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ORIGINAL PAPER

Molecular identification and functional characterization of cytochrome P450 monooxygenases from the brown-rot basidiomycete *Postia placenta*

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Abstract We explored the molecular diversity and functional capabilities of cytochrome P450 monooxygenases (P450s) from the brown-rot basidiomycete *Postia placenta*. Using bioinformatic and experimental data, we found 250 genes of P450s in the whole genome, including 60 putative allelic variants. Phylogenetic analysis revealed the presence of 42 families, including 18 novel families. Comparative phylogenetic analysis of P450s from *P. placenta* and the white-rot basidiomycete *Phanerochaete chrysosporium* suggested that vigorous gene duplication and molecular evolution occurred after speciation of basidiomycetes. Among the 250 gene models, 184 were isolated as full-length cDNA and transformed into *Saccharomyces cerevisiae* to construct a functional library in which recombinant P450s were co-expressed with yeast NADPH-P450 oxidoreductase. Using this library, the catalytic potentials of P450s against a wide variety of compounds were investigated. A functionomic survey allowed the discovery of novel catalytic properties of *P. placenta* P450s. The phylogenetic diversity of the CYP53 family in *P. placenta* was clear, and CYP53D2 is capable of converting stilbene derivatives. This is the first report of this peculiar function of the CYP53 family. Our increased understanding of the molecular and functional diversity of P450s in this fungus will facilitate comprehension of metabolic diversity in basidiomycetes and has future biotechnology applications.

Keywords Cytochrome P450 · Brown-rot basidiomycete · Diversity · Functional screening · CYP53

Introduction

Cytochromes P450 (P450s) comprise a superfamily of heme-containing monooxygenases, which are widely distributed in living organisms, and play numerous physiological roles in the metabolism of endogenous and exogenous compounds (Ortiz de Montellano 2005). The vast majority of P450s are thought to have emerged and diversified during the evolution of species, implying that a multitude of secondary metabolic systems are likely to be associated with a large-scale divergence of P450s. Within the last few years, a series of genome projects have accelerated sequence compilation of P450s, and as a result, the sequence database of P450s has greatly enlarged and continues to increase (Park et al. 2008; Nelson 2009). Thus, it becomes a challenging task to exploit the catalytic functions of numerous P450s to gain a better understanding of

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metabolic diversity in living organisms. In addition to the biological importance of P450s, it will be of great interest to utilize their catalytic potentials in biotechnology, because regio- and stereo-specific oxidations by P450s promise practical advantages (Guengerich 2002; Ro et al. 2006; Urlacher and Eiben 2006; Chang et al. 2007; Gillam 2008; Grogan 2011). Hence, increase in both molecular and functional information relating to P450s would facilitate advanced research into fungal biology and applied biotechnology (Lamb et al. 2002; Yadav et al. 2006; Sabbadin et al. 2010; Nazir et al. 2011; Hirosue et al. 2011).

Wood-rotting basidiomycetes, often categorized into white-rot and brown-rot basidiomycetes, are common inhabitants of forest litter and play crucial roles in the biospheric carbon cycle (Eriksson et al. 1990). Brown- and white-rot basidiomycetes employ independent strategies for the biodegradation of woody components, despite showing some phylogenetic relationship and taxonomic similarity. A significant ligninolytic potential, for example, is found in white-rot, but not in brown-rot basidiomycetes. However, the molecular mechanisms of brown-rot decay are poorly understood relative to those of white-rot decay (Eriksson et al. 1990; Kirk and Farrell 1987; Gold et al. 1989; Hammel and Moen 1991). There is great interest, however, in the biological performance of brown-rot basidiomycetes (Yelle et al. 2011; Wei et al. 2010; Niemenmaa et al. 2008). Recently, the whole genomes of the white-rot basidiomycete *Phanerochaete chrysosporium* (Martinez et al. 2004) and the brown-rot basidiomycete *Postia placenta* (Martinez et al. 2009) have been sequenced and are openly available (http://www.jgi.doe.gov/). Many researchers have taken advantage of these genomic projects and have carried out various "Omics" studies in the post genomic era (Matsuzaki et al. 2008; Sato et al. 2009; Vanden Wymelenberg et al. 2010). Based upon genome-wide comparison, it has been elucidated that the gene number of P450s in *P. placenta* (PpCYP) is larger than that of the P450s in *P. chrysosporium* (PcCYP) (Martinez et al. 2009; Hirosue et al. 2011). It could be hypothesized that brown-rot basidiomycetes have invested more heavily in the diversification of the P450 molecule than white-rot basidiomycetes. Thus, it would be of great interest to better understand the metabolic diversity and capabilities of brown-rot basidiomycetes through a comprehensive survey of PpCYPs.

