Heterologous Expression of *Rhizopus Oryzae* CYP509C12 Gene in *Rhizopus Nigricans* Enhances Reactive Oxygen Species Production and 11α-Hydroxylation Rate of 16α, 17-Epoxyprogesterone

Chaohui Shen¹, Xiyang Gao¹, Tao Li¹,², Jun Zhang³, Yuqian Gao¹, Liyou Qiu¹ and Guang Zhang¹

¹College of Life Sciences, Key Laboratory of Enzyme Engineering of Agricultural Microbiology, Ministry of Agriculture, Henan Agricultural University, Zhengzhou, China; ²Medicine and Nursing College, Sanmenxia Polytechnic Institute, Sanmenxia, China

ABSTRACT

The 11α-hydroxylation of 16α, 17-epoxyprogesterone (EP) catalyzed by *Rhizopus nigricans* is crucial for the steroid industry. However, lower conversion rate of the biohydroxylation restricts its potential industrial application. The 11α- steroid hydroxylase CYP509C12 from *R. oryzae* were reported to play a crucial role in the 11α-hydroxylation in recombinant fission yeast. In the present study, the CYP509C12 of *R. oryzae* (RoCYP) was introduced into *R. nigricans* using the liposome-mediated mycelial transformation. Heterologous expression of RoCYP resulted in increased fungal growth and improved intracellular reactive oxygen species content in *R. nigricans*. The H₂O₂ levels in RoCYP transformants were approximately 2-fold that of the *R. nigricans* wild type (RnWT) strain, with the superoxide dismutase activities increased approximately 45% and catalase activities decreased approximately 68%. Furthermore, the 11α-hydroxylation rates of EP in RoCYP transformants (C4, C6 and C9) were 39.7%, 38.3% and 38.7%, which were 12.1%, 8.2% and 9.4% higher than the rate of the RnWT strain, respectively. This paper investigated the effect of heterologous expression of RoCYP in *R. nigricans*, providing an effective genetic method to construct the engineered strains for steroid industry.

1. Introduction

Steroid compounds are terpenoids lipids containing the gonane nucleus of four cycloalkane rings [1]. In recent years, steroid hormones and their derivatives have been widely used in the pharmaceutical industry, mainly as immunosuppressive, anti-inflammatory, anti-rheumatic, prostagastional, diuretic, sedative, anabolic and contraceptive agents [2,3]. In addition, they are also used to treat hormone-dependent forms of breast and prostate cancer [1], osteoporosis, HIV infection and declared AIDS [4]. Currently, steroids constitute a vital part of the active ingredients in pharmaceuticals and intermediates used to produce medicines, and represent one of the largest sectors in the pharmaceutical industry [1].

Hydroxylation is one of the most important and commonly used reactions in the steroid industry, with the introduction of 11α-hydroxy to steroids by *Rhizopus* spp. being a great success in steroid biotransformation [1,5]. The 11α-hydroxylation performed by *R. nigricans* has been used in the pharmaceutical industry for more than a half century. However, the low conversion rate of 11α-hydroxylation conducted by *R. nigricans* is not sufficient for production scales [2].

