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ARTICLE

Cloning and Characterization of the Lanosterol 14α-Demethylase Gene from *Antrodia cinnamomea*

Chen-Hsien Lee,[†] Keng-Hao Hsu,[†,§] Sheng-Yang Wang,[§] Tun-Tschu Chang,[#] Fang-Hua Chu,*[,†] and Jei-Fu Shaw*[,⊥]

[†]School of Forestry and Resource Conservation, National Taiwan University, Taipei, Taiwan, [§]Department of Forestry, National Chung Hsing University, Taichung, Taiwan, [#]Division on Forest Protection, Taiwan Forestry Research Institute, Taipei, Taiwan, and [⊥]Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan

Sterol 14α-demethylase (CYP51) is one of the key enzymes for sterol biosynthesis in fungi; it is widely distributed in all members of the cytochrome P450 superfamily. In this study, *AcCyp51*, encoding a cytochrome P450 sterol 14α-demethylase, was obtained from the sequences of EST libraries of *Antrodia cinnamomea* by using 5′ RACE and genome walking methods. The open reading frame of *AcCyp51* is 1635 bp and encodes 544 amino acids. The recombinant protein of AcCYP51 fused with glutathione-*S*-transferase from *Escherichia coli* revealed the demethylating activity by using lanosterol as substrate and GC-MS analysis. Gene expression levels of *AcCYP51* were higher in natural basidiomes than in other cell types. Transcription of AcCYP51 increased in various culture conditions including adding squalene, lanosterol, itroconazole, and oleic acid as inducers. These reveal the important functions of AcCYP51 in basidiomatal formation and suggest that it might participate in other biological processes.

KEYWORDS: ***Antrodia cinnamomea*; *Cyp51*; sterol 14α-demethylase**

INTRODUCTION

Antrodia cinnamomea is a brown-rot fungus that is a host-specific saprophytic fungus of a conserved endemic tree, *Cinnamomum kanehirai* Hay (Lauraceae), in Taiwan. It has been shown that the fruiting bodies of *A. cinnamomea* possess several diverse bioactivities, including anticholinergic, antiserotonergic, blood pressure regulatory, antitumor, anticancer, and anti-inflammatory activities (*1*−*7*). Polysaccharides and secondary metabolites such as triterpenoids are believed to contribute to the bioactivities of *A. cinnamomea* (*8*). Crude methanol extract of fruiting bodies, which contain a number of unsaturated fatty acids and ergosterol, was significantly cytotoxic against transitional cell carcinoma (TCC) cancer cell lines (*9*). However, in nature, the fruiting bodies of *A. cinnamomea* grow extremely slowly in the heartwood cavity, and cultivation in an artificial culture system is very difficult. Although the artificial culture of mycelia is feasible, certain specific compounds that are considered to be the medically effective ones were not secreted from fruiting bodies (*10*). The natural basidomes of *A. cinnamomea* are thus rare and highly valuable. Numerous investigations focused on submerged culture to enhance the biomass of the valid compounds or on forming the fruiting bodies artificially are underway (*6*, *11*−*13*), but the molecular mechanism of the fruiting body formation of *A. cinnamomea* is still unclear.

*Corresponding authors [(F.-H.C.) telephone +886-2-33665261, fax +886-2-23654520, e-mail fhchu@ntu.edu.tw; (J.-F.S.) telephone +886-4-22840201, fax +886-4-22853813, e-mail boplshaw@gate.sinica.edu.tw].

Our previous analyses of specific gene expression of basidiomatal formation in *A. cinnamomea* revealed that several cytochrome P450 genes are significantly expressed in natural basidiomes including a lanosterol 14α-demethylase (*CYP51*) (*14*, *15*). CYP51 (EC 1.14.13.70) is a necessary enzyme for sterol biosyntheses and is regarded as a unique P450 that is functionally conserved in most biological kingdoms (*16*, *17*). The preferred substrates of CYP51 in yeast and fungi are lanosterol and 24-methylene-dihydrolanosterol (eburicol) (*18*−*20*). The first study of a basidiomycete CYP51 was from *Ustilago maydis* (*18*), in which activity was assayed using eburicol but not lanosterol as substrate. Ergosterol, synthesized from lanosterol by CYP51, is an important component in membranes that affects membrane rigidity, fluidity, and permeability (*21*). Many related cytochrome P450 genes have been suggested to be involved in fruiting body development: *CYPA* from *Agaricus bisporus* was found to be strongly induced in the generative phase compared with vegetative grown mycelium (*22*), *fbg14* from *Lentinula edodes* and two P450 homologous cDNA from *Flammulina velutipes* were specifically expressed in fruiting body (*23*, *24*), and mutation of *eln2* in *Coprinus cinereus* caused a defect of fruiting body formation (*25*). Although a putative CYP51 of *Pleurotus ostreatus* is also specifically expressed in the mature fruiting body stage, its detailed characteristics and function are not yet known (*26*). In the present study, a *CYP51* was cloned from *A. cinnamomea*. The recombinant protein expressed in *Escherichia coli* was functionally characterized, and regulation of the gene's transcription was investigated.