We performed a genome-wide survey and functionomic investigation of PpCYPs. Using bioinformatic and experimental data, we identified 250 gene candidates of PpCYPs including 60 allelic variants. Using RT-PCR techniques, we confirmed gene expression in 210 species and isolated full-length cDNA from 184 species. Furthermore, we developed a functional screening system for PpCYPs in which 184 isoforms were co-expressed with yeast NADPH-P450 reductase in *Saccharomyces cerevisiae*. A functionomic survey resulted in the discovery of novel catalytic potentials of PpCYPs, providing new insight into their fascinating fungal biology and potential for biotechnology.

Materials and methods

Chemicals

Anthracene, carbazole, dibenzo-*p*-dioxin, 4-ethoxybenzoic acid, 7-ethoxycoumarin, pyrene, testosterone, and 3,5,4′-trimethoxy-*trans*-stilbene were purchased from Wako Pure Chemicals (Osaka, Japan). 7-Ethoxycoumarin was purified before use, using a silica gel column (hexane/ethyl acetate). 5-Aminolevulinic acid was purchased from Cosmo Bio Co. (Tokyo, Japan). DO supplement without Leu was purchased from TaKaRa Bio (Shiga, Japan). All other chemicals were reagent grade. Deionized water was obtained using a Milli-Q System (Millipore Japan Co Ltd., Tokyo, Japan).

Bioinformatic annotation of P450 from *P. placenta*

A possible coding sequence for PpCYPs was found in the US Department of Energy Joint Genome Initiative database, based upon sequence similarity to known P450s (http://genome.jgi-psf.org/Pospl1/Pospl1.home.html). To evaluate annotation accuracy, we identified the P450 signature sequence (F-x-x-G-x-x-x-C-x-G) in the heme-binding domain, the E-x-x-R motif in the K-helix, a conserved Thr in the center of the I-helix, and the hydrophobic transmembrane domain (TMD) at the N-terminal region. TMD sequences were analyzed using SOSUI (Hirokawa et al. 1998; http://bp.nuap.nagoya-u.ac.jp/sosui/). If candidates lacked sequences corresponding to these regions, their capability to encode P450 was judged by overall sequence similarity to known P450s.

Isolation of cDNA by RT-PCR

Postia placenta strain MAD-698 (ATCC 44394) was grown from hyphal inocula at 27°C in a stationary culture (10 mL medium) under aerobic conditions. The medium (pH 6.0) used in this study was, as previously described, utilizing 1% glucose and either 1.2 or 12 mM ammonium tartrate as the carbon and nitrogen sources (Kirk et al. 1978; Nazir et al. 2010). Total RNA was extracted individually from 10, 15, 20, 25, and 28-day-old mycelia using the acid guanidium-phenol–chloroform method (Sambrook and Russel 2001) and further purified using an RNeasy Plant Mini Kit (QIAGEN). The concentration of RNA was calculated from the absorbance at 260 nm. Equal quantities of RNA isolated from mycelia of the five different ages were

then mixed and used for RT-PCR, as previously described (Nazir et al. 2010). Gene-specific primers used for PCR amplification were designed to anneal to 5′- and 3′-untranslated regions; to the 2–30 bp upstream or downstream flanking sequences from the putative start and stop codons (Table S1). Target cDNAs were cloned into pBluescript plasmid and sequenced using an automated DNA Sequencer (CEQ 8000; Beckman).

Sequence alignment and phylogenetic analysis

Multiple sequence alignment was carried out using the ClustalX program with a gap penalty of 10 and a gap extension penalty of 0.2 (Thompson et al. 1994). Our phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), with the Jones-Taylor-Thornton matrix using PHYLIP software (Felsenstein 1989), and visualized using the FigTree program (http://tree.bio.ed.ac.uk/).

Heterologous expression and functional screening of PpCYPs

The open reading frame of each PpCYP was re-amplified from the gene library (cloned in pBluescript vector) using gene-specific primers (Table S2). The resultant gene fragments were used for construction of expression plasmid, as previously described (Nazir et al. 2010). The expression plasmid harboring PpCYP was then transformed into *S. cerevisiae* AH22, using the *Fast*™-Yeast Transformation Kit (G-Biosciences). Carbon monoxide (CO) difference spectra of the transformants were recorded on a UV–Vis spectrophotometer equipped with a head-on photomultiplier (Hitachi; U3900H) (Omura and Sato 1964; Nazir et al. 2011; Hirosue et al. 2011). To construct a functional library, *S. cerevisiae* harboring each expression plasmid was inoculated into 0.5 mL of synthetic dextrose liquid (SDL) medium (8% glucose, 2.68% yeast nitrogen base without amino acids, 0.1% DO supplement without Leu, and 0.5 mM 5-aminolevulinic acid), and these were simultaneously grown in a 96-deep-well plate. After 4 days incubation, transformants were harvested by centrifugation (1,300×*g*) and resuspended in 2 mL potassium phosphate (10 mM, pH 7.0) containing 10% glycerol. The 96-well plates accommodating transformants were stored at −80°C. For bioconversion, a 20 µl solution containing transformants was inoculated into 0.5 mL of SDL medium containing substrate (0.5 mM) and 5-aminolevulinic acid and incubated in a Micro Bio Shaker (TAITEC) at 28°C for 2 days. Reactions were stopped by the addition of methanol/acetone (0.5 mL), and metabolic products were analyzed by high-performance liquid chromatography (HPLC), after removal of cell debris by centrifugation (1,300×*g*) and filtration (0.45 mm, Whatman). If necessary, the metabolic products were extracted using ethyl acetate, purified by preparative HPLC, and analyzed by liquid chromatography electron spray ionization mass spectrometry (LC–ESI–MS) and/or ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectrometry.

