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Insight into functional diversity of cytochrome P450 in the white-rot basidiomycete *Phanerochaete chrysosporium*: Involvement of versatile monooxygenase

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ABSTRACT

To elucidate functional diversity of cytochrome P450 monooxygenases from the white-rot basidiomycete *Phanerochaete chrysosporium* (PcCYPs), we conducted a comprehensive functional screening using a wide variety of compounds. A functionomic survey resulted in characterization of novel PcCYP functions and discovery of versatile PcCYPs that exhibit broad substrate profiles. These results suggested that multifunctional properties of the versatile PcCYPs would play crucial roles in diversification of fungal metabolic systems involved in xenobiotic detoxification. To our knowledge, this is the first report describing multifunctional properties of versatile P450s from the fungal kingdom. An increased compilation of PcCYP functions will facilitate a thorough understanding of metabolic diversity in basidiomycetes and provide new insights that could also expedite practical applications in the biotechnology sector.

1. Introduction

White-rot basidiomycetes are the only organisms known to be responsible for the complete mineralization of woody components including lignin, which is one of the most recalcitrant biomaterials on Earth [1–4]. White-rot basidiomycetes commonly share unique extracellular enzymes such as lignin, manganese peroxidases, and laccase. These enzymes facilitate decomposition of polymeric lignin via non-specific oxidation, resulting in the formation of a wide variety of aromatic fragments [3–6]. Since the aromatic fragments are further metabolized and mineralized intracellularly, basidiomycetes should possess versatile intracellular metabolic systems to achieve superior ligninolytic efficiency. Besides studies on the ligninolytic system, utilization of basidiomycetes and/or their metabolic systems for a variety of applications has attracted much research attention [7–13].

Cytochromes P450 (P450s) constitute a large superfamily of heme-containing monooxygenases, which are distributed in a wide variety of organisms. The vast majority of P450s are thought to have emerged and diversified during the evolution of individual organisms. Therefore, a comprehensive survey of P450 functions is valuable to better understand metabolic diversity in each organism, especially of secondary metabolic pathways such as detoxification of xenobiotics and synthesis of secondary metabolites [14–16]. As well as the biological impacts of P450s, utilization of their catalytic functions in the biotechnology sector is of interest because of their catalytic advantages for *regio*- and *stereo*-specific hydroxylation [17–21]. Recently, a large-scale divergence of P450s within the fungal kingdom has been explored [22–26]; however, the majority of catalytic potentials and physiological functions of fungal P450s still remain obscure. A rational comprehensive approach towards elucidating catalytic potentials and utilities of P450s is therefore required to facilitate advanced research on both their basic biology and applied biotechnology.

Molecular diversity of P450s in the white-rot basidiomycete *Phanerochaete chrysosporium* (PcCYP) has been elucidated by whole-genome sequencing [23,27], in which 154 PcCYP genes were identified (see also Supplementary information). Recently, we constructed a functional library of PcCYPs in which 120 isoforms are coexpressed with yeast cytochrome P450 oxidoreductase (CPR) in *Saccharomyces cerevisiae*. In a previous study, we applied the functional library for target-driven screening of PcCYPs capable of converting dibenzo-*p*-dioxin [28], anthracene [29], flavone [30], and naringenin [31]; consequently, we proposed biotechnological

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applications such as PcCYP-based bioremediation of environmental pollutants and production of value-added flavonoids.

Thus, the functional screening system is a robust method to elucidate catalytic potentials of PcCYPs and a powerful tool to achieve a thorough understanding of metabolic diversity and capability of white-rot basidiomycetes. Herein, we demonstrate a wide range of PcCYP functions explored by comprehensive functional screening using structurally varied compounds, such as steroids, pharmacochemicals, petrochemicals, and plant-related compounds. A functionomic survey resulted in characterization of novel PcCYP functions and discovery of versatile PcCYPs that exhibit broad substrate profiles. The multifunctional properties of versatile PcCYPs suggested their physiological impacts on the fungal metabolic system involved in xenobiotic detoxification. Versatile P450s from the fungal kingdom are reported for the first time.

