Wang et al. Annals of Microbiology (2020) 70:45
https://doi.org/10.1186/s13213-020-01577-6

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

ORIGINAL ARTICLE Open Access

Determination of steroid hydroxylation specificity of an industrial strain *Aspergillus ochraceus* TCCC41060 by cytochrome P450 gene *CYP68J5*

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Abstract

Purpose: The use of *Aspergillus ochraceus* TCCC41060 for synthesis of 11α-OH-ethylgonendione, an important intermediate for synthesis of desogestrel-a major ingredient of the "third-generation" oral contraceptives, is hampered by its low regioselectivity of hydroxylation. In the present study, we sought to characterize gene(s) involved in steroid hydroxylation specificity in strain TCCC41060.

Methods: Taking advantage of the fact that expression of the 11α-hydroxylase, a member of the cytochrome P450 family, is highly induced by steroid substrates, we combined RNA-seq, qRT-PCR, and yeast functional expression to search for responsible steroid hydroxylase gene(s).

Results: Two highly inducible P450 genes (*CYP68L8* and *CYP68J5*) were isolated and recombinant yeast cells expressing *CYP68J5* were capable of 11α-hydroxylating both 16,17α-epoxyprogesterone and D-ethylgonendione. Disruption of *CYP68J5* in strain TCCC41060 resulted in complete loss of hydroxylation activities towards D-ethylgonendione, indicating that *CYP68J5* was solely responsible for hydroxylation activity on D-ethylgonendione in TCCC41060.

Conclusion: The above results demonstrated that low hydroxylation specificity of *CYP68J5* on D-ethylgonendione fully accounted for high by-product contents in TCCC41060, thus pointing to a strategy to engineer 11α-hydroxylase variants with higher hydroxylation specificity.

Keywords: *Aspergillus ochraceus*, Steroid, Hydroxylase, Cytochrome P450, D-ethylgonendione, Glucocorticoids

Introduction

Steroid drugs are a very important class of clinical drugs widely prescribed for the treatment of inflammation, cancer, rheumatic arthritis, allergy as well as contraception (Hogg 1992; Mahato et al. 1989; Tong and Dong 2009). Selective hydroxylation of steroid compounds is crucial for their appropriate pharmacological activities since hydroxylated steroids typically show higher biological activity compared with their less polar non-hydroxylated analogs (Choudhary et al. 2005; Donova and Egorova 2012; Smith et al. 1993; Tong and Dong 2009). Microbial steroid transformation is a powerful tool for introduction of oxygen to nonactivated carbons of a steroid molecule, a challenging reaction to implement using conventional chemical methods (Carballeira et al. 2009; Donova and Egorova 2012; Mahato and Garai 1997).

In the industrial setting, C11α, β-hydroxylations of steroids, one of the most important steroid microbial transformations for the production of key intermediates of corticosteroids, are performed by employing various

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filamentous fungi including *Aspergillus ochraceus*, *Rhizopus nigricans*, *Aspergillus niger*, *Absidia coerulea*, and *Curvularia lunatus* (Samanta et al. 1978; Shirasaka and Tsuruta 1960; Žnidaršič et al. 1998). It has been well documented that in filamentous fungi, the hydroxylases (monooxygenases) involved in steroid hydroxylation are members of the cytochrome P450 superfamily (Črešnar and Petrič 2011; Den Besten et al. 1990). The fungal steroid hydroxylation system consists of a cytochrome P450 monooxygenase (CYP) and a cytochrome P450 reductase (CPR) that mediate the transfer of electrons from NADPH to the P450 monooxygenases (Tong and Dong 2009; Wachenfeldt and Johnson 1995). However, it is still a challenge to assign a specific steroid hydroxylation ability to individual genes, given the existence of dozens or even hundreds of CYP genes in many filamentous fungi (Moktalil et al. 2012; Petrič et al. 2010) and the low homology between cytochrome P450s catalyzing steroid hydroxylation reaction even at the same position among closely related species (Nebert and Gonzalez 1987; Nebert et al. 1989). In many fungi, the enzymes involved in steroid hydroxylation are known to be inducible (Breskvar and Hudnik-Plevnik 1981; Irrgang et al. 1992; Jia et al. 2017; Lin and Smith 1970; Okey 1990; Wang et al. 2017) and are often localized on the endoplasmic reticulum membrane (Wachenfeldt and Johnson 1995). Microbial transformations have proven to be an efficient alternative to chemical methods in the regio- and stereoselective functionalizations of steroid drugs (Borges et al. 2009; Donova and Egorova 2012). But often low specificity and productivity associated with biotransformation severely limit their industrial applications because of low yields and expensive downstream purification (Collins et al. 2001; Hu et al. 2002). As a result, engineering highly efficient industrial strains are often required to implement a more economical production process. However, the molecular basis of steroid hydroxylation in many filamentous fungi is poorly understood.

Desogestrel is a major ingredient of the "third-generation" oral contraceptives which have a better side-effect profile for most women except for those at increased risk of venous thrombosis (LeBlanc and Laws 1999). 11α-OH-ethylgonendione is a key intermediate in the production of desogestrel (Gao et al. 1997). However, 11α-hydroxylation of D-ethylgonendione by conventional chemical route is economically impractical. Although various *A. ochraceus* strains have been used for large-scale 11α-hydroxylation of progesterone (Bihari et al. 1984; Houng et al. 1994; Samanta et al. 1978), the process using *A. ochraceus* TCCC41060 for commercial preparation of 11α-OH-ethylgonendione suffers from low specificity and significant side product formation (about 40%), mainly 10α-hydroxy-ethylgonendione and 10β-, 11α-dihydroxy-ethylgonendione (unpublished data) (Fig. 1). Because of high costs of substrate D-ethylgonendione, the development of an economical industrial process for 11α-hydroxy-D-ethylgonendione production will depend on the availability of strains with improved hydroxylation specificity.

Though an 11α-hydoxynlase gene (*CYP68J5*) in *A. ochraceus* was previously isolated from a cDNA library by co-expressing the *CYP68J5* gene with a human NADPH-cytochrome P450 reductase gene in *Spodoptera frugiperda* (Sf-9) (Petrič et al. 2010), its functional role in steroid hydroxylation in vivo remains to be elucidated. Furthermore, the genetic basis underlying the differential hydroxylation specificities of *A. ochraceus* TCCC41060 towards 16,17α-epoxyprogesterone and D-ethylgonendione is unclear, which hampers efforts to breed strain with higher hydroxylation specificity.

In the present study, to gain insights into the mechanistic aspects of steroid hydroxylation in *A. ochraceus*, we isolated two highly inducible P450 genes, *CYP68L8* and *CYP68J5*, by transcriptome sequencing and qRT-PCR, and we provide definitive genetic evidence that in *A. ochraceus* TCCC41060 *CYP68J5* is solely responsible for 11α-hydroxylation activity on 16,17α-epoxyprogesterone and D-ethylgonendione.

