BNL-223713-2022-BOOK

Biocatalytic system for comparatively assessing the functional association of monolignol cytochrome P450 monooxygenases with their redox partners

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To be published in "Biochemical Pathways and Environmental Responses in Plants: Part A"

August 2022

Biology Department

Brookhaven National Laboratory

U.S. Department of Energy

USDOE Office of Science (SC), Basic Energy Sciences (BES) (SC-22)

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Running title: Monolignol P450s and redox partners

Biocatalytic system for comparatively assessing the functional association of monolignol cytochrome P450 monooxygenases with their redox partners

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Abstract

Lignin is a complex heterogenous polymer derived from oxidative radical polymerization of three monolignols, i.e., *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These lignin monomeric precursors structurally differ in their methoxy groups of the benzene rings. In phenylpropanoid-monolignol biosynthetic pathway, the endoplasmic reticulum (ER)-resident cytochrome P450 monooxygenases, cinnamate 4-hydroxylase, coumaroyl ester 3′-hydroxylase and ferulate 5-hydroxylase, establish the key structural characteristics of monolignols. The catalysis of cytochrome P450 monooxygenase requires reducing power, which is supplied by the ER electron transfer chains, composed of cytochrome P450 oxidoreductase (CPR), cytochrome b_5 reductase (CBR) and/or cytochrome b_5 protein (CB5), from cofactor NADPH or NADH. While NADPH-dependent CPR serves as the typical electron donor for most P450 enzymes, in some cases, the CBR-CB5 or CPR-CB5 electron transfer system also transfers electrons to the terminal P450 enzymes. There are tremendous studies focusing on the discovery and characterization of cytochrome P450 monooxygenases. However, very limited attention has been paid to the versatility and the roles of electron transfer components in the P450 catalytic system. Due to the membrane-residence property of both P450 enzymes and electron transfer components, it is challenging to establish an effective experimental system to evaluate the functional association of P450s with their redox partners. This chapter describes a yeast cell biocatalytic system and the related experimental procedures for comparatively assessing the functional relationship of monolignol biosynthetic P450 enzymes and different redox partners in their catalysis.

1. Introduction

Cytochrome P450 monooxygenases are heme-thiolate enzymes, constituting one of the largest and oldest protein superfamilies found in main life kingdoms, including bacteria, yeasts, mammals and plants. The enzymes catalyze extremely diverse reactions leading to the precursors of structural macromolecules such as lignin, cutin, suberin and sporopollenin, or are involved in biosynthesis or catabolism of small molecule natural products such as pigments, odorants, flavors, antioxidants, allelochemicals and defense compounds, plant hormone and signaling molecules, and in the xenobiotics metabolism (Bak et al., 2011; Bernhardt, 2006; Degtyarenko & Archakov, 1993; Nelson, 2009). The P450-catalyzed reactions insert one of the oxygen atoms from active O_2 into the substrate and reduce the other to water; therefore, the enzymes are classified as monooxygenases. In each catalytic cycle, P450 enzyme coupled with its redox partner(s) acquires two electrons from reduced pyridine nucleotides (NADPH or NADH) to generate iron-oxygen intermediates that in turn oxidizes the substrates (Hannemann, Bichet, Ewen, & Bernhardt, 2007; Krest et al., 2013; Porter & Coon, 1991). In eukaryotic cells, there are two primary electron transfer systems that interact with microsomal P450s and deliver electrons from reductant cofactors to the prosthetic heme group of the P450 enzyme: NADPH-cytochrome P450 oxidoreductase (CPR) chain, and NADH-cytochrome b_5 reductase (CBR)-cytochrome b_5 (CB5) chain (Hannemann et al., 2007; Iyanagi, 2019) (Fig. 1). Since 1971, the involvement of CB5 in the CPR electron transfer system has been evidenced (Hildebrandt & Estabrook, 1971). However, whether CB5 is obligatory for the catalysis of P450s and how CB5 participates in the CPR electron transfer chain remain controversial. Most P450 activities could be