2. Materials and methods

2.1. Chemicals

Benzoic acid, biphenyl, carbazole, cinnamic acid, dibenzofuran, flavone, fluorene, and naphthalene were purchased from Sigma Aldrich (Tokyo, Japan). Dibenzothiophene and 4-ethoxybenzoic acid were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Compactin, dehydroabietic acid, diclofenac, 7-ethoxycoumarin, (*S*)-naproxen, progesterone, and testosterone 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 (5ALA) 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).

2.2. Functional characterization of PcCYPs

Recombinant PcCYPs were heterologously expressed in *S. cerevisiae* strain AH22 (see Supplementary information). A single colony of a positive transformant was inoculated into 0.5 mL synthetic dextrose liquid (SDL) medium (8% glucose, 2.68% yeast nitrogen base without amino acids, 0.01% DO supplement without Leu, and 0.5 mM 5ALA), and 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 SDL medium containing substrate (0.5 mM), and incubated in a Micro Bio Shaker (TAITEC) at 28 °C. After incubation for 2 days, reactions were stopped by the addition of methanol/acetone (0.5 mL). After removal of cell debris by centrifugation at 1,300*g* and filtration (0.45 μm, Whatman), metabolic products were analyzed by high-performance liquid chromatography (HPLC). 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), gas chromatography–mass spectrometry (GC–MS), and/or ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectrometry.

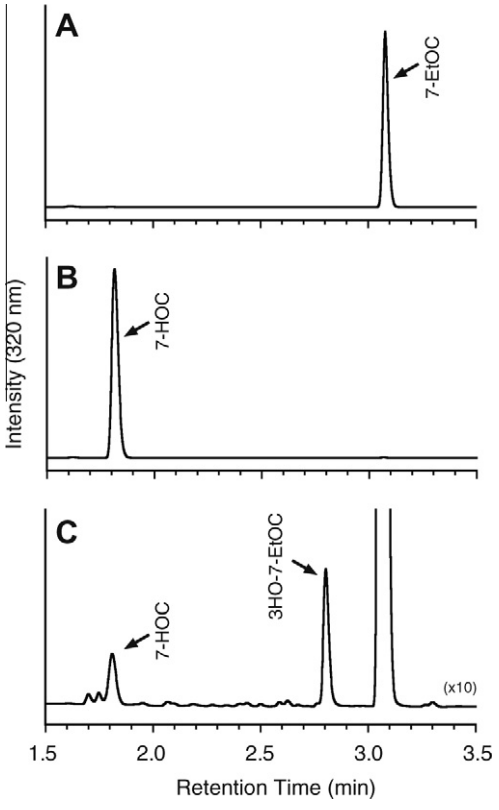

Fig. 1. HPLC analysis of 7-ethoxycoumarin conversion catalyzed by PcCYPs. Metabolic products observed from *S. cerevisiae* expressing (A) no PcCYP, (B) PcCYP1c, and (C) PcCYP38a were analyzed by HPLC at 320 nm. The chromatogram for PcCYP38a is plotted as a 10-fold expanded scale. 7-EtOC, 7-HOC, and 3-HO-7EtOC represent 7-ethoxycoumarin, 7-hydroxycoumarin, and 3-hydroxy-7-ethoxycouamrin, respectively.

Table 1
Catalytic properties of *P. chrysosporium* cytochrome P450 monooxygenases.