Materials and methods

Materials

D- ethylgonendione and 11α-hydroxy-D-ethylgonendione were obtained from Zizhu Pharmaceutical (Beijing, China). 16,17α-epoxyprogesterone and 11α-hydroxy-16,17α-epoxyprogesterone were purchased from Toronto Research Chemicals.

Bacterial strains, plasmids, chemicals, and culture conditions

Aspergillus ochraceus TCCC41060 was obtained from the microbial strain collection of the applied microbiology lab of Tianjin University of Science and Technology (TUST). The fungus was routinely maintained on potato dextrose agar (PDA) or in steroid transformation medium (STM) (20 g/l glucose, 20 g/l yeast extract, 20 g/l tryptone, pH 5.8). The diploid yeast strain *Saccharomyces cerevisiae* INVSc1 (*his3Δ1*, *leu2*, *trp1-289*, *ura3-52*) was used for heterologous expression of the putative CYP genes. The *S. cerevisiae* INVSc1 was cultivated at 30 °C in YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose). *Escherichia coli* JM109 was used for plasmid construction. PCR products were cloned into pUC18-T vector (TaKaRa, Dalian, P.R. China). The pYES2 expression plasmid (Invitrogen) was used for functional expression of the candidate CYP genes. The plasmid pCSN44 is kindly provided by Prof.

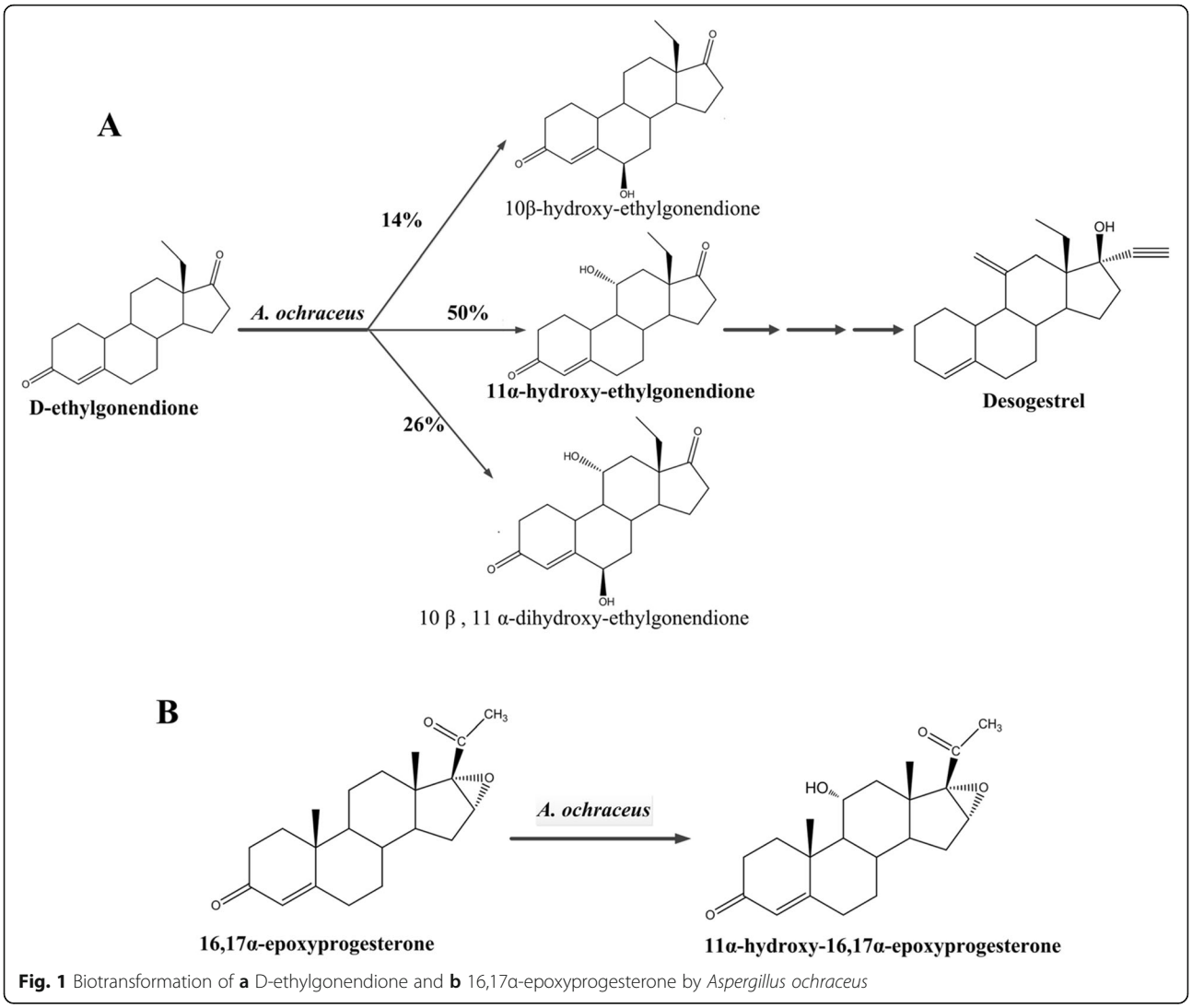

Fig. 1 Biotransformation of **a** D-ethylgonendione and **b** 16,17α-epoxyprogesterone by *Aspergillus ochraceus*

Shaojie Li (Institute of Microbiology, Chinese Academy of Sciences), and plasmid pAg1-h3-ble is a generous gift from Prof. Hao Liu (College of Biotechnology, Tianjin University of Science and Technology). Unless noted, all enzymes for DNA molecular manipulations in this study were purchased from the TaKaRa (Dalian, China) and all primers were from AuGCT (Beijing, China).

Induction of 11α-hydroxylase activity in *A. ochraceus*

To determine whether the fungal steroid 11α-hydroxylase activity is induced by steroid substrate at the transcription level, *A. ochraceus* was inoculated into 50 ml liquid STM medium in a 250-ml Erlenmeyer flask at 28 °C for 24 h. Steroid induction assays were conducted as follows: (1) 16,17α-epoxyprogesterone dissolved in methanol was added to the fungal culture at a final concentration of 0.1 mg/ml and incubated for 3 h, and for the control, equal volume of methanol only was added; (2) collected mycelia then were filtered, rinsed three times with saline water, and about 5 g wet mycelia was resuspended in 20 ml of fresh phosphate buffer (1 mM sodium phosphate, 0.2 mM EDTA, 0.04 mM glutathione, pH 5.5); (3) 16,17α-epoxyprogesterone and cyclohexmide (Solarbio) were added together to respective final concentrations of 1 mg/ml and 0.03 mg/ml and incubated for 24 h at 28 °C with shaking at 180 rpm; and (4) 1 ml fermentation broth was extracted with ethyl acetate and analyzed by thin layer chromatography.