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MATERIALS AND METHODS

Fungal Strains and Media. *A. cinnamomea* strain TFRIB 470 was identified and provided by Tun-Tschu Chang (Taiwan Forestry Research Institute) and cultured as described by Chang and Wang (*6*). The natural basidiomes were obtained from the infested wood of *C. kanehirai*. The cells were cultured in liquid containing 2% malt extract broth and 2% D-(+)-glucose in darkness without shaking at 24 °C for 20 days and treated for different experimental conditions. For sterol induction analysis, the 5 mm diameter mycelium was inoculated at the center of a Petri dish; squalene (4 g/L) and lanosterol (10 mg/L) as inducers were added into cultural medium (2% malt extract broth, 2% D-(+)-glucose, and 2% agar) and incubated at 24 °C. For heat shock experiments, cells were cultured at the higher temperature of 39 °C for 0, 10, 30, 60, and 90 min. The mycelia for different carbon source and nitrogen source treatments were cultured in medium containing 1% ethanol, 1% glycerol, 0.2% oleic acid, 40 mM KNO_3, 20 mM L-glutamine, or 2% D-(+)-glucose. The samples were harvested by vacuum pumping and collected quickly, immediately frozen in liquid nitrogen, and kept at −80 °C until used.

RNA and Genomic DNA Extraction. Total RNA was extracted from *A. cinnamomea* according to the CTAB RNA extraction method (2% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide [CTAB], 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2.0 M NaCl, 2% PVP, 0.5 g/L spermidine, 2% β-mercaptoethanol) following the two chloroform/isoamyl alcohol (24:1) extractions as previously described (*15*). Genomic DNA was isolated from the liquid-cultured mycelia, harvested, and ground in liquid nitrogen. The powder was transferred to a centrifuge tube and mixed gently and thoroughly with CTAB buffer as described elsewhere (*27*).

Cloning and Sequencing. 5′-Rapid amplification of cDNA ends (RACE) was performed using the 5′ RACE System (Invitrogen) using specific primer ACyR8 (5′-CTGATAGACATCTTCC-3′) for synthesis of first-strand cDNA, primer ACyR4 (5′-CTTGCCACCGAATACAAA-GTCATTTCC-3′) for first PCR reaction, and primer ACyR9 (5′-GCAT-GACTTGAAGAACTCGACGGGATC-3′) for nested PCR reaction.

The promoter sequence of AcCYP51 was obtained with GenomeWalker Kits (BD Biosciences) using the specific primers ACyR4 (5′-CTTGCCA-CCGAATACAAAGTCATTTCC-3′) and ACyR9 (5′-GCATGACTT-GAAGAACTCGACGGGATC-3′). The PCR products were cloned using the pGEM-T Easy vector system (Promega) and sequenced using an ABI 377 automatic sequencer (Perkin-Elmer).

Bioinformatics Analysis. The *AcCYP51* sequence was compared with the nonredundant (nr) database at the NCBI by the BLASTX algorithm. Signal peptide and transmembrane region prediction were carried out with the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), respectively. The cis-acting regulatory elements of the promoter were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) MOTIF Search web service (http://motif.genome.jp/).

Protein Expression and Purification. To confirm and characterize the protein function, a full-length and a mature open reading frame of *AcCYP51* were generated by PCR with specific primer pairs, ACyF18 (5′-CCGGAATTCATGTCGCTGAACATGAACGTCAG-3′)−ACyR19 (5′-TCCCCTCGAGCTAGTCAAACTTCCTGCGGCGATA-3′) and ACyF25 (5′-CCGGAATTCGTGGTCTTCAATGTCTTATACCAA-TTAG-3′)−ACyR19 (5′-TCCCCTCGAGCTAGTCAAACTTCCTG-CGGCGATA-3′), respectively. The PCR products were subcloned into the fusion protein expression vector, pGEX4T-1 (Pharmacia), and the resultant construct was expressed in *E. coli* BL21(DE3). Soluble glutathione *S*-transferase (GST) fusion proteins were purified using GST-Bind Kits (Novagen). Purified protein was stored in 100 mM potassium phosphate (pH 7.4) buffer exchanged with an Ultra-15 PLGC centrifugal filter unit with 10000 NMWL (Amicon).

Catalytic Activity Assay. Lanosterol was prepared with the detergent Tween 80 in 100 mM potassium phosphate buffer (pH 7.4) by vortexing the mixture to an almost clear micellar suspension. The activity reaction was carried out by incubating 1 nmol of AcCYP51p with 18 nmol of spinach ferredoxin, 2 nmol of ferredoxin-NADP reductase, and 2 mM reduced nicotinamide adenine dinucleotide phosphate (all from Sigma) at 37 °C for 16 h. Sterols were extracted in *n*-hexane several times and evaporated to dryness, followed by silylation with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Sigma) in toluene at 85 °C for 1 h. GC-MS analysis used a DB-5 gas column (J&W Scientific), with heating initially at 280−300 °C increasing by 10 °C min^{-1} and then held for a further 15 min, using helium as the carrier gas at 1 mL min^{-1}. The injector temperature was 280 °C. Identification of monitoring compounds, that is, silylated lanosterol and silylated demethylated lanosterol, were carried out by using the Wiley/NBS Registry of Mass Spectral Data and the National Institute of Standards and Technology (NIST, V.2.1) search and authentic reference compounds.