Instruments

HPLC analysis was carried out using a Prominence UFLC system (Shimadzu) consisting of two pumps (LC-20AD), an auto-injector (SIL-20AC HT), UV-detector (SPD-20A), and a column oven (CTO-20A). Chromatographic separation was performed using a Shima-pack XR-ODS II column (Shimadzu; 3.0 mm I.D. × 75 mm) with a column temperature of 40°C. The mobile phases for HPLC were (A) water with 0.05% phosphoric acid and (B) acetonitrile. The mobile phase gradient was as follows: 0–0.2 min, 10% B; 0.2–3.2 min, 10–40% B; 3.2–3.6 min, 40–100% B; 3.6–4.0 min, 100% B. The flow rate was 1.4 mL/min. An ultraviolet (UV) monitor was utilized for product detection. ^{1}H-NMR (400 MHz) spectra were obtained using a JEOL JNM-AL400 spectrometer with the chemical shift expressed as parts per million downfield from an internal standard of tetramethylsilane. Samples were dissolved in deuterated methanol or chloroform.

Results and discussion

Genome-wide survey and molecular identification of PpCYPs

The whole genome of *P. placenta* strain MAD-698 has recently been sequenced (Martinez et al. 2009) and released by the US Department of Energy Joint Genome Initiative (http://genome.jgi-psf.org/Pospl1/Pospl1.home.html). In the current database, 17,173 gene models, over 300 PpCYPs, were predicted in the dikaryotic genome of this fungus; however, the gene model remains complicated due to the allelism of this strain and unidentified and/or unassembled segments. Moreover, it was presumed that gene annotation and prediction errors would be included in the current draft release, and in fact, several PpCYP candidates seem to have unexpected truncations at their N-and/or C-terminal region(s). We further refined the gene annotation accuracy, therefore, and selected gene candidates of PpCYP based on consideration of the conserved sequence feature in eukaryotic P450s (Ortiz de Montellano 2005; Nazir et al. 2010; Hirosue et al. 2011).

A bioinformatic survey was used to identify possible coding sequences of 242 PpCYPs from the whole genome sequence (Table 1; see also Figure S1, Figure S2, and Table

Table 1 Summary of PpCYP numbers

Description	Number
Gene candidate	
Bioinformatically predicted	242
Experimentally isolated[a]	8
Total	250
CYP family	
Existing family	24
Novel family	18
Total	42
cDNA amplification	
Full-length cDNA[b]	175
Frame-shifted cDNA	43
Functional library	
Applied for heterologous expression	184
Functionally expressed[c]	116

[a] cDNA unexpectedly amplified by RT-PCR, but not found in the genome database

[b] Including eight species unexpectedly isolated by RT-RPR, but not found in the database

[c] Confirmed by carbon monoxide difference spectra and bioconversion experiments

S3). Among the 242 candidates, several gene pairs showed high sequence similarity; over 98% at amino acid level. Although this striking sequence homology implies allelic features, it could be expected that *P. placenta* possesses at least 190 haploidal PpCYP genes showing sequence identity of lower than 90%. In addition, we found several gene fragments showing significant homologies to P450s; however, we were not able to identify their full-length coding sequences because these sequences were connected to unassembled and/or uncharacterized segments. Although the dataset could be slightly enlarged after development of the database, we initiated further studies using the 242 PpCYP candidates.