Steroid fungal biotransformation

A *ochraceus* TCCC41060 conidia were inoculated into 50 ml STM in a 250-ml flask with 106 condia/ml and cultivated for 22–24 h at 28 °C with shaking at 180 rpm. Then the steroid substrate dissolved in methanol was added to a final concentration of 2 g/l and allowed transformation to proceed at 28 °C for 24 h on a rotary shaker. Reaction products were analyzed by TLC and HPLC.

TLC and HPLC analyses

To identify the transformation products, 1 ml of fungal transformation broth was extracted with 200 μl ethyl acetate. The product profile was monitored by TLC using 0.15–0.2 mm silica gel with petroleum and ethyl acetate (1:1–2:1) as a solvent system and 11α-hydroxy-16,17α-epoxyprogesterone and 11α-hydroxy -D-ethylgonendione as the standards. The spots on the TLC plates were visualized under ultraviolet light at 254 nm. The products were further analyzed by HPLC. The extracts (100 μl) were allowed to dry completely and then were re-dissolved in 200 μl acetonitrile. Samples (5 μl) were analyzed on an Agilent 1200 system equipped with a single-wavelength UV detector set at 240 nm. The HypersilTM ODS C18 columns (250 mm × 4.6 mm. D Column, 5 μm; Thermo Scientific) were used and the mobile phase consists of 80% acetonitrile and 20% H_2O with a flow rate of 0.8 ml/min. 11α-OH-16,17α-epoxyprogesterone and 11α-OH-D-ethylgonendione standards were used to confirm the authenticity of the product.

Transformation products separation

To determine the structure of transformation products, respective large-scale bioconversions of 16,17α-epoxyprogesterone and D-ethylgonendione were performed. The collected fermentation broth (1 l) was extracted 2 times with ethyl acetate. The extracts were concentrated by rotary evaporation, followed by addition of 2 g silica gel and mixing until powder was formed. Silica gel (200–300 mesh) was used for the chromatography column (26 mm × 30 cm) packing and an isocratic solvent system comprising petroleum ether: ethyl acetate = 3:1 – 1:1 was employed to purify the target product, followed by vacuum distillation.

Measurements of nuclear magnetic resonance (NMR)

To further verify the identity of the biotransformation products, the purified target products were subjected to NMR analysis. The purified target products dissolved in dimethyl sulfoxide (DMSO-d6) were used for NMR spectroscopy (^{1}H NMR with 400 MHz and 21 °C; ^{13}C NMR with 100 MHz and 25 °C).

C11α-OH-16,17α-epoxyprogesterone

^{1}H NMR included signals at δ 4.38 (d, 1H, C-11H), 3.86 (s, 1H, J = 4, OH) ppm. 13C NMR δ: 205.1 (C-20), 199.1 (C-3), 171.7 (C-5), 124.1 (C-4), 70.3 (C-11), 67.2 (C-17), 60.9 (C-9), 59.2 (C-14), 44.6 (C-12), 43.2 (C-13), 41.6 (C-10), 40.6 (C-1), 37.2 (C-2), 34.3 (C-8), 33.2 (C-6), 32.5 (C-7), 31.6 (C-21), 27.1 (C-15), 26.2 (C-16), 18.4 (C-19), 16.4 (C-18).

C11α-hydroxy-D-ethylgonendione

^{1}H NMR included signals at δ 4.48 (d, 1H, J = 8, C-11H), 3.63 (s, 1H, OH) ppm. 13C NMR δ: 217.8 (C-17), 199.5 (C-3), 168.7 (C-5), 123.8 (C-4), 70.1 (C-11), 54.5 (C-13), 51.1 (C-2), 49.8 (C-9), 43.5 (C-16), 38.8 (C-6), 38.2 (C-10), 36.0 (C-12), 35.8 (C-14), 35.8 (C-7), 31.3 (C-8), 27.3 (C-1), 21.2 (C-18), 18.2 (C-15), 8.0 (C-19).

RNA isolation and cDNA synthesis

To isolate total RNAs, *A. ochraceus* TCCC41060 was cultured for 24 h at 28 °C in liquid STM medium, followed by addition of 0.1% 16,17α-epoxyprogesterone for induction of 3 h. Total RNAs were extracted using Trizol reagents (Promega, USA) from the resultant mycelia and synthesis of the first cDNA strand was performed using 5 μg total RNAs with PrimeScript Reverse Transcriptase in a 20-μl reaction volume according to the manufacturer's instructions.

Transcriptome sequencing and qRT-PCR

Total RNA was extracted from fungal culture treated with the steroid substrate for profiling expressed CYP genes in *A. ochraceus* under substrate induction conditions. The RNA quality was evaluated by the Nano Drop (NanoDrop Technologies, USA) and RNA sequencing was conducted by BGI-Beijing (Beijing Genomics Institute) using Illumina HiSeq™2000 (Illumina, Inc., USA). Analyses of the returned RNA sequencing data were conducted to identify expressed CYPs under substrate induction. To verify the RNA sequencing data, real-time quantitative PCR (qRT-PCR) was performed to quantify the expression levels of candidate CYP genes under induction and no induction conditions. PCR reaction components consisted of 10 μl of MasterMix with SYBR (Solarbio, Beijing, China), 300 nmol/l of both primers (Table S1) and 1 μl of cDNA template in a final volume of 20 μl. The qRT-PCR amplification procedure included denaturation (95 °C, 10 min), 40 cycles of denaturation (95 °C, 30 s), and annealing (60 °C, 30 s) (Applied Biosystems, USA). The transcript level of glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was used as an internal control, and the amounts of target transcripts were calculated based on $2^{-\Delta\Delta CT}$ ((Livak and Schmittgen 2001)).

Heterologous expression of *CYP68J5* and *CYP68L8* in *S. cerevisiae*

The full length cDNAs of two highly induced genes *G7750* and *G8957* (renamed as *CYP68J5* and *CYP68L8*, which were submitted to NCBI GenBank with Accession Numbers: MN508259 and MN508258, respectively, were PCR-amplified with Pyrobest DNA polymerase with primers sets (*G7750*, forward: 5′-GAATTCATGCCCTTCTTCACTG GGCT-3′, reverse: 5′-CTCGAGCTACACAGTTAAACTC

GCCAT-3′; *G8957*, forward: 5′-GAATTCATGATGCT CCCAGTATTCAC-3′, reverse: 5′-TCTAGATCATAGTT CAATGTCGGAGTT-3′), which contain appropriate restriction sites and their respective PCR products were cloned into the SmaI site of pBluescript II KS+ to produce pB-J5 and pB-L8 plasmids, followed by double enzyme digestion with EcoRI/XhoI and EcoRI/XbaI, respectively. The released *CYP68J5* EcoRI/XhoI and *CYP68L8* EcoRI/XbaI fragments were gel purified and ligated into the corresponding sites of pYES2, respectively, to generate expression vector pYES2-*CYP68J5* and pYES2-*CYP68L8*, which were transformed into *S.cerevisiae* INVSc1 by the lithium chloride method. The resulting transformants were selected on SC-Ura agar plates containing 6.7 g/l yeast nitrogen base, 20 g/l glucose, and appropriate amino acids and confirmed by PCR with the primer sets (*G7750*, forward: 5′-TCTAGAATGCCCTTCTTC ACTGGGCT-3′, reverse: 5′-TTAATTAACTACACAGTT AAACTCGCCAT-3′; *G8957*, forward: 5′-GAATTCATGA TGCTCCCAGTATTCAC-3′, reverse: 5′-TCTAGATCAT AGTTCAATGTCGGAGTT-3′).