RT-PCR Analysis. The first-strand cDNA library of *A. cinnamomea* was obtained by reverse transcription PCR (RT-PCR) using SuperScript II Reverse Transcriptase (Invitrogen); 1 unit of RNase H was added after the reaction. RT-PCR analysis was performed using the specific primer pair ACyR30 (5′-GTGCCTTTACTGAAGATGAAG-3′)−ACyF29 (5′-CAAGAACAAGTGGAGCATTTTG-3′), which corresponds to the partial coding region and 3′-noncoding region of *AcCyp51*. The internal control was 18S rRNA of *A. cinnamomea* (*15*), analyzed using a pair of specific primers as previously described: TM82N (5′-ACTGTGAAACTGCGAA-TGGCTC-3′) and TM82C (5′-GACTTGCCCTCCAATTGTTCCTC-3′).

RESULTS

Gene Cloning and Sequence Analysis. The complete nucleotide sequences of *AcCyp51* and its flanking sequence including 59 nucleotides of the 5′ UTR and 228 nucleotides of the 3′ UTR were obtained. Full-length *AcCyp51* consists of 2045 bp (GenBank accession no. EU149948). The gene codes for a 544 amino acid protein with a predicted M_r of 61.9 kDa and a theoretical isoelectric point (p*I*) of 6.12. A signal peptide with 40 amino acid residues at the N-terminus of *AcCyp51* was predicted by SignalP, and three possible transmembrane helices were predicted by TMPRED.

Sequence analysis showed that the deduced protein of *AcCyp51* was closest to the sterol 14α-demethylase gene (GenBank accession no. AAU01160) of *Phanerochaete chrysosporium*, the model white rot basidiomycetous fungus, with an identity of 65% (**Figure 1**). AcCyp51 also exhibits close homology with other eukaryotic CYP51 genes, such as *Coprinopsis cinerea* lanosterol 14α-demethylase (GenBank accession no. AAU01159), *P. chrysosporium* CYP51 (GenBank accession no. AAU01160), *Aspergillus fumigatus* Af293 CYP51A (GenBank accession no. XP_752137), *Aspergillus fumigatus* Af293 CYP51B (GenBank accession no. XP_752134), and *Saccharomyces cerevisiae* cytochrome P450 51 (GenBank accession no.EDN62241). The conserved amino acids of CYP51s were clustered into several regions including six putative substrate recognition sites (SRS) and a heme binding site (*28*, *29*). The putative SRS 1−5 and heme-coordinating cysteine of AcCyp51 are highly conserved among other CYP51s of various origin (**Figure 1**).

Examination of intron/exon organization revealed that there was a relatively good correlation between intron numbers, phases, and positions compared with the phylogenetic relationship among the members of the P450 subfamilies (*30*). Supporting Information Figure 1 demonstrates the comparison of genomic sequence of intron number, intron phase, and position of introns in CYP51 with eight fungal homologues generated by Gene Structure Display Server (GSDS) (*31*). A splice site between codons was phase 0 (i.e., NNN|), a splice site one base up from the 5′ end was called phase 1 (i.e., N|NN), and that two bases away was called phase 2 (i.e., NN|N). As with the three other basidiomycetes (*F. neoformans*, *C. cinerea*, and *P. chrysosporium*), the number of introns in *AcCYP51* is higher than in ascomycetes (Supporting Information Figure 1). The first five introns in four basidiomycetes present the same phase, 00002, but the later phases are varied.

Protein Expression in BL21 and Activity Assay. To further study the catalytic activity and estimate the molecular mass of the encoded protein, we used an *E. coli* GST system of which the vector is pGEX4T-1 for expression and enzymatic activity assay.