Insight into PpCYP phylogenies

To better understand the molecular evolution of P450s within brown-rot basidiomycetes, we conducted a phylogenetic analysis using the 190 haploidal PpCYPs. The P450 nomenclature committee recommended that families should be considered to share greater than 40% identity, and subfamilies should be considered to share greater than 55% identity of amino acid sequences (Nelson et al. 1996). Based on sequence comparisons, PpCYPs were assigned to 42 families including 18 novel families. A comparative phylogenetic analysis of PpCYPs and PcCYPs revealed interesting aspects, including the revelation that CYP5027, CYP5350, and CYP5348 are large families in *P. placenta* but are absent from *P. chrysosporium* (Table 2). These results may highlight an evolutionary trajectory of vigorous gene duplication and molecular evolution within a short evolutionary period after the speciation of basidiomycetes, to meet the requirements of species. In fact, PpCYPs and PcCYPs shared few subfamilies (Table 2) and branched into unique clusters on our phylogenetic tree (Fig. S3). In addition, it was noteworthy that the gene number of the CYP53 family (widely distributed in the fungal kingdom and well known to catalyze benzoate hydroxylation) was significantly higher than in other organisms (Faber et al. 2001). Indeed, *P. chrysosporium* encodes one CYP53C2 in its genome (Martinez et al. 2004; Matsuzaki and Wariishi 2005). In *P. placenta*, the CYP53 family was shown to include orphan species assigned to the CYP53C subfamily, and 6 species assigned to a novel CYP53D subfamily. As shown in Fig. 1, PpCYPs CYP53D subfamily from *P. placenta* consisted of distinctive clusters showing significant phylogenetic distance from other subfamilies, suggesting a possibly unique evolutionary trajectory in the CYP53D subfamily. In addition, it should be noted that several PpCYPs were phylogenetically closer to P450s from ascomycetous fungi than to PcCYPs. *P. placenta*, for example, possesses a CYP537 family that is also found in *Aspergillus* species, but not in *P. chrysosporium* (Kelly et al. 2009; Nazir et al. 2010). PpCYPs assigned to the CYP5148 family showed higher sequence homology with CYP5148B1 from *A. clavatus* (74% identity) than to CYP5148A1 from *P. chrysosporium* (52% identity). Thus, one can assume that *P. placenta* possessed some evolutionary interaction such as gene transfers, at least in part, with ascomycetous fungi.

cDNA isolation and heterologous expression of PpCYPs

In addition to bioinformatic studies, experimental approaches are compulsory to facilitate advanced research into basic biology and applied biotechnology. We performed isolation and characterization of full-length PpCYP cDNAs. Since several allelic P450s are known to exhibit different catalytic properties (Ingelman-Sundberg 2001; Yu et al. 2002), we aimed to isolate all possible PpCYPs found in the database, including allelic variants, and aimed for heterologous expression. We have previously demonstrated that the gene expression of fungal P450s is affected by cultural conditions (Ichinose et al. 1999, 2002; Chigu et al. 2010). In particular, it has been strongly suggested that the transcriptional regulation of fungal P450s may respond to nitrogen limitation or starvation (Ichinose et al. 1999; Nazir et al. 2010). Therefore, total RNA was extracted from *P. placenta* grown in a synthetic liquid medium; in which it has been shown that the white-rot basidiomycete *P. chrysosporium* and the ascomycetous fungus *Aspergillus oryzae*