Biotransformation of steroid by recombinant *S.cerevisiae*

To assess 11α-hydroxylation activities of *CYP68J5* and *CYP68L8* recombinant yeast cells were respectively seeded in 50 ml yeast extract peptone dextrose medium (YPD) in 250 ml flasks at 30 °C and 180 rpm. Induced expression was initiated by adding 1 g D-galactose when the cell density reached 2.5 OD_{600}. After induction for 6 h, substrate (16,17α-epoxyprogesterone or D-ethylgonendione) dissolved in methanol was added to the culture at a final concentration of 1 g/l, followed by further incubation for 48 h at 28 °C. Fermentation broth of 1 ml was extracted by ethyl acetate and analyzed by thin layer chromatography and HPLC methods as described above.

Genomic DNA isolation

To isolate fungal genomic DNA, mycelium powder of 1 g was added to 700 ml lysis buffer (30 mmol/l Tris-acetate, pH 7.5; 30 mmol/l EDTA; 5 g/l SDS; 14 g/l NaCl; 50 μl/l 2-mercaptoethanol), followed by vigorous vortexing for 5 min. After spinning at 13,000×*g* for 10 min, the supernatant was transferred to a 1.5-ml microcentrifuge tube, and Proteinase K (10 mg/ml, Solarbio) was added with a final concentration of 30 μl/ml and incubated for 30 min at 37 °C, followed by sequential equal volume phenol, and chloroform extraction. DNA was precipitated with an equal volume of isopropanol and rinsed 3 times with 70% ethanol, dried and dissolved in 50 μl TE buffer (10 mmol/l Tris-HCl, 0.1 mmol/l EDTA; pH 7.8), and stored at − 20 °C.

Protoplast preparation and transformation

Preparation of A. ochraceus protoplasts

Conidia of *A. ochraceus* were harvested from PDA slants cultivated at 28 °C for 4–6 days using 1 M sorbitol and adjusted to 1.0×10^7 conidia/ml. Fungal mycelia were prepared by inoculating 100 μl conidia into 100 ml YPD in a 500-ml flask and incubated for 20–28 h at 28 °C with shaking, followed by still incubation for 10–15 h at 28 °C. Mycelia were washed thoroughly with 1 M sorbitol and resuspended in enzyme mixture [consisting of 10 g/l cellulase (Solarbio, Beijing, China), 1 g/l lysing enzymes (Solarbio, Beijing, China), and 10 g/l snailase (Solarbio, Beijing, China)] solutions containing 1 M sorbitol and incubated at 30 °C for 2 h with shaking. Protoplasts were collected by centrifugation at 3000×*g* for 15 min at 4 °C and then washed twice using 0.6 M KCl + STC (1 M sorbitol, 10 mM Tris-HCl and 50 mM CaCl2; pH 7.5) and resuspended in STC to > 5 × 106 protoplasts/ml.

For protoplast transformation, an aliquot of 200 μl of the protoplasts suspension was added to a 10-ml microcentrifuge tube, followed by gently mixing with 5–10 μg of linearized pko-J5 and 100 μl of PEG solution (300 g/l PEG 8000; 50 mM CaCl2; 10 mM Tris-HCl; pH 7.5; Sangon Biotech) and incubating for 30 min on ice. PEG solution (2 ml) was added, gently mixed with the protoplasts and then kept at room temperature for 5 min before the addition of STC (4 ml). The transformation reaction was then added to 50 ml liquid regeneration medium (PDA containing 0.6 M KCl and 300 μg/ml hygromycin B or 30 μg/ml bleomycin; Solarbio) and plated on PDA. Transformants appeared after 3–5 days incubation at 28 °C and transferred to PDA slant containing hygromycin. Selected transformants were propagated for 3–5 generations to assess their mitotic stability before used for further analysis.

Construction of the *cyp68j5* mutant

To delete *CYP68J5* by homologous recombination, about 1.5 kb genomic fragments of upstream and downstream of *CYP68J5* ORF were amplified by PCR using LA Taq polymerase with the primer sets (forward: 5′-TCTAGA TTCTGGATTGAATCAGC-3′, reverse: 5′-CCCGGG GATAATGAGCTGTCAGCTT-3′;forward: 5′-GTCGA CGTCGAACACGAAGTCCTG-3′, reverse: 5′-GGTAC CGGACTTTGTGAAGTGG-3′), in which appropriate restriction enzyme sites are respectively incorporated. The respective PCR products were then ligated with the vector pUC18-T. The 1.5 kb 5' arm AOHL was released by XbaI/SmaI double digestion and the 1.5 kb 3' arm AOHR was obtained by SalI/KpnI double digestion. Digestion of pCSN44 with SmaI and XhoI generated hygromycin B resistance gene (*HYG*) fragment. The pBluescript II KS+ was linearized with XbaI and KpnI. The four restriction fragments (AOHL, AOHR, HYG and the linearized pBluescript II KS+) were assembled by a designed order to generate the deletion vector pko-J5 (Fig. 3a). The pko-J5 linearized with the restriction

enzyme NotI and KpnI was used for fungal protoplast transformation. Transformants were selected on PDA containing 300 μg/ml hygromycin B and a PCR approach used for screening *AOH* deletion mutant using two forward primers (13F1, forward: 5′-ATCATCTCTAGGCGTTCTGC-3′; 13F2, forward: 5′-TGGACAGACCATCAGTTTGG-3′) just outside the deletion vector and a reverse primer based on the *HYG* gene sequence (13R1, reverse: 5′-TTCTAGAGGATCCTCTACGC-3′) (Fig. 3a).

Southern blot analysis of the *cyp68j5* mutant

To further verify the inactivation of the *CYP68J5* gene, southern blot was conducted. Extracted genomic DNA of 10-15 μg of *A. ochraceus* TCCC41060 strain and the *cyp68j5* mutant was respectively digested with EcoRV/BglII, followed by separation on 0.8% agarose gel, and blotting was performed with the N + -Magaprobe Nylon Transfer Membrane according to the manufacturer's instruction (GE Osmonics Inc., MN, USA). The hybridization probe containing the ~ 2.3 kb EcoRV/EcoRV fragment encompassing the promoter region and part of the *CYP68J5* gene (Fig. 4a) was PCR-amplified with primers Prob-F (forward: 5′-GATATCACTTGCTGTCCTTG-3′) and Prob-R (reverse: 5′-GATATCATGTGAGCAGGCG-3′). The probe was labeled with digoxigenin (DIG) using the high prime DNA labeling and detection starter kit II according to the manufacturer's protocol (Roche Diagnostics; Mannheim, Germany).