reconstituted with only CPR and P450 itself, which argues against an obligatory role for cytochrome b_5 in this pathway (Hannemann, Bichet, Ewen, & Bernhardt, 2007)(Jensen & Moller, 2010); whereas many studies also reveal that a subset of P540-catalyzed reactions can be stimulated by CB5 protein (Porter, 2002). Moreover, CB5 displays multifaceted effects on P450 catalysis. The protein shows stimulation, no effect, or inhibition effect to the P450 monooxygenase activity in drug metabolism (Porter, 2002; Schenkman & Jansson, 2003). Collectively, two hypothetic roles are proposed to the CB5 protein in P450 catalysis: allosteric modulator or electron donor (Iyanagi, 2019; Porter, 2002; Schenkman & Jansson, 2003). CB5 is readily reduced by NADH-dependent CBR and NADPH-dependent CPR, suggesting CB5 can act as an electron carrier to shuttle reducing equivalent from either reductases to the terminal acceptors (Enoch & Strittmatter, 1979; Fukuchi-Mizutani, Mizutani, Tanaka, Kusumi, & Ohta, 1999). Cases from mice, human and fungi evidence that the CBR-CB5 electron transfer chain can donate entire electrons to some P450s (Estrada, Laurence, & Scott, 2016; Henderson, McLaughlin, & Wolf, 2013; Ichinose & Wariishi, 2012; Stiborová et al., 2016); whereas in CPR-CB5 pathway, CB5 was proposed to donate the second electron to oxyferrous P450 (Porter, 2002; Duggal et al., 2016). Furthermore, although CB5 can accept electron from CPR, the turnover efficiency is lower than that of CBR (Bhatt, Khatri, Rodgers, & Martin, 2017; Enoch & Strittmatter, 1979; Niu et al., 2017). Therefore, CB5 as an electron carrier to augment P450-catalyzed reactions probably have two different modes: 1) direct electron transfer of both required electrons from NADH-CBR to P450 in a pathway separate and independent of NADPH-CPR; 2) transfer of the second electron to oxyferrous P450 from either CPR or CBR (Porter, 2002) (Fig. 1). In addition to the documented electron shuttle role, some studies also suggest the allosteric stimulation effect of CB5 protein to cytochrome P450 activity. The activities of some P450s could be stimulated with incorporation of an apo-CB5, devoid of the heme group (Yamazaki, Johnson, Ueng, Shimada, & Guengerich, 1996; Yamazaki et al., 2002); in P450 17A1-catalyzed reaction, the P450 conformation is changed with the binding of CB5 protein, which either alters the selective substrate affinity or increases the catalytic efficiency of the enzyme (Estrada et al., 2016). These data suggest an allosteric stimulation of CB5 to P450 enzyme without electron transfer.

[Insert Fig. 1 here]

Fig. 1 Electron transfer systems supplying reducing equivalence to microsomal cytochrome P450.

While a subset of mammalian P450s involved in xenobiotics and drug metabolism were found to be affected by CB5 protein in their catalyses (Porter, 2002), CB5 proteins have been recognized to functionally associate with plant P450-catalyzed reactions in just a couple cases so far (de Vetten et al., 1999; Gou et al., 2019). Nevertheless, those *in planta* evidence unambiguously demonstrate the indispensable role of CB5 as an electron donor in stimulating P450-catalzyed reactions in plant phenolic biosynthesis. An early genetic study discovered a petunia *cb5* locus, *Dif F,* that predominantly expresses in the flower of petunia and is required for the full activity of flavonoid 3′,5′-hydroxylase (F3′5′H) for the synthesis of 3′, 5′ substitution of anthocyanins and the purple/blue coloration of flora. Disruption of *Dif F* gene by targeted transposon mutagenesis reduced F3′5′H enzymatic activity and the accumulation of 5′-substituted anthocyanins, resulting in an altered petunia flower colour (de Vetten et al., 1999). Interestingly the loss of cb5 locus does not influence the activities of other cytochrome P450s, including the functionally close homolog flavonoid 3′-hydroxylase in the anthocyanin biosynthetic pathway (de Vetten et al., 1999). These data suggest that cytochrome b_5 regulates the activity of specific P450s in plant metabolism. Recently, among four ER-localized CB5 isoforms in Arabidopsis, AtCB5D was discovered from the immunoprecipitated proteins with monolignol biosynthetic P450s as bait (Gou et al., 2019). The protein was then found to specifically functionally associate with ferulate 5-hydroxylase (F5H) for syringyl lignin synthesis (Gou et al., 2019). The heterogenous biopolymer lignin plays a crucial role in plant growth and development and in the resistance to environmental stress. It confers

