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Comprehensive Survey of Cytochrome P450 Function from *Aspergillus oryzae*

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Research aims

Cytochromes P450 (P450s) constitute a large superfamily of heme-containing monooxygenases, which are distributed in a wide variety of organisms[1]. The vast majority of P450s are thought to have specifically emerged and individually diversified during evolution of each organism, implying P450s play crucial roles in specialization to meet the metabolic requirements of each organism, especially secondary metabolic pathways such as detoxification of xenobiotics and synthesis of secondary metabolites. As well as the biological impacts of P450s, it is of great interest to utilize their catalytic functions in biotechnology processes. Within the last few years, the sequence database of P450s has enlarged exponentially, and continues to increase. Although a compilation of P450 sequences is compulsory to increase understanding of metabolic diversity and evolutionary history of living organisms, their biological roles and functions are still poorly understood.

A wide variety of filamentous fungi are used to produce economically valuable consumer items. The filamentous fungus *Aspergillus oryzae* is one of the most widely used microorganisms, and has been used for more than 1000 years in Japanese fermentation industries to produce indigenous products such as *sake* (rice wine), *miso* (soybean paste), and *shoyu* (soy sauce). Besides fermentation technologies, many application studies have been focused on the utilization of *A. oryzae* for production of recombinant enzymes and primary and secondary metabolites. Recently, the whole genomic sequence of *A. oryzae* (strain RIB40) was determined and made available to the public[2]. The increased gene number in *A. oryzae* is mainly due to a gain of extra genes involved in secondary metabolic pathways, including P450s, suggesting that *A. oryzae* has unique metabolic processes that are absent in other *Aspergillus* species.

In the present study, we explored the molecular diversity of *A. oryzae* P450s (AoCYPs) by bioinformatic genome analysis and experimental validation. Furthermore, we developed a comprehensive functional screening system of AoCYPs in which 121 isoforms were co-expressed with yeast NADPH-P450 reductase in *Saccharomyces cerevisiae*. The functional screening system promises rapid and comprehensive characterization of the catalytic potential of AoCYPs. Using the functional screening system, a series of catalytic properties of AoCYPs were characterized and suggested to be useful for bioindustrial applications.

Methods

Molecular characterization and cDNA isolation of AoCYPs

Possible coding sequences for AoCYPs were searched against the *A. oryzae* genome database at the National Institute of Technology and Evaluation (NITE) based upon sequence similarity to known P450s[3]. 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. For gene amplification by RT-PCR, total RNA was isolated from the fungal cells grown in synthetic culture media containing 1% glucose and 1.2 mM ammonium tartrate[4,5]. PCR amplifications were carried out using gene-specific primers designed to anneal to 5′- and 3′-untranslated regions; basically, to the 20–30 bp upstream or downstream flanking sequence from the putative start and stop codons. The amplified cDNAs were cloned into pUC18 plasmid and sequenced with an automated DNA Sequencer.

Construction of a functional screening system

The isolated cDNA of AoCYPs were cloned into yeast expression plasmid pGYR, which allows co-expression of NADPH-cytochrome P450 reductase and P450s[6]. The expression plasmid harboring cDNA was transformed into *S. cerevisiae* AH22. A positive transformant of *S. cerevisiae* was selected on synthetic dextrose (SD) agar plate (2% glucose, 0.67% yeast nitrogen base without amino acid, 20 mg/L L-histidine, 1.5% agar). A single colony of transformant was then inoculated in 4×SD liquid medium (0.5 ml) and simultaneously grown using 96-deep-well plates. After 4-days incubation, transformants were harvested by centrifugation and resuspended in 2 ml potassium phosphate buffer (10 mM, pH 7.0) containing 10% glycerol. The 96-well plates were stored at −80°C until use. Then, 20 µl solution containing transformants were simultaneously inoculated into 0.5 mL of 4×SD liquid media supplemented with 0.5 mM substrate. The plates were further incubated (12,000 rpm, 28 °C) for bioconversion. After 2-days incubation, reactions were stopped by an addition of methanol/acetone (0.5 ml). After removal of cell debris, metabolic products were analyzed by HPLC. If necessary, the metabolic products were extracted by ethyl acetate, purified by preparative HPLC, and analyzed by GC-MS, LC-