Complementation experiments

To complement the *cyp68j5* mutant, a ~ 3.0 kb genomic fragment (CGF) encompassing the full ORF of *CYP68J5* plus 873 bp of the 5′ promoter region and 334 bp of 3′ terminator region was PCR-amplified using a primer set (forward: 5′-GATATCACTTGCTGTCCTTG-3′, reverse: 5′-GGTAATGATAGGAGGGGAGC-3′) with Pyrobest DNA polymerase and then ligated with the SmaI site of pBluescript II KS+ to produce vector pB-CGF. The CGF fragment was obtained by double digestion of pB-CGF with XhoI and EcoRI. The bleomycin resistance marker gene (*BLE*) fragment was obtained by digestion of pAg1-H3-b with SalI and HindIII and then cloned into SalI and HindIII sites of the pBluescript II KS+ vector to generate plasmid pB-BLE, which was digested with XhoI and HindIII to release the XhoI/HindIII *BLE* fragments. The CFG and *BLE* fragments were then cloned into the HindIII and EcoRI sites of pBluescript II KS+ to generate the complementation construct, which was then introduced into the *cyp68j5* mutant cells by protoplast transformation. Transformants were selected on bleomycin-containing PDA agar plates and then screened for complemented strains by PCR amplification of the *BLE* and *CYP68J5* gene with the primer sets (*BLE*, forward: 5′-CCAATGGCTCATAGTACCAG-3′, reverse: 5′-GACCTAGACTTCAGGTTGTC-3′; *CYP68J5* forward: 5′-ATGCCCTTCTTCACTGGGCTT-3′, reverse: 5′-CTACACAGTTAAACTCGCCAT-3′).

Results

Substrate induction of the 11α-hydroxylase gene(s) in *A. ochraceus*

Induction of steroid hydroxylase activities by steroid compounds has previously been reported for several fungal strains of diverse species (Breskvar and Hudnik-Plevnik 1981; Irrgang et al. 1992; Lin and Smith 1970). To determine whether the expression of 11α-hydroxylase (CYP) gene(s) in *A.ochraceus* TCCC41060 was induced by substrate 16,17α-epoxyprogesterone, mycelia were exposed to 0.001% 16,17α-epoxyprogesterone for 3 h before the addition of the protein synthesis inhibitor cyclohexmide (see "Materials and methods" section). As shown in Fig. 2a, 16,17α-epoxyprogesterone-induced cultures formed appreciable amounts of 11α-hydroxylated product after 6 h incubation, whereas the non-induction culture control showed no detectable levels of 11α-hydroxylated product. The fact that protein synthesis inhibitor cycloheximide could completely block 11α-hydroxylation of 16,17α-epoxyprogesterone indicates that the expression of the target CYP gene(s) in this fungal strain was induced at the transcriptional level.

Search for candidate CYP genes by transcriptomic profiling and qRT-PCR. To identify CYP genes expressed in strain TCCC41060 under induction conditions, a RNA-seq analysis was performed and 44 CYP gene sequences identified (Table S2). Given that transcription of target steroid hydroxylase gene(s) in *A. ochraceus* TCCC41060 was highly induced by substrate 16,17α-epoxyprogesterone, qRT-PCR analysis was conducted for the 44 identified CYP gene sequences to pinpoint candidate CYP genes. Of the 44 CYP genes, the expression of two genes, 8957 and 7750, was highly induced (> 500), which were named as *CYP68L8* and *CYP68J5*, respectively, according to the recommendation of the Cytochrome P450 Nomenclature Committee (http://drnelson.uthsc.edu/CytochromeP450.html). *CYP68J5* was previously described as an 11 alpha-hydroxylase gene isolated from a cDNA library of *A. ochraceus* conidia and functionally expressed in Sf-9 insect cells (Suzanne et al. 2003).

Functional expression of *CYP68J5* in *S. cerevisiae*

Taking advantage of the fact that the cytochrome P450 reductase in *S. cerevisiae* cells can transfer electrons from NADPH to most eukaryotic cytochrome P450 monooxygenases, the respective full length cDNA of *CYP68L8* and *CYP68J5* was cloned and inserted into the yeast expression vector pYES2 (Invitrogen) to generate