hydrophobicity of vasculature for water conductance, mechanical strength for upright growth, and physical barrier against microbial and herbivory attacks (Boerjan, Ralph, & Baucher, 2003). *P*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol are three major lignin monomeric precursors (i.e., monolignols) that undergo oxidative radical polymerization to form *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin subunits, respectively (Boerjan et al., 2003). Three cytochrome P450 monooxygenases catalyze the hydroxylation of the benzene ring of phenylpropanoid derivatives to yield structurally characteristic monolignols. They are *trans*-cinnamic acid 4-hydroxylase (C4H; CYP73A5), *p*-coumaroyl ester 3′-hydroxylase (C3′H; CYP98A3), and ferulate/coniferyl alcohol/coniferaldehyde 5-hydroxylase (F5H; CYP84A1). With *t*-cinnamic acid as starting precursor, C4H catalyzes the 4-position hydroxylation of the phenyl ring yielding *p*-coumaric acid that is required for the formation of all three types of lignin monomeric precursors. C3′H and F5H catalyze the hydroxylation reactions at C3- and C5-positions of benzene ring, sequentially, leading to the biosynthesis of G and S lignin monomers. *Arabidopsis* genome encodes two ER-localized CPRs (ATR1 and ATR2) and two CBR proteins with only one located to the ER (Shockey et al., 2005; Wayne, Wallis, Kumar, Markham, & Browse, 2013). ATR2 protein has been demonstrated to physically interact with monolignol biosynthetic C4H and C3′H (Guo, Ran, Martin, & Liu, 2018) and be involved in lignin biosynthesis (Gou et al., 2019; Sundin et al., 2014). The composition of Arabidopsis stem lignin and phenolic metabolites changed significantly in *atr2* mutants, indicating the involvement of ATR2 in lignin formation (Sundin et al., 2014). Notably, disruption of *ATR2* resulted in the accumulation of the biosynthetic precursors of all three monolignol biosynthetic P450 enzymes (Sundin et al., 2014), implicating that ATR2 likely functionally associates with C4H, C3′H and F5H-catalyzed reactions without discrimination. In addition to the genes encoding both reductases, six *CB5* and *CB5-like* genes are annotated in *Arabidopsis* genome which encode five canonical CB5 isoforms (AtCB5A~AtCB5D) and a CB5-like protein (AtCB5LP). Four of them locate to the ER membrane (Hwang et al., 2004; Maggio, Barbante, Ferro, Frigerio, & Pedrazzini, 2007). CB5 is conventionally regarded to couple with CBR, and is implicated in the fatty acid desaturation or elongation (Kumar, Tran, Neelakandan, & Nguyen, 2012; Wayne et al., 2013). Nevertheless, our recent study unveiled that disruption of AtCB5D, one of the ER-localized CB5 family members, significantly impaired the biosynthesis of S-lignin subunits but had no detrimental effect on G-lignin formation. Consistently, the loss of CB5D reduced microsomal F5H activity but not the C4H activity, suggesting that AtCB5D is an indispensable electron donor for F5H-catalyed 5-hydroxylation of lignin monomeric precursor (Gou et al., 2019). However, it is still not fully resolved which electron transfer chain(s) that AtCB5D might be involved in for delivering reducing equivalence to augment F5H-catalyzed reaction. Due to the complexity of plant genetics and the long-life cycle of plant, it is difficult to assess P450 monooxygenase-electron transfer system with an *in planta* system, a relatively clean and effective heterologous system is much desired.