The hydroxylation of the steroid skeleton structure is catalyzed by a family of enzymes, mainly the cytochrome P450s (CYPs) of the fungi [6]. The steroid hydroxylation systems in fungi are typical eukaryotic two-component systems that consist of a NADPH-CPR (cytochrome P450 reductase) and a cytochrome P450 monoxygenase [7,8]. The CYPs are external monoxygenases, in that they catalyze the incorporation of a single atom of molecular oxygen into a substrate with the concomitant reduction of the other atom to water [8]. The two main classes of CYPs are the mitochondrial/bacterial type and the microsomal type [7]. Microsomal CYPs are membrane bound and accept electrons from a microsomal CPR, which is an essential redox partner of CYPs, and whose function is to accept two electrons from NADPH and transfer them sequentially to CYPs [9,10]. The activities of CYP and CPR are crucial for various oxidation reactions performed by microsomal enzymes [11]. To date, many CYPs and CPRs have been cloned from fungal species, such as *R. oryzae* [12]. However, attempts to functionally characterize the purified or cloned steroid monoxygenases of *R. nigricans* have been unsuccessful so far.
The 11α-hydroxylation of 16α, 17-epoxyprogesterone (EP) with \textit{R. nigricans} (but not with \textit{R. oryzae}) is an essential and important step in the process to produce 11α-hydroxy-16α, 17-epoxyprogesterone (HEP), and for decades, this has been a crucial step in the production of glucocorticoids in China [9]. Improving the conversion rate of 11α-hydroxylation is of great importance for the pharmaceutical industry. The 11α-steroid hydroxylase CYP509C12 from \textit{R. oryzae} were reported to play a crucial role in the 11α-hydroxylation in recombinant fission yeast [12]. Considering the crucial role of CYPs in steroid hydroxylation systems, the function of CYP509C12 of \textit{R. oryzae} (RoCYP) was investigated in \textit{R. nigricans} in the present study.

2. Materials and methods

2.1. Strains and culture conditions

\textit{Rhizopus nigricans} LH21 from Henan Lihua Pharmaceutical (Anyang, Henan, China) was used as the wild type (RnWT) strain. \textit{Rhizopus oryzae} CGMCC3.4826 was purchased from the China General Microbiological Culture Collection Center (CGMCC). The regeneration complete medium (RCM) for transformant screening contained (per liter, pH 4.5): glucose, 30 g; corn meal, 25 g; silkworm meal, 2 g; and (NH4)2SO4, 1.6 g. \textit{Escherichia coli} (DH5x) was used for plasmid amplification and was grown in lysogeny broth containing ampicillin (100 µg/ml) or kanamycin (50 µg/ml) as needed.

2.2. Cloning of RoCYP from \textit{Rhizopus oryzae}

The sequence of CYP509C12 (RO3G-05077.1) of \textit{R. oryzae} were downloaded from the Broad Institute Database (http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/MultiHome.html, 7.9.2009) and was used to design primers for amplification of RoCYP. The cDNA was synthesized from the total RNA isolated from \textit{R. oryzae} treated with 300 µg/ml neomycin at 28°C for 3 days. Colonies on the plate were randomly picked, and were used as the putative RoCYP transformants.

2.3. Construction of fungal expression vectors

Plasmid pEGFP-C1 was used for the construction of expression vector (pC1-CYP) based on previously described study [13]. Briefly, the pMD19-T-CYP vector was digested with \textit{Age} I and \textit{Kpn} I, and then CYP fragments were inserted into the pEGFP-C1 vector at the corresponding restriction sites, yielding the final expression vector pC1-CYP (Figure 1).

2.4. Transformation of \textit{Rhizopus nigricans}

The pC1-CYP were introduced into \textit{R. nigricans} using liposome-mediated mycelial transformation as described previously [13]. Briefly, 50 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and 50 µl of 2 µg/ml pC1-CYP were mixed and placed on ice for 30 min. Then, 1 ml of the mycelial fragments suspension from \textit{R. nigricans} mycelia ground with 0.6 M mannitol were added into the mixture of pC1-CYP and Lipofectamine 2000, mixed and placed on ice for another 30 min. Finally, the 100 µl of the transformation solution were plated on RCM medium supplemented with 300 µg/ml neomycin, and cultivated at 28°C for 3 days. Colonies on the plate were randomly picked, and were used as the putative RoCYP transformants.

2.5. Transformants screening and subculture

The putative RoCYP transformants were subcultured on PDA plates containing 300 µg/ml neomycin at 28°C three times, followed by purification through single spore isolation on PDA plates with 150 µg/ml neomycin. The isolates obtained were the RoCYP transformants and were used for further analysis.