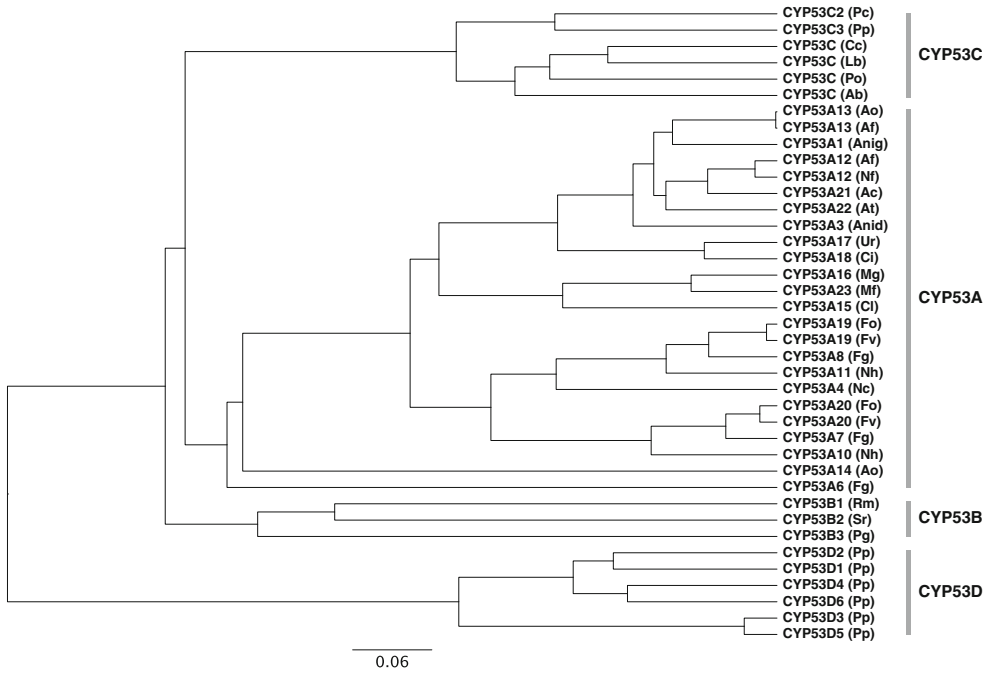

Fig. 1 Phylogenetic tree of the CYP53 family. CYP number is represented by an abbreviated fungal species name. Ab *Agaricus bisporus*, Ac *Aspergillus clavatus*, Af *Aspergillus flavus*, Anid *Aspergillus nidulans*, Anig *Aspergillus niger*, Ao *Aspergillus oryzae*, At *Aspergillus terreus*, Cc *Coprinus cinereus*, Ci *Coccidioides immitis*, Cl *Cochliobolus lunatus*, Fg *Fusarium graminearum*, Fo *Fusarium oxysporum*, Fv *Fusarium verticillioides*, Lb *Laccaria bicolor*, Mf *Mycosphaerella fijiensis*, Mg *Mycosphaerella graminicola*, Nc *Neurospora crassa*, Nf *Neosartorya fischeri*, Nh *Nectria haematococca*, Pc *Phanerochaete chrysosporium*, Po *Pleurotus ostreatus*, Pp *Postia placenta*, Rm *Rhodotorula minuta*, Sr *Sporobolomyces roseus*, Ur *Uncinocarpus reesii*. P450s from Ab, Cc, Lb, and Po are able to be classified into the CYP53C subfamily but have not been named

express a high number of P450s (Chigu et al. 2010; Nazir et al. 2010). Using RT-PCR technique, we isolated 167 species as full-length cDNA. Nevertheless, there are several nucleotide substitutions in some of the isolated *P. placenta* genes presumably attributable to polymorphisms. In addition, 43 species were amplified as immature cDNA whose open reading frames were shifted by illegal splicing events. During RT-PCR experiments, we obtained 8 allelic variants that could not be found in the current database, but that could share primer sequences with known PpCYPs. Eventually, we identified 250 possible sequence of PpCYPs and experimentation and isolated 175 species as full-length cDNA (Table 1; see also Table S3). cDNA and deduced amino acid sequences are listed in Figure S1 and Figure S2.

Using the isolated cDNA, we performed heterologous expression of PpCYPs in *S. cerevisiae.* In addition to the 175 full-length cDNAs isolated by RT-PCR, 9 species were generated to encode a theoretical open reading frame by removing unexpected introns from their frame-shifted variants. Thus, 184 PpCYPs could be used for further experimentation. We have successfully expressed a number of fungal P450s from *P. chrysosporium* and *A. oryzae* in *S. cerevisiae* using the expression plasmid pGYR (Hirosue et al. 2011; Nazir et al. 2011). We therefore employed the pGYR vector system for heterologous expression of PpCYPs. To evaluate and optimize heterologous expression, we analyzed the CO difference spectra of transformants. A typical CO difference spectrum for P450s can be observed with an absorption maximum of around 450 nm (Omura and Sato 1964). We clearly demonstrated that an exogenous addition of heme precursor, 5-aminolevulinic acid, to liquid culture medium, can elevate protein concentration of several PpCYPs (Fig. 2); this supports the finding of earlier studies using a plant P450 (Jiang and Morgan 2004). Each transformant was therefore grown in culture medium supplemented with 5-aminolevulinic acid and was applied to spectroscopic analysis. We confirmed the substantial expression of 113 species based upon CO difference spectra. Furthermore, several PpCYPs showed catalytic activities even though we could not confirm their expression using CO difference spectra, due to low levels of expression. Combining the results of spectroscopic analysis and bioconversion, we concluded that at least 116 PpCYPs were functionally expressed in *S. cerevisiae* (Table 1).

Functional screening of PpCYPs

The expression plasmid pGYR is useful for screening of P450-dependent reactions because P450 is co-expressed

Table 2 Comparison of P450 genes and subfamilies in *P. placenta* and *P. chrysosporium*

Family	Gene number		Subfamily number		Common subfamily
	PpCYP	PcCYP	PpCYP	PcCYP	
CYP51	1	1	1	1	51F
CYP53	7	1	2	1	53C
CYP61	1	1	1	1	61A
CYP63	5	7	3	3	63A, 63B
CYP502	4	1	1	1	502B
CYP505	2	7	1	1	505A
CYP512	16	15	7	8	None
CYP537	2	0	1	0	None
CYP5027	10	0	2	0	None
CYP5035	3	13	1	5	None
CYP5036	0	5	0	3	None
CYP5037	14	6	3	3	5037B
CYP5136	0	5	0	1	None
CYP5137	6	2	2	1	5137A
CYP5138	1	1	1	1	5138A
CYP5139	8	1	1	1	None
CYP5140	1	1	1	1	5140A
CYP5141	4	8	2	4	5141A
CYP5142	0	7	0	5	None
CYP5143	0	2	0	2	None
CYP5144	3	35	3	9	None
CYP5145	0	3	0	1	None
CYP5146	0	6	0	3	None
CYP5147	0	6	0	3	None
CYP5148	1	2	1	1	None
CYP5149	1	1	1	1	None
CYP5150	24	6	3	2	None
CYP5151	1	1	1	1	5151A
CYP5152	2	2	1	1	5152A
CYP5154	0	1	0	1	None
CYP5155	0	1	0	1	None
CYP5156	1	1	1	1	5156A
CYP5158	2	2	1	1	None
CYP5339	2	0	2	0	None
CYP5340	1	0	1	0	None
CYP5341	3	0	2	0	None
CYP5342	1	0	1	0	None
CYP5343	1	0	1	0	None
CYP5344	3	0	2	0	None
CYP5346	1	0	1	0	None
CYP5347	2	0	2	0	None
CYP5348	35	0	18	0	None
CYP5349	2	0	1	0	None
CYP5350	11	0	2	0	None
CYP5351	1	0	1	0	None
CYP5352	1	0	1	0	None