Among heterologous expression systems, for example, *Escherichia coli*, mammalian cell lines, and insects cells, yeast is a preferential choice for expressing microsomal P450s (Pompon, Louerat, Bronine, & Urban, 1996). The insufficiency of endogenous CPR present in yeast cells and the low sequence similarities of yeast CPR and CB5 with mammalian or plant counterparts limit the capacity of yeast cells to be directly used in determining catalytic-efficiency of P450s (Pompon et al., 1996). Nevertheless, these unique features of yeast cells offer opportunities for assessing the involvement of different electron transfer systems in a specific P450 monooxygenase-catalyzed reaction through heterologous expression of exogenous redox components. Furthermore, the availability of yeast mutants (partially) deficient in its endogenous electron transfer component(s) provides robust host strains with clean genetic background for heterologously determining the dependence of a particular P450 enzyme on electron transfer systems. Therefore, we adopt yeast cells deficient in their endogenous *CPR*, *CBR,* or *CB5* gene to establish a cell culture-based biocatalytic system, in which the plant-origin electron supply chains are selectively co-expressed with monolignol biosynthetic P450 enzymes, then biotransformation rate of the fed phenolic substrate is monitored and used as read-out to evaluate the functional association of the monolignol P450s with their electron donors. This established experimental system can be applied to evaluating the functional relationship of other cytochrome P450 monooxygenases from different metabolic processes with their electron donors.

This chapter describes the experimental procedure to establish and utilize the yeast biocatalytic system for comparatively assessing Arabidopsis C4H and F5H1 in the requirements for their redox partners *in vivo* and *in vitro*. C3´H was not included in the study due to the unavailability of its substrate. To determine the preferred electron transfer chains of C4H and F5H1, we co-expressed both enzymes with different combinations of *Arabidopsis thaliana* ATR1, ATR2, CBR1 and CB5D in yeast cells. A mutant version of AtCB5D, 2muCB5D, was adopted as a control. 2muCB5D has it's His-40 and -64 substituted with Ala; both His residues are known to be the key axial ligands for heme binding and are critical for transferring electrons to the iron center of heme (Cowley et al., 2002; Schenkman & Jansson, 2003; Vergeres & Waskell, 1995). Therefore, H40A and H64A mutations devoid the electron donor property of the mutant variant (Gou et al., 2019). Aided with the self-cleaving 2A peptide, polycistronic gene expression strategy was employed to integrate both C4H and F5H1 with the selected redox components into a same operon (Fig. 2). This strategy therefore ensures the co-expression of the targeted P450s and electron transfer components in the same yeast cells under the control of a same promoter. With this design, C4H and F5H1 operate in the exact same redox condition, thus facilitating the precise and reliable comparation of the needs of two P450 enzymes for particular electron transfer chains. The assays evidence that C4H couples with NADPH-ATR electron transfer chain for its catalysis; whereas F5H1 through CB5D associates with both NADH-CBR1 and NADPH-ATR pathways to fulfil its functionality.

2. Plasmid construction

pYeDP60 vector, developed for the effective P450 protein expression under the control of inducible *GAL10-CYC1* promoter (Pompon et al., 1996), was adopted in the whole yeast cell biocatalytic system. The vector was digested with *BamHI* and *EcoRI* restriction enzymes for subcloning. The coding sequences of *Arabidopsis* P450 genes, *C4H* and *F5H1*, and the electron transfer components *ATR1*, *ATR2*, *CBR1* and *CB5D* were amplified by polymerase chain reaction (PCR) using Phusion High-Fidelity DNA Polymerase (NEB) with gene specific primers and fusion primers where T2A or P2A sequence was incorporated into gene specific primers (Table 1). The purified PCR products were subcloned to pYeDP60 vector with Gibson assembly method (Gibson et al., 2009), which generated a set of expression cassettes, including pYeDP60-F5H1-2muCB5D-C4H (as control), pYeDP60-F5H1-CB5D-C4H, pYeDP60-F5H1- CBR1-2muCB5D-C4H, pYeDP60-F5H1-CBR1-CB5D-C4H, pYeDP60-F5H1-ATR1-2muCB5D-C4H, pYeDP60-F5H1-ATR1-CB5D-C4H, pYeDP60-F5H1-ATR2 -2muCB5D-C4H and pYeDP60-F5H1-ATR2 -CB5D-C4H plasmids (Fig. 2).

[Insert Fig. 2 here]

Fig. 2 The schema of T/P2A-based polycistronic expression cassettes for heterologous yeast protein production.