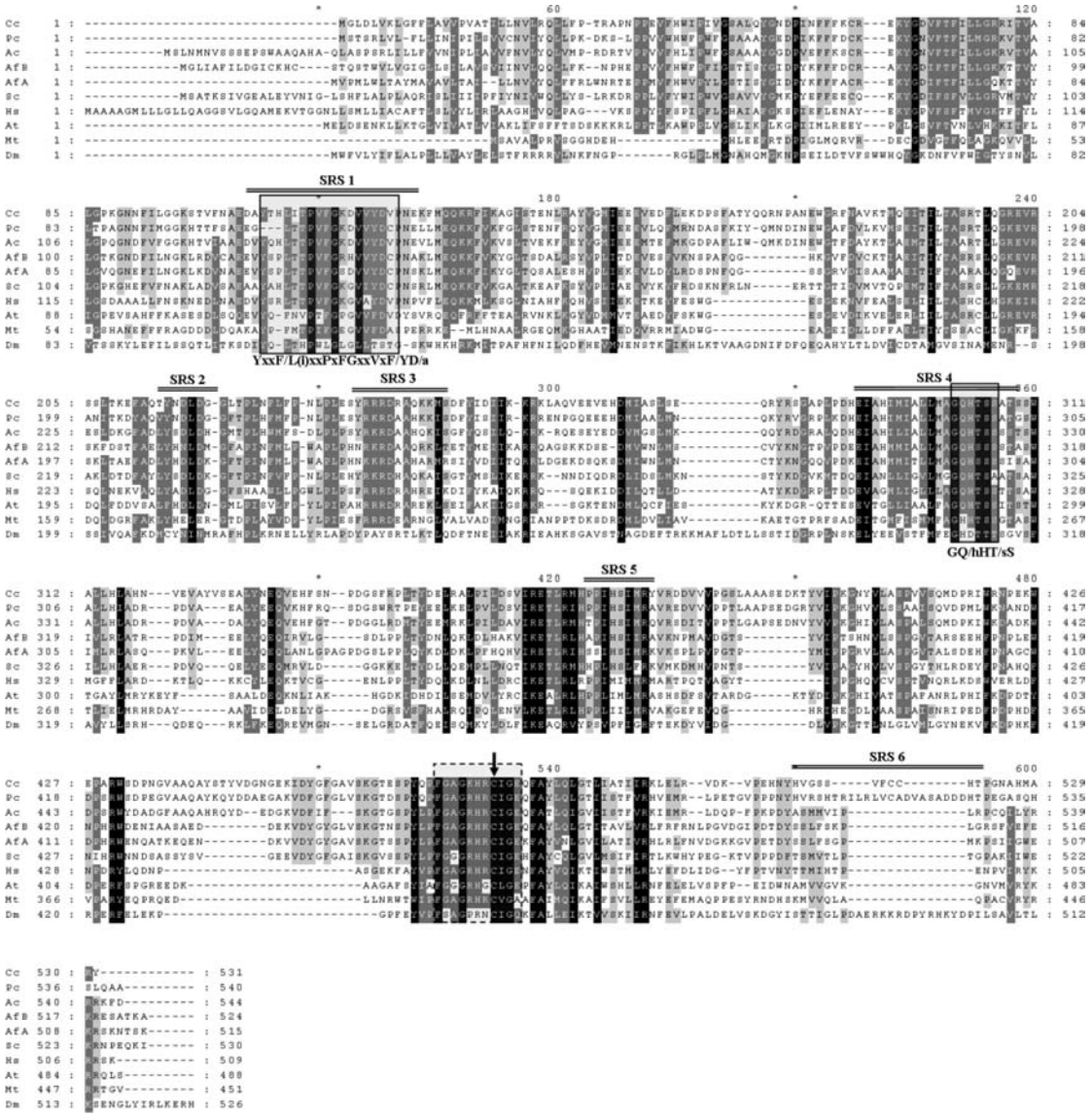

Figure 1. Amino acid alignment of *AcCYP51* from *A. cinnamomea* with other homologous *CYP51* sequences, by ClusterIX and GeneDoc. The residues conserved throughout the sequences of CYP51 from prokaryota to eukaryota are shaded to show the degree of conservation: 100% (black), 70% (dark gray), and 60% (light gray). Six predicted substrate recognition sites (SRS) are marked with a double-line above the sequences. The highly conserved regions, SRS1 and 4, are denoted in a single-line box, and the signatures are written below the alignment. The heme-binding region is delimited by a dashed-line box, and the iron-binding cysteine is shown by arrowheads. *Cc*, *Coprinopsis cinerea* (accession no. AAU01159); *Pc*, *Phanerochaete chrysosporium* (AAU01160); Ac, *A. cinnamomea*; AfA, *Aspergillus fumigatus* Af293 CYP51A (EAL90099); AfB, *Aspergillus fumigatus* Af293 CYP51B (EAL87096); Sc, *Saccharomyces cerevisiae* (AAB68433); Hs, *Homo sapiens* (Q16850); At, *Arabidopsis thaliana* (AAD30254); Mt, *Mycobacterium tuberculosis* (P0A512); Dm: *Drosophila melanogaster* (NP_524771).

The full length of AcCYP51 and mature AcCYP51 without predicted signal peptide recombinant proteins were overexpressed and purified (Supporting Information Figure 2). The molecular masses of recombinant full-length and mature AcCYP51 that were fused with the 26 kDa GST were approximately 87.9 and 84.6 kDa, respectively.

Lanosterol was selected as a substrate to investigate the activity of AcCyp51 in this study. The demethylated products that were catalyzed by AcCYP51 were analyzed by GC-MS. In the presence of reduced NADPH, full-length and mature AcCYP51 both showed lanosterol demethylase activity (**Figure 2**). A shoulder peak (demethylation product) appeared at 0.2−0.3 min after 11.59 min (lanosterol) in GC-MS spectra (**Figure 2A**, middle and lower panels) but not in vector control reaction (**Figure 2A**, upper panel). The demethylation product was identified by comparison of spectral data with those reported in Kahn et al. (*32*). The molecular ion of the silylated lanosterol was m/z 498 with a retention time of 11.59 min as control, and the molecular ion of the silylated demethylated lanosterol was m/z 482 plus one molecule of hydrogen with a retention time of 11.74 min. Together with the function of 14-carbon demethylation demonstrated earlier (*33*), these data demonstrate that the AcCYP51 functions as a lanosterol 14-demethylase.