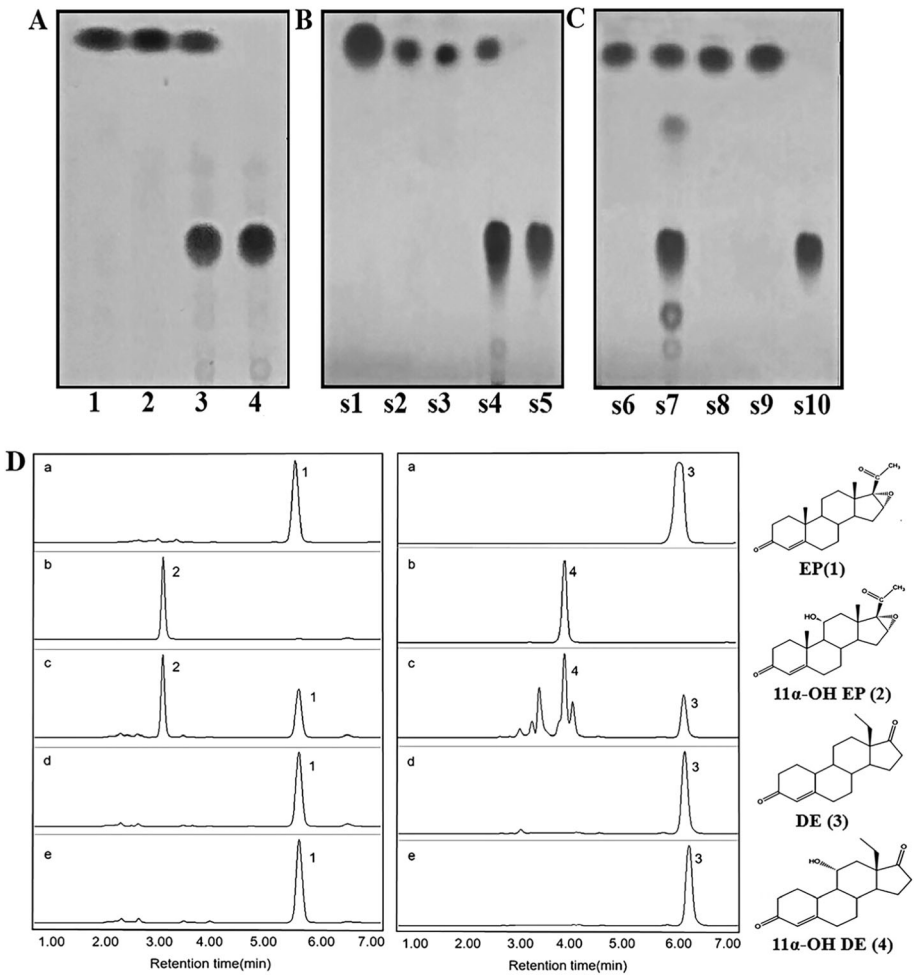

Fig. 2 Identification of steroid 11α–hydroxylase gene *CYP68J5*. **a** TLC analysis of induction of steroid 11α–hydroxylase activities in TCCC41060. 16,17α-epoxyprogesterone (EP) was used for induction and also as transformation substrate, and petroleum and ethyl acetate (3:2) was used for product separation. 1, EP standard; 2, induction for 0 h; 3, induction for 3 h; and 4, 11α-OH EP standard. **b**, **c** Analysis of bioconversion products of the recombinant *S. cerevisiae* cells expressing either *CYP68L8* or *CYP68J5*. **b** EP as substrate, petroleum and ethyl acetate (3:2) as the solvent system for TLC; s1, EP standard; s2, *S. cerevisiae* wild-type; s3, recombinant INVSc1-*CYP68L8*; s4, INVSc1-*CYP68J5*; and s5, 11α-OH EP standard. **c** DE as substrate and petroleum and ethyl acetate (1:1) as the solvent system; s6, DE standard; s7, INVSc1-*CYP68J5*; s8, *S. cerevisiae* wild-type; s9, recombinant INVSc1-*CYP68L8*; and s10, 11α-OH DE standard. **d** HPLC assays of biotransformation product of recombinant *S. cerevisiae* cells, acetonitrile: H_2O 80:20 (v/v) as the mobile phase. (a) substrate standards (1) EP, (3) DE; (b) 11α-hydroxy-products standards (2) 11α-OH EP, (4) 11α-OH DE; (c) recombinant *S. cerevisiae* INVSc1-*CYP68J5*, (d) *S. cerevisiae* wild-type, and (e) recombinant *S. cerevisiae* INVSc1-*CYP68L8*

recombinant expression plasmid pYES2-*CYP68L8* and pYES2-*CYP68J5*, which were then transformed into *S. cerevisiae* INVSc1 to obtain recombinant yeast strains INVSc1-*CYP68L8* and INVSc1-*CYP68J5*, respectively. The ability of recombinant *S. cerevisiae* strains INVSc1-*CYP68L8* and INVSc1-*CYP68J5* to 11α-hydroxylate 16, 17α-epoxyprogesterone and D-ethylgonendione was examined by TLC and HPLC analyses as described in "Materials and methods" section. Only recombinant *S. cerevisiae* INVSc1-*CYP68J5* had the ability to 11α-hydroxylate 16,17α-epoxyprogesterone and D-ethylgonendione. As shown in (Fig. 2b-d), the major transformation products of recombinant *S. cerevisiae* INVSc1-*CYP68J5* were respectively identified as 11α-hydroxy-16,17α-epoxyprogesterone and 11α-hydroxy-D-ethylgonendione, which were further confirmed by NMR analysis.

Functional characterization of *CYP68J5* in vivo

To evaluate the potential role of *CYP68J5* in vivo, we disrupted the *CYP68J5* gene locus using a 2.4-kb *HYG* fragment to replace the *CYP68J5* ORF by the homologous recombination strategy. A deletion vector pko-J5 was constructed which includes a 2.4-kb fragment containing the hygromycin B resistance gene (*HYG*) and two 1.5 kb homologous arm fragments. The linearized pko-J5 vector was introduced into *A. ochraceus* cells by protoplast transformation, and selection of

transformants on agar plates containing 300 μg/ml hygromycin B resulted in 6 transformants. PCR analysis using the primer pair 13F1 and 13R1 (Fig. 3a) to detect *CYP68J5* ORF replacement event showed that a PCR product of 2.9 kb rather than the expected 2.0 kb (Fig. 3b), suggesting that the *HYG* cassette was not inserted via homologous recombination. To determine the insertion position of *HYG*, the full ORF of *CYP68J5* of the mutant was PCR-amplified and a 4.3 kb PCR product was obtained (Fig. 3c) and sequenced. Sequence alignment showed that the *HYG* maker was inserted into the position of 914 bp up from the ATG start code (Fig. S11). Southern blot analysis confirmed the *HYG* insertion event as evidenced by the hybridization bands of 1.6 kb and 3.1 kb in the *cyp68j5* mutant as opposed to the hybridization bands of 1.6 kb and 0.7 kb in the *A. ochraceus* TCCC41060 recipient strain (Fig. 4b).

To investigate the effect of *CYP68J5* deletion on steroid hydroxylation activity, biotransformation of the *cyp68j5* mutant on 16,17α-epoxyprogesterone was conducted and the product was analyzed by TLC and the HPLC. As shown in (Fig. 5), the hydroxylation activity of the *cyp68j5* mutant strain on both 16,17α-epoxyprogesterone and D-ethylgonendione was completely lost, demonstrating that the *CYP68J5* gene was solely responsible for the 11α-hydroxylation activities towards the above two steroid substrates.

To ensure that *CYP68J5* accounts for the observed phenotype, the *cyp68j5* mutant was complemented by a ~ 3 kb genomic fragment containing the full ORF of the *CYP68J5* gene plus ~ 800 bp of the 5′ promoter region using the 1.5 kb bleomycin resistance gene as a selection marker. Two transformants were obtained, as shown in Fig. S12. Biotransformation experiments showed that the