Substrate	Reaction	PcCYP
Steroids		
Testosterone	2β-Hydroxylation	16g, 24q, 81b, 142c
	6α-Hydroxylation	154a
	6β-Hydroxylation	15a, 15b, 30a, 154a
	7β-Hydroxylation	16g
	11β-Hydroxylation	15a, 16d, 16g, 30a, 81b, 121a, 142c
	12β-Hydroxylation	15a, 16g, 24q, 154a
Progesterone	Uncharacterized	15a, 15b, 16g, 24q, 30a, 81b, 142c, 154a
Petrochemicals		
Carbazole	3-Hydroxylation	5b, 11a, 16c, 16g, 81b, 121a, 128a, 142c
Dibenzofuran	2-Hydroxylation	11a, 16g, 121a, 142c
Fluorene	9-Hydroxylation	16g, 121a, 142c
Dibenzothiophene	S-Oxidation	5b, 11a, 15a, 16c, 16d, 16g, 38a, 50c, 121a, 142c
	2-Hydroxylation	11a, 16g, 121a, 142c
Biphenyl	4-Hydroxylation	11a, 65a, 83b, 121a, 142c
Naphthalene	1-Hydroxylation	16g, 38a, 65a, 81b, 121a, 128a, 142c
	2-Hydroxylation	16g, 81b, 121a, 128a, 142c
Plant-related compounds		
7-Ethoxycoumarin	O-Deethylation	1b, 1c, 1d, 5b, 11a, 16g, 16d, 38a, 50c, 65a, 81b, 121a, 142c
	3-Hydroxylation	16d, 38a, 81b, 142c
4-Ethoxybenzoic acid	O-Deethylation	16g, 121a, 142c
Dehydroabietic acid	Uncharacterized	30d, 54b, 66a, 81b, 121a, 142c
Flavone	Uncharacterized	1a, 50c, 66a, 73d, 79c, 81b, 121a, 142c
Pharmacochemicals		
Diclofenac	4′-Hydroxylation	66a, 121a, 142c
Compactin	Uncharacterized	16g, 83a
Naproxen	Uncharacterized	12a, 38a, 54b, 81b, 97a, 121a, 142c

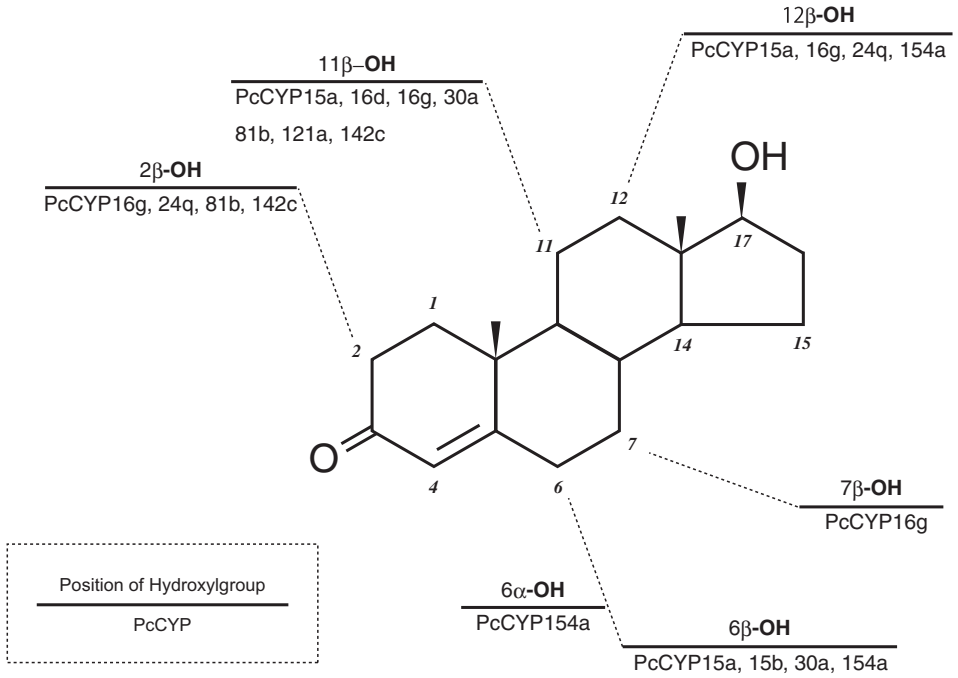

Fig. 2. Summary of catalytic potentials of PcCYPs converting testosterone.

2.3. Instrumentation

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 Shimapack 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^{-1}. 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.