Table 2 continued

Family	Gene number		Subfamily number		Common subfamily
	PpCYP	PcCYP	PpCYP	PcCYP	
CYP5353	1	0	1	0	None
CYP5354	2	0	1	0	None
CYP5355	1	0	1	0	None
CYP5356	1	0	1	0	None
CYP5445	1	0	1	0	None
(Total)	190	151	82	69	15 subfamily

Table 3 Catalytic potentials of PpCYPs against various compounds

Substrate	PpCYP
7-Ethoxycoumarin	CYP5037B5v2, CYP5139D2, CYP5139D3v1, CYP5139D5v1
4-Ethoxybenzoic acid	CYP5152A3v1, CYP5152A3v2, CYP5152A4, CYP5027B6v2
Dehydroabietic acid	CYP512N6v1, CYP512N6v2, CYP512P2, CYP5027B4v1, CYP5150D18, CYP5150D12v1,
Testosterone	CYP512N6v1, CYP512N6v2, CYP512P1v2, CYP512P2, CYP5139D2, CYP5150D1
Anthracene	CYP5027B1, CYP5150D1, CYP5150D7, CYP5139D5v1, CYP5350B2v1, CYP5350B9
Carbazole	CYP63A6v1, CYP5027B1, CYP5139D3v1, CYP5139D3v2, CYP5139D6, CYP5150D1, CYP5350B2v1, CYP5350B9
Pyrene	CYP5027B1, CYP5144K1v1, CYP5150D1, CYP5150D7, CYP5350B2v1,
Phenanthrene	CYP5027B1, CYP5150D1, CYP5139D3v1, CYP5139D3v2, CYP5139D5v1, CYP5144K1v1, CYP5348J3, CYP5350B2v1, CYP5350B9
trans-Stilbene	CYP5139D3v1, CYP5139D5v1, CYP5141A6, CYP5150D1, CYP5350B1, CYP5350B2v1, CYP5350B3v1, CYP5350B3v2
3,5-Dimethoxy-*trans*-stilbene	CYP53D2v1, CYP53D2v2, CYP5144K1v1, CYP5158B2,
3,5,4′-Trimethoxy-*trans*-stilbene	CYP53D2v1, CYP53D2v2, CYP5144K1v1, CYP5158B2, CYP63A6v1

with its redox partner cytochrome P450 oxidoreductase (Murakami et al. 1990; Sakaki et al. 1992, 2002; Hirosue et al. 2011; Nazir et al. 2011). To facilitate high-throughput screening, we constructed a functional library in which each transformant was separately inoculated into 0.5-mL culture medium and grown in 96 DeepWell plates. Transformants accommodated in the 96-well plates were easily replicated and used for functionomic surveys, in which each transformant was incubated with a wide variety of compounds, and the resultant metabolic products were comprehensively analyzed. The catalytic potentials of the PpCYPs revealed are summarized in Table 3.

Through comprehensive functional screening, it was demonstrated that CYP53D2 exhibits *O*-demethylation activity against 3,5,4′-trimethoxy-*trans*-stilbene to produce 3-hydroxy-5,4′-dimethoxy-*trans*-stilbene and 3,5-dihydroxy-4′-methoxy-*trans*-stilbene (Fig. 3 and Table S4). *O*-demethylation activity was also shown from bioconversion experiments using 3,5-dimethoxy-*trans*-stilbene. In contrast, no reaction proceeded when *trans*-stilbene was used as substrate for CYP53D2. This may suggest that CYP53D2 recognizes the methoxyl group(s) in the stilbene derivatives. Although further investigation should be directed at understanding the mechanisms involved, it was noteworthy that substantial activities by CYP53D2 against stilbene derivatives were observed, because the CYP53 family has generally been considered to exhibit substrate specificity against benzoate, and the carboxyl group in benzoate is essential for enzyme-substrate binding (Matsuzaki and Wariishi 2005; Podobnik et al. 2008). To the best of our knowledge, this is the first report describing catalytic potentials of the CYP53 family against stilbene derivatives that seem structurally dissimilar to benzoic acid. However, we could not determine catalytic potentials of CYP53D2 against benzoic acid because *S. cerevisiae* metabolized benzoate without PpCYP. Thus, it would be of great interest to better understand biochemical aspects of CYP53D2 using purified enzymes. The finding of peculiar functions of CYP53D2 would also support unique evolutionary histories within the CYP53D2 subfamily (Fig. 1). These results should