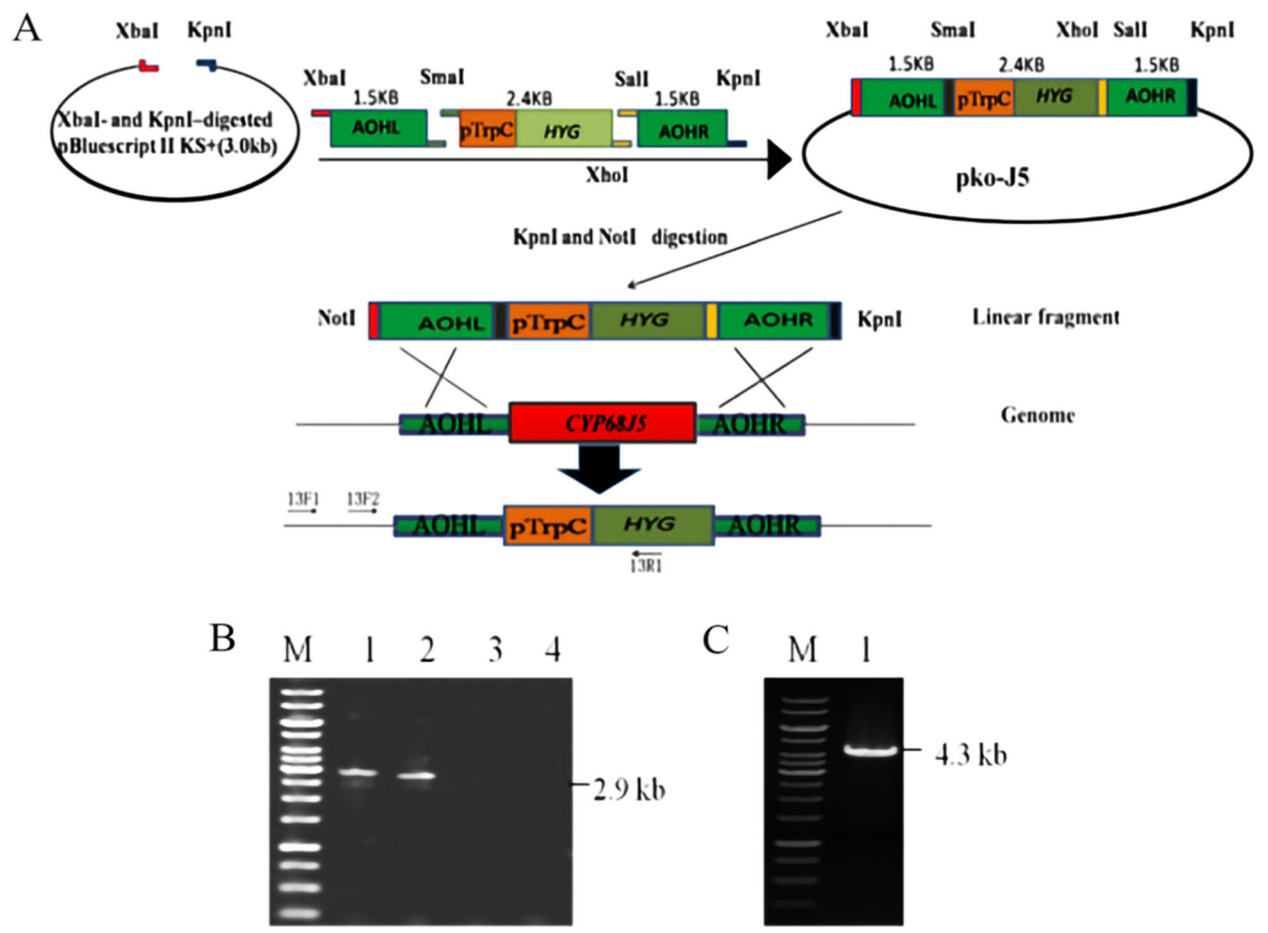

Fig. 3 Disruption of *CYP68J5* and mutant verification. **a** Diagram of the strategy for *CYP68J5* mutation and mutant verification. **b** Verification of the *cyp68j5* mutant by PCR analysis. PCR products were separated on a 0.8% agarose gel. Lanes 1 and 2, with primers 13F1 and 13R1, 13F2 and 13R1, respectively, using the *cyp68j5* mutant genomic DNA as a template and lanes 3 and 4, with primers 13F1 and 13R1, 13F2 and 13R1, respectively, using the recipient strain TCCC41060 genomic DNA as a template, which serves as a control. **c** PCR amplification of the full ORF of the *cyp68j5* mutant locus with primers X and Y

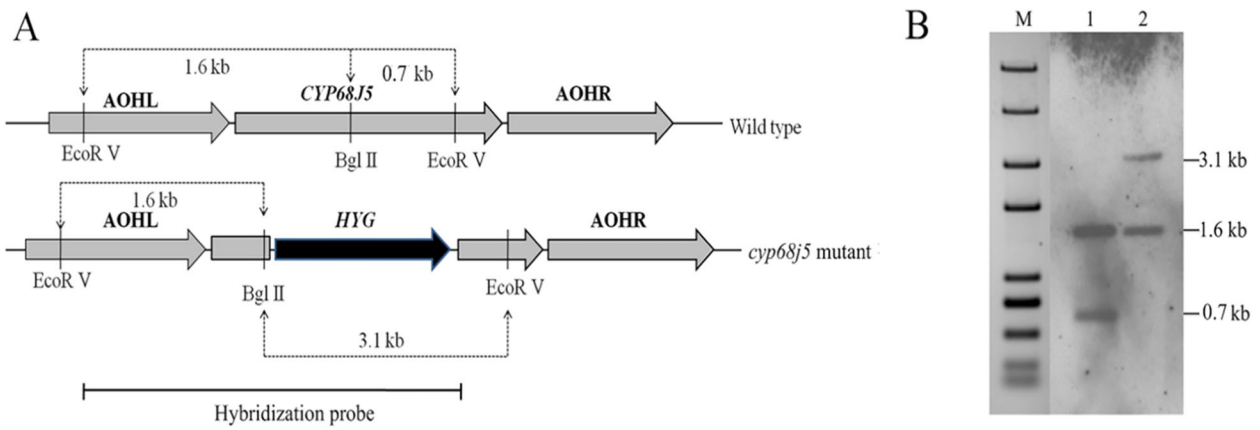

Fig. 4 Confirmation of the disruption of *CYP68J5* by southern blot. **a** The strategy for southern blot, total DNA from each strain was digested with EcoRV/BglII and probed with a hybridization probe. **b** Detection of the disruption of *CYP68J5* in the *cyp68j5* mutant by southern blot analysis: lane 1, EcoRV/BglII digested genomic DNA of *A. ochraceus* TCCC41060; 2, EcoRV/BglII digested genomic DNA of the *cyp68j5* mutant