2.6. Transformants identification

The integration of RoCYP gene in the RoCYP transformants was confirmed by PCR and RT-PCR using

| Primer | Sequence (5’-3’) | Description |
|--------|-----------------|-------------|
| CYP-F  | ATGATGAAATGGGCTGAATTTGC | Get the full length of RoCYP gene |
| CYP-R  | TTAAATACCCGTTTTGGATAATTAGTCAA | Get RoCYP fragments for construction of pC1-CYP |
| CYP-AgeI-F | CGCTACCCGGTCGACCATGGGAAATGGGCTGAATTTGC | Detects the RoCYP expression |
| CYP-KpnI-R | GGGTATCCATTTAAACGCTTTGGAATATTAGTCAA | Detects the S6β1 expression |
| sqRT-CYP-F | TCTTGTGTCATCTGCTTCTG | |
| sqRT-s6β1-F | GCTTCAATTGCCAACGTA | |
| sqRT-s6β1-R | CTTCCGTGCTCTCCTG | |

Table 1. Oligonucleotide primers used.
their genomic DNA and cDNA synthesized from their total RNA as templates, respectively. The expected PCR product size was 1,581 bp for RoCYP transformants. In addition, semi-quantitative RT-PCR was further carried out to screen transformants as described previously [13]. The housekeeping gene Ssb1 (AY257252) was used as a control according to previous study [14].

2.7. ROS (reactive oxygen species) detection and enzymatic activity assays

The H$_2$O$_2$ content in the hyphae of RoCYP transformants from liquid PDA cultures was quantified by a commercial Hydrogen Peroxide assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). H$_2$O$_2$ bound to molybdenic acid forms a complex, measured at 405 nm, from which the content of H$_2$O$_2$ was calculated.

For the determination of enzymatic activity, mycelia of RoCYP transformants from liquid PDA cultures were ground in liquid N$_2$. Frozen mycelia (0.3 g) were homogenized in 5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 $g$ for 20 min at 4°C, and the supernatant was immediately used for the following enzymatic assays. The protein content was determined according to the Bradford method, using BSA as a standard. The superoxide dismutase (SOD) activity and catalase (CAT) activity were measured as previously described [15]. To assay the SOD activity, the inhibition of the photochemical reduction of NBT was monitored at 560 nm. One unit of SOD activity was defined as the amount of enzyme required for 50% inhibition of the reduction of NBT. The CAT activity was determined as the decomposition rate of H$_2$O$_2$ per minute at 240 nm as previously described [16].

2.8. Biotransformation reaction and HPLC measurements

The 1-ml spore solution (approximately $2 \times 10^8$ spores/ml) of the RnWT and RoCYP transformants was transferred into the fermentation medium (99 ml) in a 500-ml Erlenmeyer flask and cultured at 150 rpm for 22 h at 28°C. Then, same amount of wet mycelia (1.0 g) were collected and transferred into new fermentation medium, followed by adding 2.0 g of EP and incubating for another 43 h. At the end of the biotransformation, the reaction products were extracted from the cells by circumfluence with acetone, and quantitatively evaluated by HPLC as described previously [17]. The HPLC measurements were performed in Xianju Pharmaceutical Company Ltd. (Taizhou, Zhejiang, China) as described elsewhere [12]. The biotransformation yield was used to evaluate the evaluation of HEP production efficiency and was calculated as the ratio between the peak areas (A) of the chromatograms of HEP and EP:

$$\text{Biotransformation yield} = \frac{A(\text{HEP})}{A(\text{EP}) + A(\text{HEP})} \times 100$$

2.9. Statistical analysis

Data were expressed as the mean ± SE averaged from three independent sample measurements. Error bars represent standard deviations from the means of triplicates. The significance of samples was determined by analysis of variance, and differences between sample means were analyzed by one-way ANOVA and Tukey’s honest significant difference test ($p < 0.05$).
3. Results

3.1. RT-PCR amplification of the RoCYP gene

With the reverse-transcribed cDNA of *R. oryzae* as the template, the coding sequences of RoCYP (MH125208) were amplified successfully. The results of sequence alignment showed that the amino acid sequence similarity was 96.96% between RoCYP cloned and CYP509C12 reported previously [12], with the conserved regions as the same (Figure S1 in Supplementary Material). This result indicates that the RoCYP obtained is highly consistent with the reported sequences and could be used for the next experiments.