RT-PCR of Different Culture Stages and Growth Test. The higher level of *AcCyp51* expression in wild type basidiome (AT) stages compared to liquid-cultured mycelia, solid-cultured mycelia, and solid-cultured basidomes was analyzed by semi-quantative RT-PCR (**Figure 3**). When cultured in liquid culture without shaking, the mycelia that grew on the top of the liquid along the flask wall became aerobic as if growing on solid medium. After separating the submerged part and the aerial part of the cultured mycelia, we found higher expression of

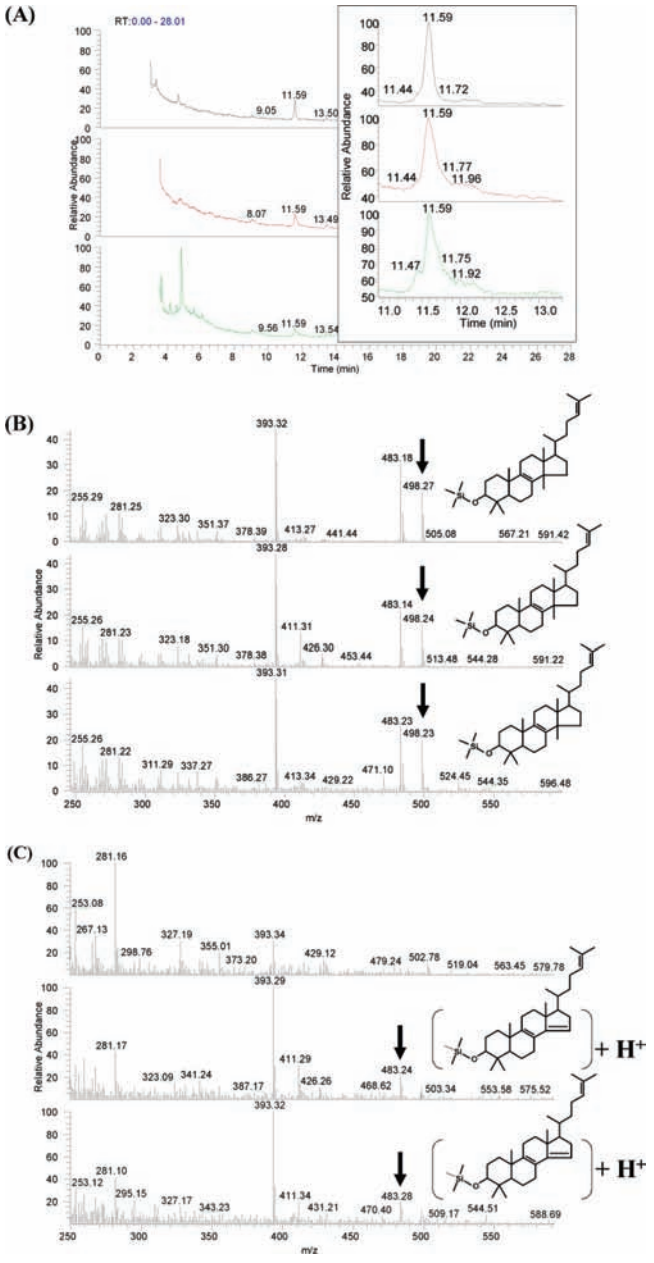

Figure 2. Catalytic properties of recombinant AcCYP51 analyzed by GC-MS using lanosterol as a substrate. (**A**) GC spectrum: (upper panel) vector control, (middle panel) full-length protein, and (lower panel) matured one. Silylated lanosterol has a retention time of 11.59 min. The inset shows that there were shoulder peaks at 0.2−0.3 min after 11.59 min in the middle and lower panels but not in the upper one. (**B**) Mass spectrum of silylated lanosterol at a retention time of 11.59 min; mass spectrum of silylated lanosterol showing the molecular ion of *m*/*z* 498. (**C**) Mass spectrum of silylated demethylated lanosterol at retention time of 11.89 min; mass spectrum of the silylated product formed from lanosterol having a molecular ion of *m*/*z* 483.

AcCyp51 in the aerial parts than in the submerged parts (**Figure 3B**).

Cells were treated with lanosterol or squalene to look for any effects on the gene expression of *AcCYP51* in response to both compounds. Significant induction of *AcCYP51* mRNA was observed with both lanosterol and squalene treatments (**Figure 3C**,**D**). To further investigate the influence on cell growth of the addition of lanosterol or squalene, radial growth assay was performed for 77 days (**Figure 4**). The mycelium development in medium with squalene was much faster than in lanosterol and control media.