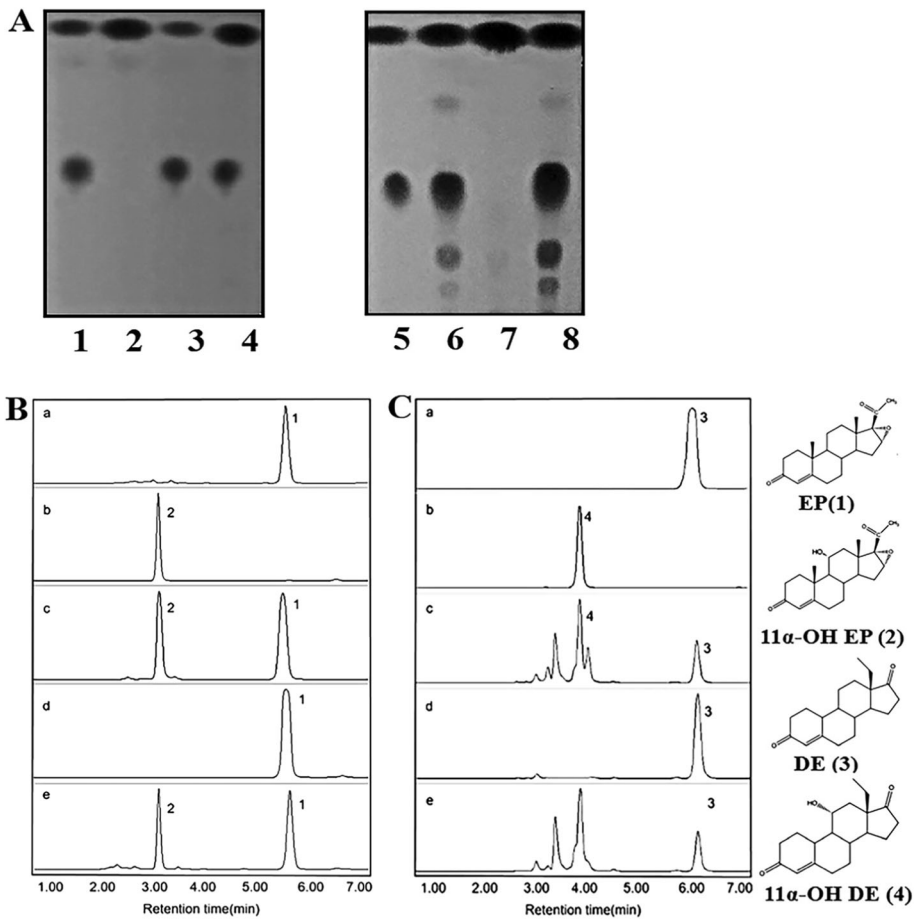

Fig. 5 TLC and HPLC analyses of biotransformation products. **a** TLC assays: lanes 1–4, EP as substrate, petroleum and ethyl acetate (3:2) as the solvent system; lanes 5–8, DE as substrate, petroleum and ethyl acetate (1:1) as the solvent system. 1, *A. ochraceus* TCCC41060; 2, the *cyp68j5* mutant; 3, the reconstituted strain; 4, EP and 11α-OH EP standards; 5, DE and 11α-OH DE standards; 6, *A. ochraceus* TCCC41060; 7, the *cyp68j5* mutant; 8, the reconstituted strain. **b**, **c** HPLC assays: acetonitrile: H_2O 80:20 (v/v) as the mobile phase, **b** EP as substrate: EP standard (a), 11α-OH EP standard (b), transformation products of recipient strain (c), the *cyp68j5* mutant (d), and the reconstituted strain (e); **c** DE as substrate: DE standard (a), 11α-OH DE standard (b), transformation products of the recipient strain (c), the *cyp68j5* mutant (d), and the reconstituted strain (e)

reconstituted strains were capable of 11α-hydroxylating both 16,17α-epoxyprogesterone and D-ethylgonendione (Fig. 5), thus further verifying the function of *CYP68J5*.

Interestingly, no appreciable amounts of products were observed when cyp68j5 mutant was used to transform D-ethylgonendione, while significant amounts of side products (~ 40%) were generated by the *A. ochraceus* TCCC41060 strain and the two CYP68J5 reconstituted strains. The above results demonstrate that it is the low regioselectivity of 11α-hydroxylase encoded by the *CYP68J5* gene that resulted in the relative low yields and significant side product formation, thus excluding the contribution of other genes in *A. ochraceus* to the production of side products. On the other hand, 11α-hydroxylase shows much higher specificity for substrate 16,17α-epoxyprogesterone. The fact that *A. ochraceus* TCCC41060 produced about 40% by-product on substrate D-ethylgonendione limits its use as a biocatalyst for the commercial manufacture of 11α-hydroxylated D-ethylgonendione, which is a key intermediate in the production of desogestrel, a major ingredient of third-generation oral contraceptives (Gao et al. 1997; LeBlanc and Laws 1999).

Discussion

Specific steroid hydroxylation by filamentous fungal cells is key to cost-effective production of a wide variety of steroid drugs. Although diverse fungal species can hydroxylate steroids (Donova and Egorova 2012), but only a few fungal species have been used in the industrial process, largely due to the fact that the majority of these fungi display low enzymatic activity and undesirable regioselectivity and stereoselectivity on substrates of interest. Expanding the use of catalytic potentials of the repertoire of the diversity of fungal species in nature for industrial applications entails the breeding of more efficient fungal strains.

Although *CYP68J5* was previously isolated from a cDNA library prepared from *A. ochraceus* spores and functionally expressed in Sf-9 insect cells (Suzanne et al. 2003), its contribution to 11α-hydroxylase activities in vivo is unclear. Deletion of *CYP68J5* allows us to definitively assess its functional role in *A. ochraceus* TCCC41060. Our current work demonstrates that *CYP68J5* is solely responsible for the hydroxylation activities for both 16,17α-epoxyprogesterone and D-ethylgonendione, since disruption of *CYP68J5* resulted in complete loss of hydroxylation activities towards both substrates. These results clearly indicate that the formation of high levels of side products when strain TCCC41060 is employed to produce 11α-OH-ethylgonendion from D-ethylgonendione is due to the inherent low substrate regiospecificity of 11α-hydroxylase *CYP68J5* rather than the contribution of additional gene(s), thus immediately suggesting a strategy to engineer CYP68J5 variants with higher regiospecificity for constructing efficient strains for preparative-scale process of 11α-OH-ethylgonendion production.

A number of fungal steroid hydroxylase genes have been identified from diverse filamentous fungi including a steroid 11α-hydroxylase gene from *Aspergillus nidulans* ((Ríos et al. 2017)), a 15α-hydroxylase gene from *Penicillium raistrickii* (Jia et al. 2017), an 11α-hydroxylase gene from *A. coerulea* (Wang et al. 2017), a 19α-hydroxylase gene from *Thanatephorus cucumeris* (Lu et al. 2018), a 14α-hydroxylase gene from *C. lunatus* (Chen et al. 2019), and an 11β-hydroxylase gene from *C. lunatus* (Felpeto-Santero et al. 2019); however, except for the *P. raistrickii* 15α-hydroxylase gene and the *A. orchraceus CYP68J5*, the in vivo functional role of other reported hydroxylase genes have not been determined.

Numerous studies have shown that cytochrome P450s are highly evolvable (Jung et al. 2011; Kille et al. 2011; McIntosh et al. 2014). In order to improve regioselectivity of *CYP68J5* towards D-ethylgonendione, direct evolution by error-prone PCR is underway in the lab and yeast functional expression platform will facilitate the screening of *CYP68J5* variants with desired substrate regiospecificity.

Conclusions and future work

The combined use of transcriptome sequencing, qRT-PCR, and yeast functional screening platform has allowed us to rapidly identify the target steroid 11α-hydroxylase *CYP68J5* gene in an industrial strain TCCC41060. The in vivo functional characterization of *CYP68J5* and the creation of a deletion mutant cyp68j5 have provided a good starting point for engineering strains with much improved regiospecificity for D-ethylgonendione and other steroids of interest, thus potentially making *A. ochraceus* TCCC41060 an efficient host for producing a variety of valuable steroid intermediates.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13213-020-01577-6.

Additional file 1: Table S2. Differentially expressed P450 candidates identified by comparing 6 h induction with non-induction transcriptomes. **Figure S12.** Verification of reconstituted strains by PCR amplification.

Authors' contributions

The authors read and approved the final manuscript.

Funding

This work was financially supported by a grant from the National High Technology Research and Development Program of China (863 Program) (No. 2011AA02A211).

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no conflicts of interest.

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Received: 17 December 2019 Accepted: 8 May 2020
Published online: 06 July 2020

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