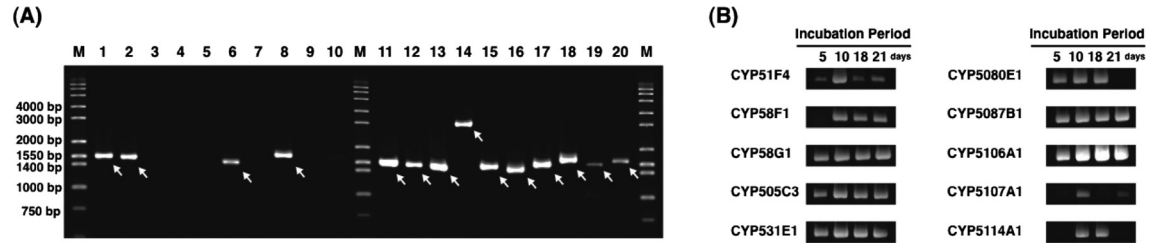

Fig. 1. Transcriptomic survey of AoCYPs encoded on chromosome VI.
A; Effects of culture conditions on gene expression of AoCYPs. RT-PCR was carried out using total RNA extracted from *A. oryzae* grown for 5-day in YPD liquid culture medium (Lane 1 to 10) or 10-day in a liquid culture medium (Lane 11 to 20). cDNA fragments; CYP51F4 (Lane 1 and 11), CYP58F1 (Lane 2 and 12), CYP58G1 Lane (Lane 3 and 13), CYP505C3 (Lane 4 and 14), CYP531E1 (Lane 5 and 15), CYP5080E1 (Lane 6 and 16), CYP5087B1 (Lane 7 and 17), CYP5106A1 (Lane 8 and 18), CYP5107A1 (Lane 9 and 19), and CYP5114A1 (Lane 10 and 20).
B; Time course of gene expression in a nitrogen-limited synthetic liquid culture medium. RT-PCR was performed with total RNA individually extracted from A. oryzae grown for 5-, 10-, 18-, or 21-day in a synthetic liquid culture medium.

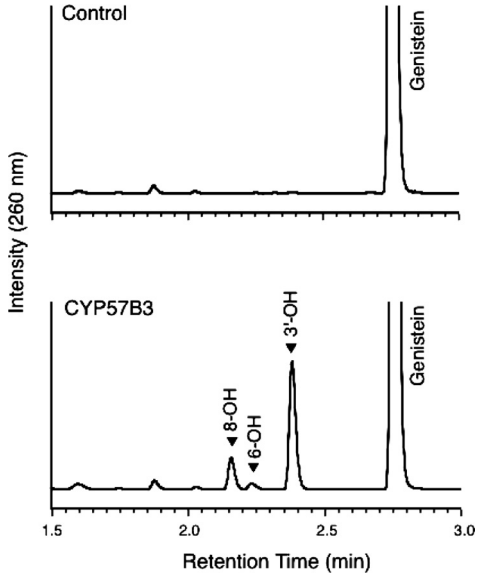

Fig. 2. Functional screening of AoCYP catalyzing genistein conversion.
Metabolic products from *S. cerevisiae* expressing CYP57B3 were analyzed by HPLC. 8-OH, 6-OH, and 3′-OH indicate 8-hydroxygenistein, 6-hydroxygenistein, and 3′-hydroxygenistein, respectively.

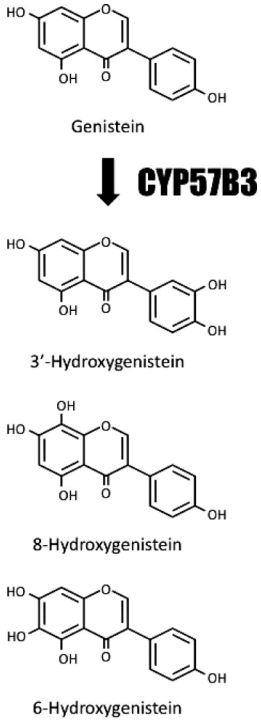

Fig. 3. Catalytic activity of CYP57B3 against genistein.

MS, and/or NMR.

Results

Molecular characterization and cDNA isolation of AoCYPs

The whole-genome sequence of *A. oryzae* strain RIB40 was released recently. According to the public database, there are several candidate genes assigned to P450s. However, some candidates have low sequence similarity to known genes for P450s but significantly higher similarity to other proteins, suggesting that annotational errors may be involved (data not shown). Several candidates have unexpected truncations of their N- and/or C-terminal sequence (s). Therefore, we further refined gene annotation accuracy based on the following sequence features: (i) conservation of F-x-x-G-x-x-x-C-x-G in the heme-binding domain, (ii) conservation of E-x-x-R in the K-helix, (iii) A/G-G-x-x-T at the center of the I-helix, and (iv) a hydrophobic transmembrane domain (TMD) at the N-terminal region. After searching the database, 155 putative genes of P450 were identified from the whole-genome sequence. However, sequence deletions and/or inframe stop codon(s) were found in 13 genes, suggesting that they are possibly pseudogenes that have originated from gene reorganizations and/or single mutations during fungal evolution. Therefore, 142 AoCYPs from the *A. oryzae* genome were selected for further investigation.