Genome Structure Analysis. It has been reported that the genes involved in the biosynthesis of diterpenoids in *Phoma betae*, including cytochrome P450, were clustered in the neighboring region of genomic DNA (*34*). Thus, in this study we used genome walking to look for genes with similar function. Three genes were found upstream of the 5′ end of *AcCyp51* including a V-ATPase subunit E, a hypothetical protein, and a metal ion transport-related protein (Supporting Information Figure 3). Although the functions of these three genes are not yet clear, a corresponding gene cluster in the *Coprinopsis cinerea* Okayama7#130 genome is present in the same arrangement as the three genes in *A. cinnamomea* (**Table 1**).

Prediction of Promoter Region. The *AcCyp51* promoter region was also obtained through the genome walking method. The putative cis-acting regulatory elements were identified using the MOTIF Search web service. Several putative elements were found within the 1503 bp length promoter of *AcCyp51*, including 13 repeats of heat shock factors (HSF), 8 repeats of positive-acting nitrogen regulator (NIT2), and 8 repeats of alcohol dehydrogenase gene regulator 1 (ADR1). HSF is believed to regulate transcription of heat shock genes. This protein binds to a regulatory sequence, the heat shock element, which is absolutely conserved among eukaryotes. NIT2 mediates the repression of its target genes when primary nitrogen sources, for example, glutamine or ammonia, are available. Expression of the nitrogen catabolic genes requires not only the presence of an inducer or substrate of a certain pathway but also the absence of primary nitrogen sources such as L-glutamine, suggesting that *nit-2* acts in a higher hierarchical level of the nitrogen regulation network (*35*, *36*). Two inverted orientation sequences of ADR1 named UAS1 (upstream activation sequence) are required for transcriptional activation to occur (*37*) and act synergistically with another transcriptional regulative element named ORE (oleate response element) (*38*−*40*). UAS1 and ORE govern the up-regulation of many related genes in the presence of oleic acid in *Saccharomyces cerevisiae* and *Candida rugosa* (*38*, *41*, *42*). Several putative UAS1 and ORE elements were identified in the *AcCYP51* promoter region and suggest the possible regulation of AcCYP51 expression by oleic acid.

To further investigate if these elements found in the *AcCYP51* promoter region regulate gene expression, *A. cinnamomea* was tested under various culture conditions. As shown in **Figure 5A**, expression of *AcCyp51* was induced when the culture temperature was increased to 39 °C. Many additives, such as ethanol, glycerol, oleic acid, KNO_3, and L-glutamine, were added as different carbon or nitrogen sources in the culture medium, and *AcCYP51* showed obvious induction of expression compared with control (**Figure 5B**). Itroconazole, a fungicide that targets CYP51 and as an inhibitor causes the accumulation of lanosterol and reduced ergosterol biosynthesis in fungi (*43*, *44*), also induced *AcCYP51* expression after 24 h of treatment (**Figure 5C**).

DISCUSSION

CYP51 is an exception to other P450s in that it is regarded as an orthologous gene in all biological kingdoms (*45*). The amino acid sequence of *AcCYP51* deduced here showed much conservation with CYP51s of other species, especially in substrate-recognition sites (SRSs) and the heme-binding site (**Figure 1**). As it is the only invariant P450 in every organism, it has been speculated that CYP51 might be the ancestor of other P450s (*46*). Although the evolutionary aspects of the CYP51 family remain unresolved, the exon−intron structures might provide important evidence in