3.2. Characterization of RoCYP transformants

To investigate the effect of the RoCYP gene on *R. nigricans*, the overexpression vector pC1-CYP was constructed (Figure 1). Liposome-mediated mycelial transformation was used to introduce pC1-CYP into *R. nigricans*. Approximately 20 transformants were screened from the neomycin-resistant RCM plates and subcultured three times. Then, six transformants were randomly selected and used for further analysis.

Multiplex PCR and RT-PCR analysis were carried out to confirm the presence of RoCYP gene in the RoCYP transformants, respectively. The results showed that there were obvious bands for the target genes from the genomic DNA and cDNA of RoCYP transformants (Figure 2), while there were no bands for the RnWT strain. These results indicate that the RoCYP gene were randomly integrated into the genome of *R. nigricans* and could be transcribed. Moreover, three RoCYP transformants (C4, C6 and C9) were further screened for the next experiments using semi-quantitative RT-PCR, as the relative expression of RoCYP in these selected transformants was higher (Figure 3).

3.3. The fungal growth was increased in RoCYP transformants

For an examination of the effect of RoCYP on the vegetative growth of *R. nigricans*, the mycelial diameters of the RoCYP transformants were determined. On PDA plates, the mycelial diameters of C4, C6 and C9 were increased by 17.8%, 16.1% and 16.7%, compared with that of the RnWT strain (Figure 4), respectively. Consistent with this result, the biomass of C4, C6 and C9 in PDB were improved by 71.1%, 59.3% and 74.1% compared with those of the RnWT strain (Figure 4). These results indicate that RoCYP increases the fungal growth of *R. nigricans*.

3.4. The intracellular ROS content was improved in RoCYP transformants

CYPs mediate ROS generation and are the main source of ROS [18]. To investigate whether RoCYP affect the intracellular ROS content of *R. nigricans*, the intracellular H$_2$O$_2$ content of the RoCYP transformants was tested using a spectrophotometer. As shown in Figure 5(A), the H$_2$O$_2$ levels in C4, C6 and C9 were 1.8-, 2.1- and 1.8-
folder that of the RnWT strain, respectively. Moreover, the activity of two major antioxidant enzymes, SOD and CAT, was analyzed to further understand how RoCYP affects the ROS content. The SOD activities of C4, C6 and C9 were significantly increased by 38%, 50% and 47% compared with that of the RnWT strain, respectively (Figure 5(B)). However, the CAT activities of C4, C6 and C9 were 61%, 69% and 73% of the CAT activity of the RnWT strain (Figure 5(C)). These results imply that RoCYP increased the intracellular ROS content of R. nigricans.

3.5. The biotransformation abilities were higher in RoCYP transformants

The effect of RoCYP on the 11α-hydroxylation rate of R. nigricans was determined. With the addition of 2% EP in the fermentation medium, the biotransformation rates of C4 (39.7%), C6 (38.3%) and C9 (38.7%) were increased by 12.1%, 8.2% and 9.4%, compared with that of the RnWT strain, respectively (Figure 6). This result indicates that RoCYP has a promotion effect on the 11α-hydroxylation rate of R. nigricans.
4. Discussion

The lower conversion rate of the biohydroxylation is the bottleneck of 11α-hydroxylation of EP by *R. nigricans* which is used in steroid industry [9]. Cytochrome P450 monoxygenase was suggested to play a crucial role in steroid hydroxylation conducted by microorganisms [19,20]. Heterologous expression has become an efficient way to improve the conversion rate of steroid hydroxylation [1]. Here, the gene of 11α-steroid hydroxylase (*RoCYP*) was introduced into *R. nigricans*, and resulted in an enhancement of the 11α-hydroxylation rate of EP.