3. Results and discussion

3.1. Relevance of the functional screening system

Recently, we identified 154 PcCYPs from the whole-genome sequence and isolated 120 full-length cDNAs including nine splicing variants by RT-PCR (Fig. S1 and S2; Table S1; see also Supplementary information). The isolated cDNAs showed significant sequence identity to their orthologs in *P. chrysosporium* strain RP78, the model fungus used for the *P. chrysosporium* genome project [27]; nevertheless, there are several nucleotide substitutions in some of the isolated genes presumably attributed to polymorphisms in this species. Using the isolated cDNAs, recombinant enzymes have been successfully coexpressed CPR in *S. cerevisiae*. To date, we have confirmed high-level expression of 70 PcCYPs in the yeast cells based on carbon monoxide (CO) difference spectra unique for active P450 species (Fig. S3; see also Supplementary information).

To achieve rapid functional screening, each transformant was separately inoculated into SDL medium (0.5 mL) and grown in 96 deep-well plates with a variety of compounds as substrate. Fig. 1 depicts an example of a bioconversion experiment using 7-ethoxycoumarin, which is a widely used prototypic substrate for P450 enzymes active in foreign compounds metabolism. When 7-ethoxycoumarin was incubated with recombinant cells expressing PcCYP1c, product formation was clearly demonstrated by HPLC analysis. The product was identified as 7-hydroxycoumarin by comparison of the retention time with GC and HPLC and mass fragmentation patterns with an authentic standard. Interestingly, catalytic conversion of 7-ethoxycoumarin to 7-hydroxycoumarin was also demonstrated by several transformants for which we could not confirm PcCYP expression by spectroscopic analysis. These results highlight the potential utility of our screening system. A functional survey revealed 7-ethoxycoumarin *O*-deethylation activity by 13 PcCYPs (Table 1). In addition to the *O*-deethylation reaction, PcCYP16d, 38a, 81b, and 142c catalyzed a hydroxylation reaction (Fig. 1C). The ^{1}H-NMR spectrum obtained from the product was identical to that of 3-hydroxy-7-ethoxycoumarin, as reported previously [32]. Although the alternative activity of *O*-deethylation and 3-hydroxylation of 7-ethoxycoumarin is reported for a human drug metabolizing P450 (CYP2A6) and engineered bacterial CYP102 [32,33], multiple product formation from 7-ethoxycoumarin by a fungal P450 was demonstrated for the first time in this study.

3.2. Functionomic survey of PcCYPs

To obtain further information about PcCYP functions, we examined bioconversion of structurally varied compounds such as steroids, pharmacochemicals, petrochemicals, and plant-related compounds. When functional screening was applied for testosterone, 10 PcCYPs showed catalytic activity and seven different products were observed (Fig. 2). Among the 10 PcCYPs, eight species also showed catalytic activity against progesterone (Table 1). Substantial activities against the provisional substrate, testosterone and progesterone, might indicate that the natural substrate(s) are structurally related to the steroids. Specifically, one can as-