(**A**) F5H1-CB5D/2muCB5D-C4H. (**B**) F5H1-CB5D/2muCB5D-C4H-CBR1. (**C**) F5H1-CB5D/2muCB5D-C4H-ATR1. (**D**) F5H1-CB5D/2muCB5D-C4H-ATR2. GAL10-CYC1 promoter and PGK terminator were located at the pYeDP60 vector. *BamHI* and *EcoRI* recognition sites were used to insert PCR fragments of expressing cassettes.

2.1 Materials

1. Bio-Rad S1000 thermal cycler
2. New Brunswick Innova 42 incubator shaker (Eppendorf)
3. Thermomixer or water bath
4. LB liquid medium: tryptone 10 g/L, yeast extract 5 g/L, sodium chloride 10g/L
5. LB agar plate: LB liquid medium supplemented with 2% agar

6. Milli-Q water
7. Phusion high fidelity DNA polymerase (NEB, #M0530)
8. OneTaq 2X Master Mix with Standard Buffer (NEB, #M0482)
9. Wizard SV Gel and PCR Clean-Up System (Promega, #A9282)
10. GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, #K0503)
11. Agarose (Thomas Scientific, #C748D75)
12. *BamHI* and *EcoRI* restriction enzymes (Bio-Rad)

2.2 Procedure

1. Design primers according to the requirement of Gibson assembly method. To facilitate this procedure, *pYeDP60-genes* final construct maps are created first with Snapgene (Dotmatics, San Diego, US), then proper primer sequences were chosen (see note 1).

2. Solubilize primers to 100 µM in milli-Q water for long-term stocks and 20 µM working solutions are prepared.

3. Amplify the DNA fragments with Phusion high fidelity DNA polymerase (NEB), resolve PCR products on 1% agarose gel and recover target DNA fragments.

4. Digest 2 µg *pYeDP60* vector plasmid with *BamHI* and *EcoRI* restriction enzymes, resolve vector fragment on 1% agarose gel and recover vector backbone.

5. Prepare 2 X Gibson assembly master mix according to the describe procedure (Gibson et al., 2009).

6. Set up Gibson assembly reaction on ice with 100 ng of vector backbone, 1~2 fold molar excess of each insert DNAs, 5 µL of 2 X Gibson assembly master mix and bring up the total volume to 10 uL with milli-Q water (see note 2).

7. Incubate the assembly reactions in a thermocycler at 50 °C for 40 min.

8. Place samples on ice or keep at -20 °C for subsequent transformation.

9. Transform DH5α competent cells with heat shock method. Mix 50 µL competent cells with 5 µL of the assembly reaction, incubate on ice for 30 min, heat shock at 42 °C for 60 s, incubate on ice for 3 min, then add 1 mL LB medium, and incubate the cells at 37 °C for 30 min,

10. Spin down *E. coli* cells at 5,000 g (Eppendorf 5424R centrifuge) for 5 min and discard supernatant.

11. Resuspend cells in 100 µL water and plate them on LB agar plates containing 100 µg/mL ampicillin or carbenicillin.

12. Incubate the plates at 37 °C, overnight.

13. Conduct colony PCR screening of positive clones. Mix 10 µL OneTaq® 2X Master Mix (NEB), 9 µL water, 0.5 µL forward and reverse primers to prepare PCR reaction (see note 3). Pick a single colony with a sterile toothpick or pipette tip, stripe on a LB agar plate with grid marking to save clones for later culture, swirl the rest strain in PCR reaction mixture to introduce PCR template. For each transformation, 4 colonies are generally enough to find positive clones.

14. Analyse PCR product size in an agarose gel.

15. Inoculate one or two positive clones to LB liquid medium and culture at 37 °C for overnight.

16. Isolate plasmid DNA from overnight *E. coli* culture and verify the insert sequence with Sanger sequencing.

2.3 Notes

1. Most suppliers will increase the rate for oligonucleotides longer than 60 bp. When designing the gene-T2 fusion primers, the primer length and Tm value should be considered simultaneously.
2. The NEB suggests 1: 1 molar ratio of the each insert DNA over the vector DNA fragment. In the practice, 1 µL of the prepared insert DNA from the described procedures works efficiently.
3. Since multi-genes are assembled into one vector backbone, each of them should be verified with PCR. But for a time-saving purpose, the method described here is to use backbone primers to amplify the entire insert and then the products are resolved on agarose gel to estimate the PCR product size.