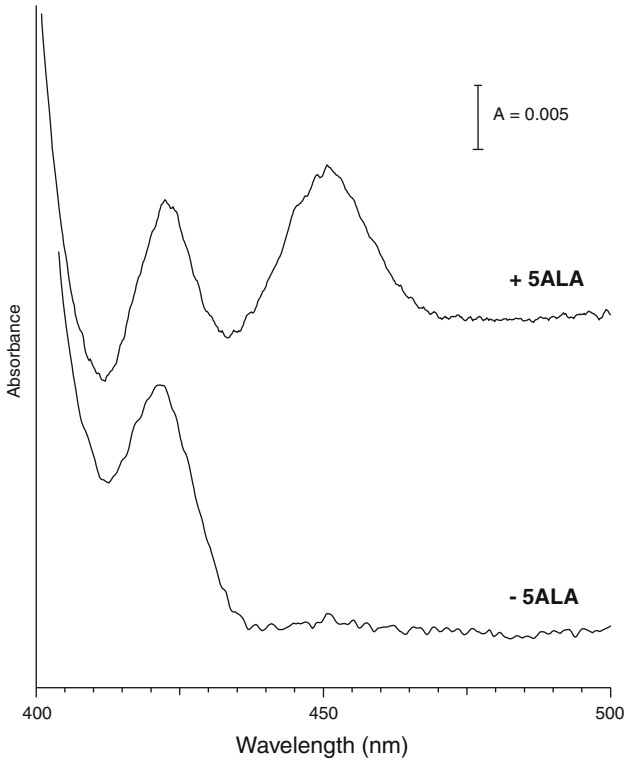

Fig. 2 Effects of 5ALA on heterologous expression of CYP5140A2. CO difference spectra were observed from transformants grown in culture media containing (+ALA) or lacking (−ALA) 5-aminolevulinic acid

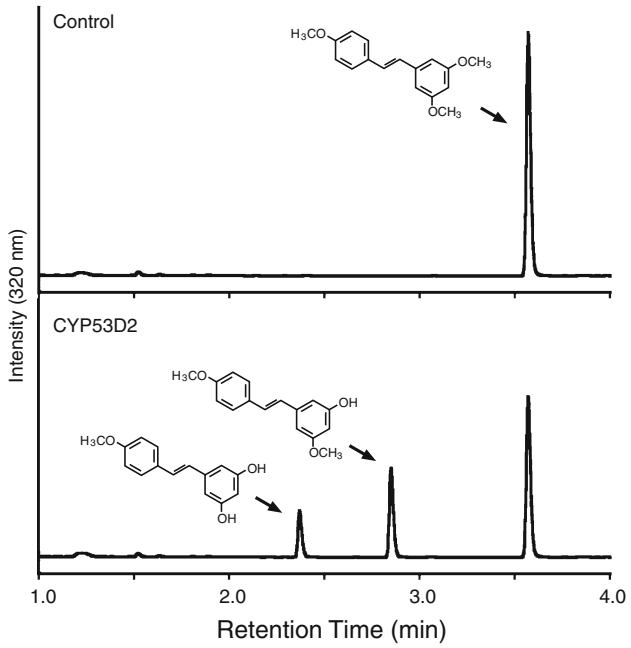

Fig. 3 HPLC analysis of bioconversion of 3,4,5′-trimethoxy-*trans*-stilbene by CYP53D2v2

encourage further research to better understand the sequence–structure–function relationships of the CYP53 family. In addition to their biological roles, it would be of interest to utilize PpCYPs for biotechnological applications, such as the production of rare and/or value-added stilbenoids (Roupe et al. 2006; Rimando and Suh 2008). A wide variety of applied researches are now under way.

We have provided a brief overview showing the phylogenetic relationships and functions of PpCYPs. Figure 4 shows a phylogenetic tree combined with functional information relating to PpCYPs. A bioconversion experiment revealed catalytic activities of CYP5139 family against aromatic compounds such as 7-ethoxycoumarin, carbazole, phenanthrene, and/or stilbene (Fig. 4 and Table 3). The ability of the CYP5139 family to convert 7-ethoxycoumarin has been previously demonstrated using CYP5139A1 from *P. chrysosporium*; this is the orphan PcCYP categorized into the CYP5139 family (Hirosue et al. 2011). These results would suggest that CYP5139 family recognize aromatic ring(s) for substrate binding. CYP512N6v1, CYP512N6v2, and CYP512P showed catalytic activity against the provisional substrates, testosterones. Several PcCYPs assigned into the CYP512 family but belonging to different subfamilies (CYP512C1, E1, F1, and G1) have been shown to convert steroid compounds. Combining the catalytic activities of PpCYPs and PcCYPs, it can be thus hypothesized that the natural substrate(s) of the CYP512 family may be structurally related to steroid compounds. The catalytic potentials of CYP512N6v1, CYP512N6v2, and CYP512P to convert both testosterone and dehydroabietic acid highlight the structural similarity of steroids and abietane diterpenoids. In addition, CYP5150D1, CYP5027B1, and CYP5350B2v1 showed multifunctional properties against a series of polycyclic aromatic hydrocarbons (PAHs), such as anthracene, carbazole, phenanthrene, and pyrene. The versatile functions would play important roles, at least in part, in fungal metabolic systems involved in xenobiotic detoxification. Although further investigation should aim to identify reaction products, our functionomic survey provides novel insights into the catalytic potentials of PpCYPs that will open the door for advanced fungal biology and biotechnology.