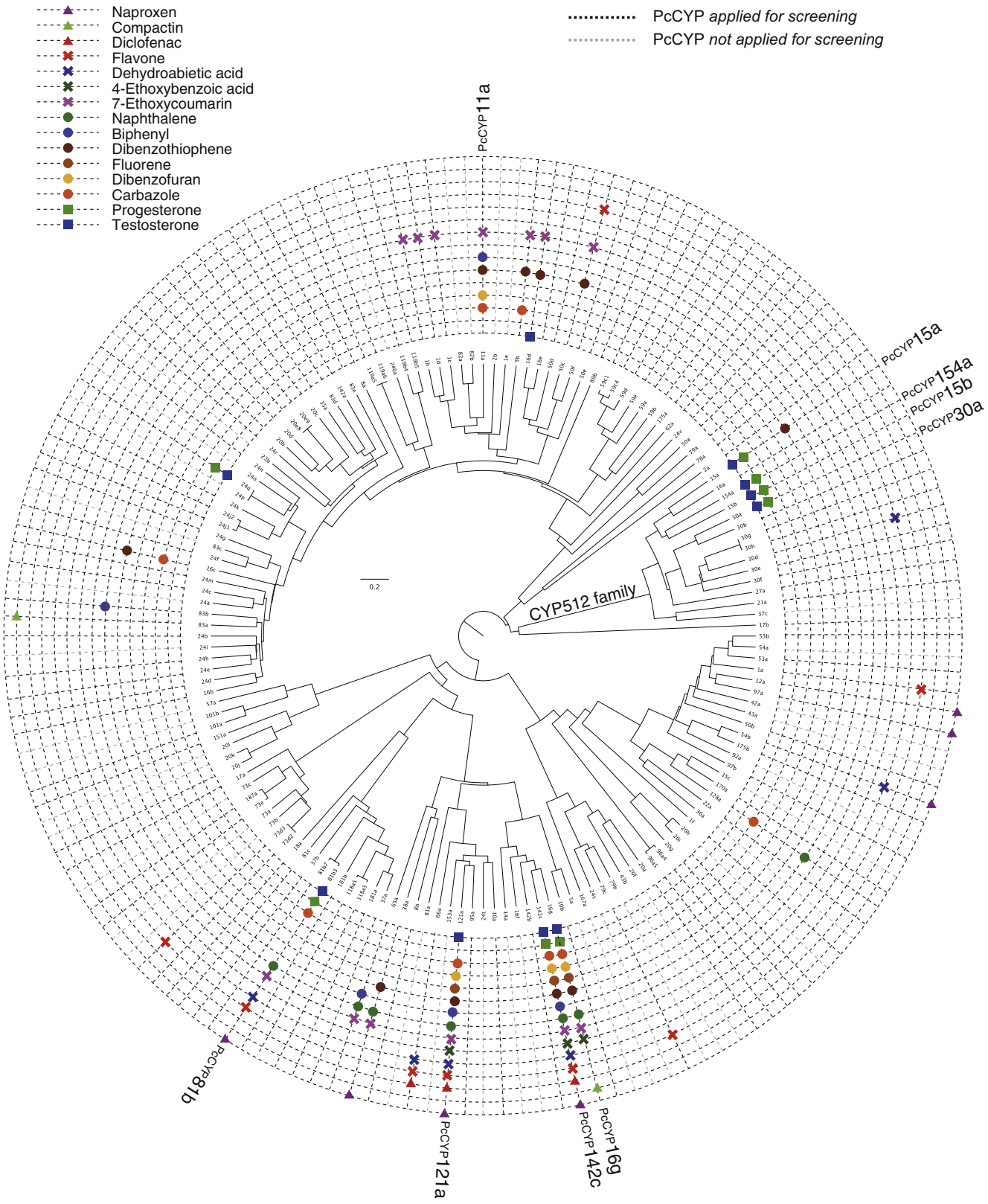

Fig. 3. Phylogenetic relationships and functions of PcCYPs. Multiple alignment of PcCYPs was carried out using the ClustalW program. The phylogenetic tree was constructed by the Unweighted Pair Group method with Arithmetic Mean method with the Jones–Taylor–Thornton matrix using PHYLIP software, and visualized using the FigTree program. Catalytic potentials of PcCYPs are depicted on the concentric-circles.

sume that some PcCYPs might be linked to biosynthetic pathways of secondary metabolites such as fungal steroids and/or lanostane triterpenoids. In addition, it was noteworthy that catalytic activities against testosterone and progesterone were widely distributed in the CYP512 family (e.g. PcCYP15a, 15b, 30a, and 154a) (Fig. 3), suggesting the CYP512 family might play important roles

in diversification of fungal steroidogenesis. Because of the pharmacological importance of the lanostane triterpenoids [34–36], the screening results provide a clear direction for further research such as metabolic engineering to produce value-added triterpenoids.