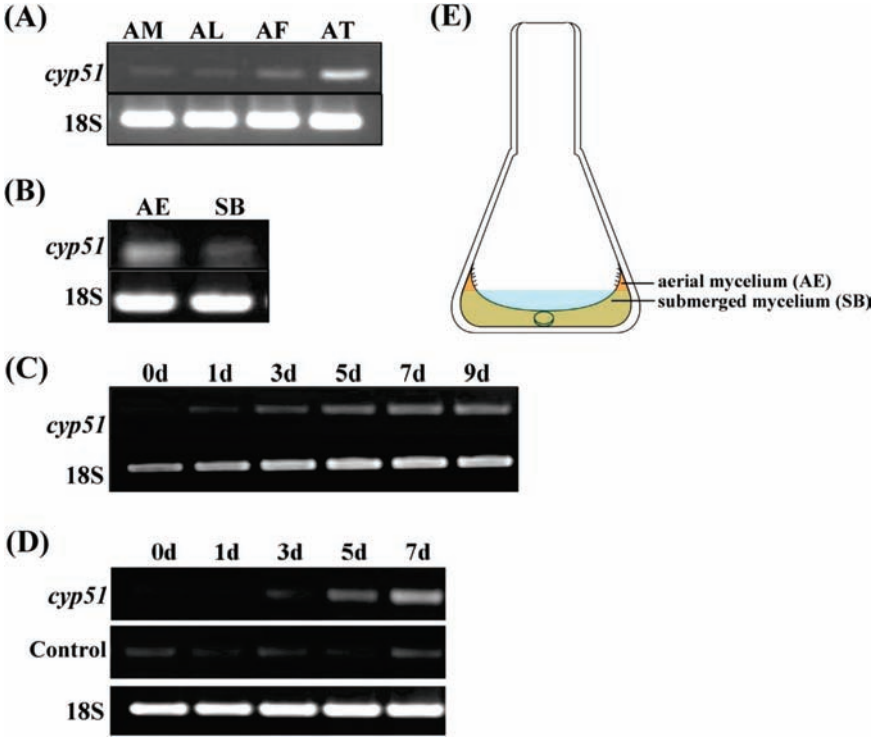

Figure 3. RT-PCR analysis of the expressions of *AcCYP51* transcripts in various culture conditions: (**A**) different culture stages (AM, liquid-culture mycelium; AL, solid-culture mycelium; AF, solid-culture basidiomes; AT, natural basidiomes); (**B**) different parts of the mycelia in liquid culture (AE, aerial mycelium; SB, submerged mycelium); (**C**) adding squalene (4 g/L) to culture medium; (**D**) adding lanosterol (10 mg/L) to culture medium (control was a Tween 80/ethanol (v/v = 1:1) solvent that was also used to dissolve lanosterol); (**E**) illustration of aerial and anaerobic parts of mycelia in liquid culture. The 18S rRNA gene is the internal control. d, days after the addition of lanosterol or squalene.

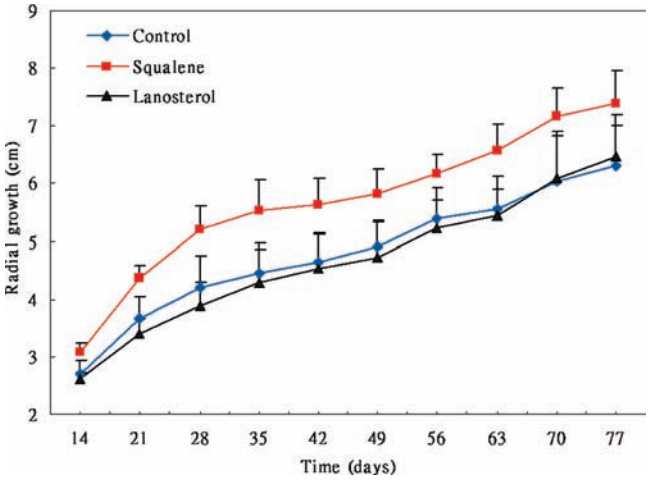

Figure 4. Radial growth curves of *A. cinnamomea* cultured in reduced nutrient media and media with lanosterol (0.1 g/L) or squalene (1 g/L).

Table 1. Gene Arrangement of *Coprinopsis cinerea* Okayama7#130 Is Similar to That of *AcCyp51* in *Antrodia cinnamomea* with Its Neighbor Genes

GenBank accession no.	putative identification	correlative to *A. cinnamomea* genome
EAU85615	hypothetical protein CC1G_06328	
EAU85616	hypothetical protein CC1G_06329	metal ion transport-related protein
EAU85617	hypothetical protein CC1G_06330	hypothetical protein CC1G_06330
EAU85618	ATP synthase subunit H	V-ATPase subunit e
EAU85619	P450	lanosterol 14-α-demethylase
EAU85620	predicted protein	
EAU85621	P450	

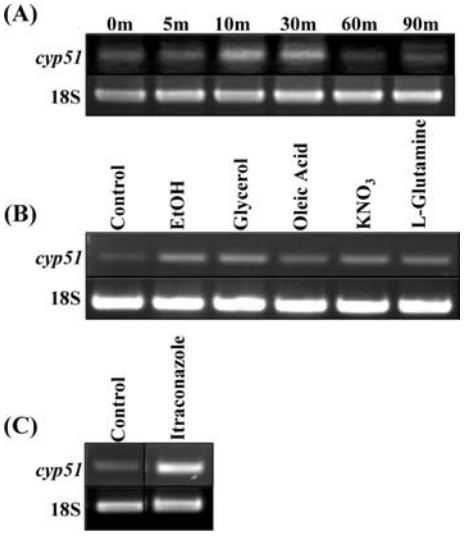

Figure 5. Differential expression analysis of *AcCYP51* under variant treatments by RT-PCR: (**A**) several heat shock incubation times at 39 °C; (**B**) exchange of carbon sources or adding nitrogen sources; (**C**) addition of itroconazole to culture medium of *A. cinnamomea*. Liquid-cultured mycelium of *A. cinnamomea* was incubated in malt extract medium with glucose in normal condition for 20 days before treatment. The 18S rRNA gene was used as an internal control.

eukaryotes (*47*,*48*). The intron arrangement of *AcCyp51* is similar to that of other basidiomycetes homologues but not to that of ascomycetes (Supporting Information Figure 1), with more introns and similar intron phases. This intron−exon sequence could provide another point of view in the evolution of P450 in these fungi.

