was used to amplify the 5' and 3' AcOSC cDNA sequences, respectively.

Homology Modeling of AcOSC Protein Structure. The homology model for AcOSC was constructed with SWISS-MODEL Automatic Modeling Mode⁴⁵ with the identified crystal structure of oxidosqualene cyclase from humans (RCSB Protein Data Bank ID: 1W6K) as a template.⁴⁶ The 3D model was displayed by using Chimera software.⁴⁷

Protein Expression in Yeast for Functional Analysis. Full-length AcOSC coding sequence was amplified by Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and inserted into the protein expression vector pYES2/CT (Invitrogen) named pYES-AcOSC. The lanosterol synthase-deficient yeast (*Saccharomyces cerevisiae*, purchased from ATCC, cell line number: 4021900, -erg7, -ura) was transformed with pYES-AcOSC by electroporation. The transformant yeast was spread on the solid synthetic complete medium without uracil (SC-Ura) for selection. The successfully transformed yeast cell line was then inoculated in 30 mL of SC-Ura medium containing ergosterol (20 $\mu\text{g mL}^{-1}$), hemin (13 $\mu\text{g mL}^{-1}$), and Tween 80 (5 mg mL^{-1}), incubated with shaking at 250 rpm and 30 °C for 2 days. The cultured yeast cells were transferred into the new SC-Ura medium with 2% galactose in place of raffinose and further cultured at 250 rpm and 30 °C for 2 days for protein induction. After induction, the yeast cells were collected and resuspended in 0.1 M potassium phosphate buffer (pH 7.0) supplemented with 2% glucose and hemin (13 $\mu\text{g mL}^{-1}$) and further cultured with shaking at 250 rpm and 30 °C for another 24 h. The yeast cells were collected by centrifugation and refluxed with 10% KOH–80% EtOH. The saponification extract was partitioned with the same volume of hexane twice, concentrated, and analyzed with GC/MS.

GC/MS Analysis. The concentrated extracts were dissolved in pyridine and derivatized with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide at 70 °C for 30 min. After silylation, the mixtures were directly used for GC/MS analysis. The analysis was performed by a Thermo Finnigan Trace GC Ultra gas chromatograph equipped with a DB-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific) and coupled with an ITQ 900TM mass instrument (Thermo Fisher Scientific).⁴⁸ The injector temperature was 280 °C with He as carrier gas (1 mL min^{-1}). The GC temperature started at 110 °C and was held for 1 min, then increased at a rate of 40 °C min^{-1} to 260 °C, held for 5 min, and further increased to 290 °C at a rate of 5 °C min^{-1} and held for 10 min.

Agrobacterium-Mediated Gene Transformation. *Agrobacterium tumefaciens* LBA4404 was used for the gene transformation and cultured in YPD medium (peptone 10 g L^{-1} , yeast extract 10 g L^{-1} , NaCl 5 g L^{-1}). Full-length AcOSC coding sequence was introduced into the gene transformation vector pCAMBIA1301 (Cambia, AU) under the regulation of either the CaMV 3SS promoter or the promoter of endoglucanase from *A. cinnamomea*⁴³ and named pCAM-AcOSC and pCAM-1112p/AcOSC, respectively.

The constructs were transformed into *A. tumefaciens* LBA4404 separately by a freeze–thaw method.⁴⁹ The submerged cultured *A. cinnamomea* mycelium was collected after 25 days and cocultured with *A. tumefaciens* LBA4404 in MEA broth medium in the dark for 2 days without shaking. The *A. cinnamomea* mycelia were collected by a filtrate with vacuum filtration on filter paper. The filtrate *A. cinnamomea* was spread on the MEA medium with hygromycin for the transformation selection. After antibiotic selection, the growing single colonies were collected and cultured separately. The genomic DNA from different colonies was prepared, and the transformed cell lines were checked using PCR with specific primer pairs on the CaMV 3SS promoter and hygromycin resistance gene.

Gene Expression Pattern. The liquid culture mycelium (AM), solid culture mycelium (AP), solid culture basidiomes (AF), wild-type

fruiting body (AT), and several successfully transformed cell line mycelia were harvested after a specific culture period. The collected samples were used for the RNA extraction and RT-PCR. RT-PCR thermal cycling conditions were 22–24 cycles with each cycle comprising denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The *AcOSC* and 18s rRNA gene were monitored.

LC-MS/MS and HPLC Analysis. Successfully transformed colonies were inoculated in MEA broth medium and incubated in the dark at 24 °C with gentle shaking for 60 days. The mycelia were collected and filtered with a vacuum pump. The metabolites were extracted using 95% EtOH with sonication for 30 min. The extracts were vacuum-dried, dissolved in MeOH (10 mg mL^{-1}), and analyzed using LC-MS/MS and HPLC. LC-MS/MS analysis was performed on a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific) equipped with a Phenomenex Luna 5 μm C₁₈ (2) column (250 × 4.60 mm, Phenomenex). The solvent program is listed in Table S3 (Supporting Information). The amaZon speed (Bruker, Fremont, CA, USA) ion trap was set to an ion source temperature of 250 °C, with N₂ as dry gas at 9.0 L min^{-1} , a capillary voltage of 4500 V, an end plate offset at 500 V, and a scan range of 70 to 600 m/z in positive and negative ionization mode. The Agilent quaternary HPLC 1200 series (Agilent) equipped with a Phenomenex Luna 5 μm C₁₈ (2) column (250 × 4.60 mm) was used for triterpenoid concentration analysis. The HPLC solvent program is outlined in Table S4 (Supporting Information).

Principal Component Analysis. The LC-MS/MS data of each transgenic line were used for the principal component analysis performed with Bruker Profile Analysis 2.0 software. The bucket generation was under the retention time range from 60 to 105 min with $\Delta t_R = 1$ min and the mass range from 450 m/z to 500 m/z with $\Delta m/z = 1$.

ASSOCIATED CONTENT

Supporting Information

Tables of AcOSC protein similarity, primers used in this study, and the LC solvent system. Figures contain the AcOSC protein homology structure, heterologous expression workflow in yeast, transformants identified, principal component analysis of transformants, and metabolite analysis of AcOSC transformants. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00020.

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Notes

The authors declare no competing financial interest.

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