We further investigated catalytic potentials of PcCYPs using aromatic petrochemicals and pharmacochemicals, which are known to be potent substrates of a series of P450s. Through functional screening, we identified a number of PcCYPs that promote the catalytic conversion of carbazole, dibenzofuran, dibenzothiophene, fluorene, biphenyl, and naphthalene (Table 1), suggesting that basidiomycetes possess alternative metabolic pathways to degrade xenobiotic compounds. In addition, we previously reported the potential activities of several PcCYPs converting hazardous chemicals such as dibenzo-*p*-dioxins and anthracene [28,29]. These results supported the earlier notions that basidiomycetes are capable of metabolizing a wide variety of aromatic petrochemicals and environmental pollutants via P450-mediated reactions [7,8,11]. The white-rot basidiomycetes *Phanerochaete sordida* and *Trametes versicolor* are capable of degrading the pharmacochemical diclofenac via intermediate formation of 4′-hydroxydiclofenac [37,38]. When diclofenac was used as a substrate in the present study, PcCYP66a, 121a, and 142c produced 4′-hydroxydiclofenac (Fig. S4 and Table 1). Although it should be further investigated whether other basidiomycetes possess their orthologs, the screening results could contribute to a better understanding of the biodegradation mechanisms. In addition, substantial PcCYP activities against compactin and naproxen were also demonstrated for the first time (Table 1), being possible candidates involved in the fungal metabolic system [39,40]. The screening results also highlighted potential activities against plant-related compounds such as dehydroabietic acid, indicating that biodegradation of woody biomass, at least in part, is associated with P450-dependent reactions. The catalytic potentials of PcCYPs revealed in this study are summarized in Table 1.

3.3. Multiple functions of versatile P450s

A functionomic approach revealed the diversity of P450-mediated reactions in *P. chrysosporium*. Functional information for each PcCYP is helpful to achieve a thorough understanding of the fascinating biology of white-rot basidiomycetes that could also be applied in the biotechnology sector, such as bioremediation, biomass refinery, and production of value-added chemicals. The functionomic approach provided a clear picture that the metabolic diversity of *P. chrysosporium* is, at least in part, associated with versatile P450s, in particular PcCYP11a, 16g, 81b, 121a, and 142c, that showed broad substrate profiles against structurally varied compounds. It is also reported that PcCYP16g is capable of converting phenanthrene to three different products, namely 3-, 4-, and 9-phenantrol, consistent with its versatile activities [41]. The multifunctional properties in single P450 species could be physiologically linked to the xenobiotic metabolism system in fungi, similar to mammalian drug-metabolizing P450s. A series of human P450s involved in xenobiotic metabolism are capable of converting steroid compounds [14]. Interestingly, some PcCYPs (16g, 81b, 121a, and 142c) showed substantial activities against steroids as well as a variety of aromatic compounds (Fig. 3), indicating they have flexible substrate recognition capacity. However, PcCYP11 showed no activities against testosterone and progesterone despite its broad substrate profile against aromatic petrochemicals. Kasai et al. [28] showed that the catalytic potential of PcCYP11a against dibenzo-*p*-dioxins is significantly higher than that of other PcCYPs. Thus, the evolutionary divergence of PcCYP11a might have been directed at metabolism of aromatic compound(s).

Based on phylogenetic analysis, it was assumed that the multifunctional PcCYPs have emerged from different ancestral genes during fungal evolution (Fig. 3); in fact, no significant sequence similarity was observed between PcCYP11a, 16g, 81b, 121a, and 142c, except for PcCYP16g and PcCYP142c (64% identity). These results indicate that molecular evolution of basidiomycetous P450 has been, at least in part, vigorously driven by survival strategies to provide a superior metabolic system to degrade exogenous chemicals, presumably involving plant-related compounds including lignin and/or its derivatives. It is thus of great interest to undertake further basic studies on sequence–structure–function relationships within the versatile fungal P450s.

In conclusion, we elucidated novel catalytic potentials of numerous PcCYPs using a wide variety of substrates. A functionomic approach resulted in discovery of versatile P450s from *P. chrysosporium*. The multifunctional properties of versatile P450s would play important roles in fungal metabolic systems involved in xenobiotic detoxification.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.121.

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