3. Yeast transformation

Yeast cells harbor endogenous electron transfer components, i.e., CB5, CBR1 and CPR (NCP1, NADPH-Cytochrome P450 reductase) on the ER membrane. To eliminate the potential disturbance of those endogenous redox components, *Saccharomyces cerevisiae* yeast mutant strains deficient in their endogenous *CB5*, *CBR1* or *NCP1* gene were adopted. Among them, the strain *ncp1* defective in *CPR* is a heterozygous diploid; while strains *cyb5* and *cbr1* deficient in *CB5* and *CBR1* are the homozygous.

3.1 Materials

1. Yeast strains: *cyb5* (MATa, BY4741 background, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cyb5Δ), *cbr1* (MATa, BY4741 background, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cbr1Δ) and *ncp1* (heterozygous diploids, BY4741/BY4742 background, his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 NCP1/ncp1Δ) (Transomic, Huntsville, US)
2. Plasmids: *pYeDP60-F5H1-2muCB5D-C4H, pYeDP60-F5H1-CB5D-C4H, pYeDP60-F5H1-CBR1-2muCB5D-C4H, pYeDP60-F5H1- CBR1-CB5D-C4H, pYeDP60-F5H1-ATR1-2muCB5D-C4H, pYeDP60-F5H1-ATR1-CB5D-C4H, pYeDP60-F5H1-ATR2 -2muCB5D-C4H* and *pYeDP60-F5H1-ATR2 -CB5D-C4H*
3. YPDA broth (Takara)
4. YPDA agar plate: YPDA broth supplemented with 2% agar
5. 200 mg/mL G418 stock solution
6. EZ-Yeast transformation kit (MP Biomedicals, Irvine, US)
7. SD-Ura agar plate containing 200 µg/mL G418
8. Eppendorf 5424R centrifuge with FA-45-24-11 rotor
9. New Brunswick Innova 42 incubator shaker

3.2 Procedure

1. Streak *cyb5*, *cbr1* and *ncp1* yeast strains on YPDA agar plates containing 200 µg/mL G418 sulfate (see note 1) and incubate at 28 °C for 2-3 days until single colonies can be observed (see note 2).
2. Inoculate a single colony into 4 mL YPDA medium containing 200 µg/mL G418 and incubate overnight at 28 °C with 250 rpm shaking.
3. Pellet the yeast cells with centrifugation at 5,000g (Eppendorf 5424R centrifuge) for 5 min.

4. Resuspend yeast pellet with Ez-yeast transformation solution (MP Biomedicals) to prepare master mix competent cells (see note 3).
5. For each transformation, add 5 µL carrier DNA (MP Biomedicals), 200 µg plasmid DNA and 70 µL yeast master mix to a 1.5 mL centrifuge tube.
6. Heat shock at 42 °C for 30 minutes; cool to 28 °C and keep for 30 minutes.
7. Pellet yeast cells at 5,000g (Eppendorf 5424R centrifuge) for 5 min and discard supernatant.
8. Resuspend in 100 µL water and plate in auxotrophic yeast medium devoid of Ura.
9. Incubate the plate at 28 °C for 2-3 days until single colonies can be observed.

3.3 Notes

1. The adopted yeast mutant strains possess KanMX4 selection marker. The addition of G418 sulfate antibiotics can prevent potential contamination and maintain the purity of the incubated strains.
2. The fresh yeast colonies from YPDA plate can be used for transformation directly. Scrape the fresh yeast colonies and resuspend in transformation solution to prepare master mix competent cells.
3. Normally, 1 mL overnight incubated yeast cells can be used for 5 transformations. Too many yeast cells used in the transformation generates more but tiny colonies on plates. Therefore, it is important to use proper amount of yeast cells to obtain discrete and healthy yeast colonies.