Four closely related endogenous compounds, lanosterol, 24,25-dihydrolanosterol, 24,25-diethylenedihydrolanosterol and obtusifoliol, are known substrates of CYP51 in general (*16*, *17*). In yeast and fungi, CYP51s prefer 24-methylenedihydrolansterol and lanosterol as substrate in the ergosterol production pathway. The activity of AcCYP51 was studied here using lanosterol as substrate, and GC-MS revealed C14 demethylation (**Figure 2**). Ergosterol, with a role similar to that of cholesterol in animal cells, has been demonstrated to play an important role in many physiological functions in *S. cerevisiae* and *Candida* species, such as membrane maintenance, protein transport, sporulation, and endocytosis (*49*−*53*). Little is known about CYP51 gene regulation in yeast and fungi, although in all mammalian cells this gene is highly expressed, especially in the liver, which is the major organ of cholesterol production (*54*). A higher expression level of *AcCYP51* was found in natural basidiomes of *A. cinnamomea* compared with others (**Figure 3**). According to our previous paper, *AcCYP51* was identified in 17 ESTs of a basidiomatal cDNA library but in only 3 ESTs of liquid-cultured mycelia cDNA library (*14*). In addition, various cytochrome P450s were detected during basidiomatal development by subtractive PCR and cDNA microarray (*15*). The oxidoreduction reactions driven by cytochrome P450 were implied to be necessary during fruiting; furthermore, it was thought to play a key role in metabolizing lignocellulose and a wide range of environmental xenobiotics in such wood parasitic fungi (*55*,*56*). The large amount of *AcCYP51* expression at the time of aerial structure formation revealed a significant role in the development of mycelia to aerial fruiting bodies when emerging into air. Although it is still unknown if ergosterol biosynthesis is required for fungi aerobic growth, the lipid composition of plasma membrane and mitochondria in an anaerobic bottom fermenting brewer's yeast, *Saccharomyces uvarum* 21, obviously had a low content of ergosterol and a high content of squalene (*57*).

In the pharmaceutical industry, CYP51 is a practical drug target to interrupt the biosynthesis of ergosterol in fungal cells. Azole fungicides (imidazole and triazole) coordinate to the heme iron binding site and inhibit the activity of CYP51 by preventing substrate binding and metabolism (*58*, *59*). An induction of *CYP51* was found in *A. cinnamomea* treated with itroconazole, a kind of azole (**Figure 5C**). Other research has shown that the transcription of *ERG11* (orthologue of *CYP51*) in *Leptosphaeria maculans*, a plant pathogenic ascomycete, increased following exposure to the triazole fungicide, flutriafol (*60*). It is reasonable to speculate that the inhibition of CYP51 causes a shortage of ergosterol, which then up-regulates the expression of *CYP51* by feedback regulation. Like the observations in mammalian cells, CYP51s are up-regulated in cholesterol-limiting conditions (*54*, *61*). Treatment with another CYP51 inhibitor, fluconazole, increased the expression of both *ERG11* and *ERG9* (squalene synthase) in *Candida albicans* (*62*). The upstream intermediates of a CYP51-like lanosterol or squalene might accumulate after the inhibition of CYP51 and act as inducers of the downstream pathway. This speculation was confirmed by the observation that lanosterol and squalene induced *AcCYP51* expression (**Figure 3**).

Regulation of CYP51 transcription is still unclear in fungi and yeasts, but the sterol regulatory elements (SRE) and cAMP regulatory element (CRE) have been found in the *CYP51* promoter in humans (*63*, *64*). Several predicted elements found in the *AcCYP51* promoter suggest particular responses to different culture conditions. The increased expression of AcCYP51 following culture in high temperature and in the presence of various carbon sources such as ethanol, glycerol, and oleic acid or nitrogen sources such as KNO_3 and L-glutamine was in agreement with the predicted regulation by the putative elements found in the promoter (**Figure 5B**). It is notable that two putative oleic acid response elements, UAS1 (ADR1 binding site) and ORE, were found in the *AcCYP51* promoter. UAS1 and ORE govern the up-regulation of genes functional in fatty acid metabolism or peroxisomal proliferation in the presence of oleic acid in *S. cerevisiae* (*38*, *41*). The peroxisome is a subcellular organelle that handles fatty acid β-oxidation; moreover, it has been found in all phyla that the hydroxylation of fatty acid depends on cytochrome P450. The response of *AcCYP51* expression to oleic acid suggests the association between the biosyntheses of fatty acids and ergosterol. Interestingly, different membrane compositions of ergosterol and oleic acid were characterized in the mycelia and conidia of *Fonsecaea pedrosoi* (*65*). Membrane fluidity is governed by the specific composition, and many stresses such as ethanol or heat shock are known to strongly promote membrane fluidity by increasing the content of sterols and fatty acids (*57*). Many ADR1-regulated genes, such as *POX11* and *SPS19*, are induced in the presence of ethanol (*38*,*39*). In addition, a unique 126 bp tandem repeat sequence found in the CYP51 promoter of *Penicillium digitatum* that contains putative ADR1 and HSF transcription factor binding sites acts as a transcriptional enhancer in the demethylation-inhibitor resistant strain (*66*). These reveal that the regulation of *AcCYP51* might not only be involved in the regulation of ergosterol biosynthesis but also, we propose, play a role in cross-talk with other important biological processes.

Supporting Information Available: Three supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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