In conclusion, we elucidated the molecular diversity of PpCYPs using bioinformatic and experimental approaches. A genome-wide survey highlighted the unique evolutionary histories of P450s in basidiomycetes. In addition, we constructed a functional library that is potentially useful for comprehensive functional screening. A functionomic approach resulted in characterization of novel catalytic properties of PpCYPs. A compilation of both molecular and functional information relating to PpCYPs will help to facilitate the study of fungal biology and applied biotechnology.

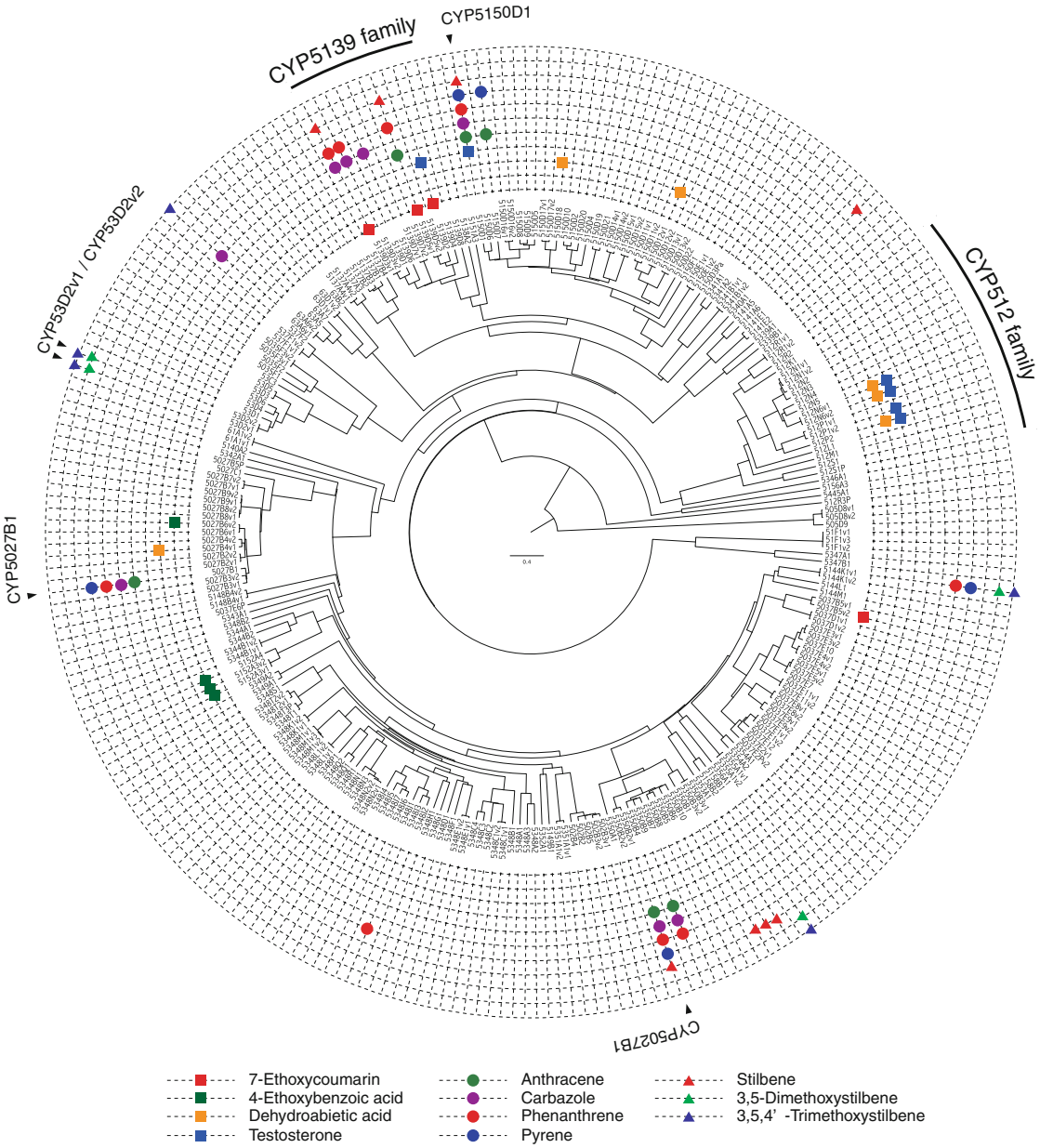

Fig. 4 Phylogenetic relationships and functions of PpCYPs. Multiple alignment of PpCYPs was carried out using the ClustalW program. The phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean, with the Jones-Taylor-Thornton matrix using PHYLIP software and visualized using the FigTree program. Catalytic potentials of PpCYPs are represented on the *concentric circles*

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