To elucidate transcriptional capability of AoCYPs, we performed gene amplification by RT-PCR. Expression profiles of genes involved in secondary metabolic systems of *A. oryzae* are very likely to be affected by cultivation conditions. Therefore, we used YPD and synthetic liquid culture media for fungal growth[4,5]. Figure 1 shows expression profiles of AoCYPs encoded on chromosome VI. When fungi were grown in YPD liquid culture medium, many genes were not amplified by RT-PCR, whereas only some genes were confirmed to be expressed (Fig. 1A). In contrast, a series of AoCYP genes were strongly expressed when *A. oryzae* was grown in a nutrient-limited synthetic liquid culture medium (Fig. 1A), suggesting that transcriptional regulation of AoCYP responds to nutrient limitation or starvation. Although AoCYPs encoded on chromosome VI

Table 1. Catalytic potentials of AoCYPs against various compounds

Substrate	AoCYP
7-Ethoxycoumarin	CYP57B3, CYP5061B5, CYP5075A1, CYP5078A5, CYP531E1, CYP620H9, CYP5080B2,
Genistein	CYP57B3
Daizein	CYP57B3
Naringenin	CYP57B3, CYP62C2
Testosterone	CYP5061B5, CYP5068A2, CYP62A1, CYP65AC2, CYP630A3

showed different time course of gene expression, a significant expression level of AoCYPs appeared after 5 days incubation and continued until 21 days incubation (Fig. 1B). Therefore, amplification and isolation of cDNAs by RT-PCR were carried out using a RNA cocktail which was prepared by mixing total RNA obtained from 5-, 10-, 18-, and 21-day-old mycelia grown in a synthetic liquid culture medium. Using an RT-PCR technique, we determined transcriptional capabilities of 133 AoCYPs experimentally. To our knowledge, this is the first report of experimental validation of AoCYPs expression, and our results provide evidence that a series of P450s can be expressed in ascomycetous fungi. So far, we isolated 121 full-length cDNAs encoding a mature open reading frame.

Functional Survey of AoCYPs

The isolated cDNA of 121 AoCYPs were cloned into yeast expression plasmid and transformed into *S. cerevisiae*. To elucidate heterologous expression of recombinant AoCYPs, carbon monoxide (CO) difference spectra of each transformant were analyzed. After 2–4 days incubation in SD media, typical CO-difference spectra for P450 species were observed with a number of transformants[7], indicating that certain recombinant AoCYP enzymes were successfully expressed in *S. cerevisiae*. Based upon CO-difference spectra, we confirmed significant expression levels for 70 AoCYPs. Then we initiated functional screening of AoCYPs using a series of substrates. To develop a functional screening system, each transformant was separately inoculated in 4×SD liquid medium (0.5 ml) and simultaneously grown using 96-deep-well plates. Transformants grown in the 96-well plates could be easily replicated and used for further investigations. Then, transformants in the 96-well plates were inoculated in 4×SD liquid medium (0.5 mL) containing substrates and further incubated for bioconversion. When genistein was utilized as a substrate, significant product formations were achieved by *S. cerevisiae* expressing CYP57B3 (Fig. 2). No reaction proceeded with the recombinant cell expressing cytochrome P450 oxidoreductase without AoCYPs, indicating that the conversion of genistein was achieved by an AoCYP-dependent reaction. Using ^{1}H-NMR analysis, the products were identified as 8-hydroxy-, 6-hydroxy-, and 3′-hydroxygenistein. To the best of our knowledge, this is the first report describing catalytic function of CYP57B3 converting genistein to its hydroxylated products. Since isoflavonoids, including hydroxygenistein, are known to show pharmacological and biological activities, further experimental efforts would be encouraged to utilize CYP57B3 for industrial production of value-added isoflavonoids. Moreover, it has been known that hydroxygenisteins is involved in fermented soybean product[8]. Thus, CYP57B3 plays important roles in production of hydroxygenisteins during soybean fermentation. In this study, a series of catalytic potentials of AoCYPs were elucidated using various compounds (Table 1). Product identification and further screening are now under way.

Conclusion

We developed a functional screening system of AoCYPs in which 121 isoforms were co-expressed with yeast NADPH-P450 reductase in *Saccharomyces cerevisiae*. Using the screening system, novel catalytic functions of AoCYPs were experimentally elucidated. The functional screening system promises rapid and comprehensive characterization of the catalytic potential of AoCYPs. Thorough understanding of AoCYP functions will open the door for advanced fungal biology and biotechnology.

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