Researchers have explored and established various methods to improve the conversion rate of steroid hydroxylation, which could be sorted into two main categories: fermentation optimization and genetic modification. For fermentation optimization, conventional approaches to improve bioconversions were based on the micronization of substrate particles [21], surfactant-facilitated emulsification [22], the application of two-phase systems with organic solvents, liquid polymers, or cyclodextrins [5], as well as combinations of different methods [1]. For genetic modification, significant progress was made in the field of heterologous expression of steroidogenic P450 enzymes in microorganisms [11]. Co-expression of 11α-hydroxylase (CYP509C12) with its natural redox partner (*RoCPR1*) from *R. oryzae* in the fission yeast host showed a 7-fold enhancement in the 11α-hydroxylation rate compared with expression of CYP509C12 alone [12]. Soon after, the *RoCPR1* of *R. oryzae* was introduced into *R. nigricans*, and resulted in an improvement of the 11α-hydroxylation rate of EP [9]. Here, we introduced the *RoCYP* into *R. nigricans*, and the 11α-hydroxylation rate of *RoCYP* transformants was significantly increased by 8.2 ~ 12.1% at the addition of 2% EP, implying that *RoCYP* also has a promotion effect on the 11α-hydroxylation rate of EP in *R. nigricans*. Co-expression of *RoCYP* and *RoCPR1* in *R. nigricans* might further improve the 11α-hydroxylation rate, which should be explored in the future.

Numerous studies have suggested that CYP metabolism plays a crucial role in intracellular ROS generation. In the endoplasmic reticulum, the major source of ROS is the microsomal monoxygenase (MMO) system, whose main function is mixed-function oxygenation of xenobiotics and some endogenous substrates [23]. The MMO system also leads to the release of large amounts of ROS from the P450 enzyme, such as superoxide anion radical and H₂O₂ [24]. CYP2E1 is believed to contribute to ROS production in different pathophysiological situations among various CYPs [25,26]. Consistently, heterologous expression of *RoCYP* in *R. nigricans* resulted in an improvement of ROS production. CYP-mediated estrogen metabolites was reported to produce high ROS concentrations [27]. Similarly, the improved biotransformation abilities in *RoCYP* transformants accompanied the increased ROS accumulation in the present study, and there might be an association between the increased biotransformation rates and improved intracellular ROS content in *RoCYP* transformants. Nevertheless, the mechanisms of *RoCYP* regulating intracellular ROS species need to be examined in depth in the future.

In summary, the *RoCYP* was introduced into *R. nigricans* using liposome-mediated mycelial transformation, and the 11α-hydroxylation rates of the *RoCYP* transformants were significantly improved. To our knowledge, this is the first report of the overexpression of *RoCYP* in *R. nigricans*, providing an effective genetic method to improve the productivity of HEP.

Acknowledgements

We are grateful to Xianju Pharmaceutical Company Ltd. for their help in the study.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Natural Science Foundation of Henan Province [grant number 112300410115].

References

[1] Donova MV, Egorova OV. Microbial steroid transformations: current state and prospects. Appl Microbiol Biotechnol. 2012;94:1423–1447.
[2] Gao Q, Qiao Y, Shen Y, et al. Screening for strains with 11α-hydroxylase activity for 17α-hydroxy progestrone biotransformation. Steroids. 2017;124: 67–71.

[3] Žnidarič-Plazl P, Plazl I. Development of a continuous steroid biotransformation process and product extraction within microchannel system. Catal Today. 2010;157:315–320.

[4] Zubair MS, Al-Footy KO, Ayyad SE, et al. A review of steroids from Sarcophyton species. Nat Prod Res. 2016;30:869–879.

[5] Wu DX, Guan YX, Wang HQ, et al. 11α-Hydroxylation of 16α,17-epoxyprogesterone by Rhizopus nigricans in a biphasic ionic liquid aqueous system. Bioresour Technol. 2011;102:9368–9373.

[6] Kristan K, Rižner TL. Steroid-transforming enzymes in fungi. J Steroid Biochem Mol Biol. 2012;129:79–91.

[7] Hannemann F, Bichet A, Ewen KM, et al. Cytochrome P450 systems-biological variations of electron transport chains. Biochim Biophys Acta. 2007;170:330–344.

[8] Cook DJ, Finnigan JD, Cook K, et al. Cytochromes P450: history, classes, catalytic mechanism, and industrial application. Adv Protein Chem Struct Biol. 2016;105:105–126.

[9] Chen X, Luo X, Cao F, et al. Molecular cloning, expression of CPR gene from Rhizopus oryzae into Rhizopus nigricans and its application in the 11α-hydroxylation of 16α, 17-epoxy-progesterone. Enzyme Microb Tech. 2014;66:28–34.

[10] Yasukochi Y, Masters BS. Some properties of a detergent-solubilized NADPH-cytochrome c(cytochrome P-450) reductase purified by biospecific affinity chromatography. J Biol Chem. 1976;251: 5337–5344.

[11] Wei Y, Ang EL, Zhao H. Recent developments in the application of P450 based biocatalysts. Curr Opin Chem Biol. 2018;43:1–7.

[12] Petrić Š, Hakki T, Bernhardt R, et al. Discovery of a steroid 11α-hydroxylase from Rhizopus oryzae and its biotechnological application. J Biotechnol. 2010;150:428–437.

[13] Chai R, Zhang G, Sun Q, et al. Liposome-mediated mycelial transformation of filamentous fungi. Fungal Biol. 2013;117:577–583.

[14] Cernila B, Crešnar B, Breskvar K. Molecular characterization of a ribosome-associated Hsp70-homologous gene from Rhizomucor. Biochim Biophys Acta. 2003;1629:109–113.

[15] Singh PK, Wang WJ, Shrivastava AK. Cadmium-mediated morphological, biochemical and physiological tuning in three different Anabaena species. Aquat Toxicol. 2018;202:36–45.

[16] Xu J, Duan X, Yang J, et al. Enhanced reactive oxygen species scavenging by overproduction of superoxide dismutase and catalase delays postharvest physiological deterioration of cassava storage roots. Plant Physiol. 2013;161:1517–1528.

[17] Zhou H, Lu W, Wen J, et al. Kinetic analysis of 11α-hydroxylation of steroids by Rhizopus nigricans. J Mol Catal B Enzym. 2009;56:136–141.

[18] Bhattacharyya S, Sinha K, Sil P. Cytochrome P450s: mechanisms and biological implications in drug metabolism and its interaction with oxidative stress. Curr Drug Metab. 2015;15:719–742.

[19] Ahmed F, Williams RA, Smith KE. Microbial transformation of steroids–IX. Purification of progesterone hydroxylase cytochrome P-450 from Phycomyces blakesleeanus. J Steroid Biochem Mol Biol. 1995;52:203–208.

[20] Cabral JMS, Aires-Barros MR, Pinheiro H, et al. Biotransformation in organic media by enzymes and whole cells. J Biotechnol. 1997;59:133–143.

[21] Fernandes P, Cruz A, Angelova B, et al. Microbial conversion of steroid compounds: recent developments. Enzyme Microb Tech. 2003;32:688–705.

[22] Kumar R, Dahiyat JS, Singh D, et al. Biotransformation of cholesterol using Lactobacillus bulgaricus in a glucose-controlled bioreactor. Bioreour Technol. 2001;78:209–211.

[23] Kim HR, Lee GH, Cho EY, et al. Bax inhibitor 1 regulates ER-stress-induced ROS accumulation through the regulation of cytochrome P450 2E1. J Cell Sci. 2009;122:1126–1133.

[24] Gonzalez FJ. Role of cytochromes P450 in chemical toxicity and oxidative stress: studies with CYP2E1. Mutat Res. 2005;569:101–110.

[25] Lieber CS. Cytochrome P-4502E1: its physiological and pathological role. Physiol Rev. 1997;77:517–544.

[26] Nieto N, Friedman SL, Cederbaum AI. Stimulation and proliferation of primary rat hepatic stellate cells by cytochrome P450 2E1-derived reactive oxygen species. Hepatology. 2002;35:62–73.

[27] Khan WA, Ali Khan MW. Cytochrome P450-mediated estrogen metabolites and autoimmunity: relationship and link to free radicals. Curr Drug Metab. 2015;17:65–74.