4. Yeast induction and *in vivo* feeding assays

4.1 Materials

1. Yeast minimal broth: SD-Ura-sugar, autoclaved at 121 °C for 15 min
2. 20% Glucose solution, autoclaved at 121 °C for 15 min or filtration sterilized
3. 20% Galactose solution, autoclaved at 121 °C for 15 min or filtration sterilized
4. TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA
5. Coniferyl alcohol (Sigma, #223735). 2 mM coniferyl alcohol solution was prepared by dissolving 3.6 mg powder in 2 mL methanol first, then adding water to final volume of 10 mL.
6. *t*-Cinnamic acid (Sigma, #C80857). 2 mM solution is prepared in the same way as above.
7. Water saturated ethyl acetate containing 0.1 mM chrysin (Sigma); chrysin is used as the extraction internal standard.
8. LUNA™ Automated Cell Counter (Logosbio, Gyeonggi-do, South Korea)
9. Thermo Scientific Labquake Tube Rotator
10. GENESYS™ 10S UV-Visible Spectrophotometer (Thermo Scientific)
11. Centrivap benchtop vacuum concentrator (Labconco, Kansas, US)
12. Eppendorf 5424R centrifuge with FA-45-24-11 rotor
13. Beckman GS-6 centrifuge with G.H. 3.8 rotor
14. New Brunswick Innova 42 incubator shaker

4.2 Procedure

1. Inoculate about 10 colonies of fresh transformed yeast cells into 4 mL of SD-Ura + 2% glucose selective broth. Incubate yeast starting culture at 28 °C with 250 rpm shaking for overnight.
2. Inoculate 0.5 mL starting culture into 15 mL SD-Ura + 2% glucose selective media in 50 mL flasks and incubate at 28 °C with 250 rpm shaking for about 6 hours.
3. Transfer the cultures to 50 mL centrifuge tubes and rinse the flasks with sterilized water once. Pellet yeast cells by centrifugation at 2,000 g (Beckman GS-6 centrifuge) for 5 min. Resuspend the yeast cell pellets with 2 mL SD-Ura + 2% galactose inductive media.
4. Measure the density of resuspended yeast cells with a LUNA™ Automated Cell Counter or with a UV-Visible spectrophotometer where 1 OD_{600} approximately equivale to 3 x 10^7 cells/mL.
5. Inoculate appropriately resuspended yeast cells into 15 mL SD-Ura + 2% galactose inductive media in the original 50 mL flasks to a final cell density of about 0.6 × 10^7 cells/mL (see note 1) Alternatively, measure the cell density of the resuspended yeast cells first, then calculate the required amount/volume of the inoculation strain.
6. Incubate at 28 °C with 250 rpm shaking for about 14 hours, until the cell density reaches 6-9 x 10^7 cells/mL (see note 2).
7. Pellet the galactose-induced yeast cells by centrifugation at 2,000 g (Beckman GS-6 centrifuge) for 5 min. Wash yeast cells once with TE buffer. Resuspend yeast cells with about 8 mL TE buffer. A cell density should be about 1.5 × 10^8 cells/ml.
8. P450 activity whole cell assays. For C4H assay, aliquot 0.5 mL yeast cells to 2 mL tubes containing one 5 mm glass bead each, add 125 µL 2 mM *t*-cinnamic acid substrate, incubate the mixture at 28 °C with slow rotation (Thermo Scientific Labquake Tube Rotator) for 0.5 hour (see note 3). For F5H1 assay, aliquot 1 mL yeast cells to 2 mL tubes containing one 5 mm glass bead each, add 125 µL 2 mM coniferyl alcohol substrate, incubate the reactions at 28 °C with slow rotation (Thermo Scientific Labquake Tube Rotator) for 3 hours (see note 4).
9. Add 0.5 mL water saturated ethyl acetate containing 0.1 mM chrysin, vortex vigorously to stop the reactions. Partition reaction products through centrifugation at 20,000 g (Eppendorf 5424R centrifuge) for 5 min.
10. 0.2 mL ethyl acetate solvent phase are taken, vacuum-dried, and stored in freezer.

4.3 Notes

1. Although most of the protocols claim the starting yeast cell density at about 5× 10^7 cells/mL or even higher (Nett, Lau, & Sattely, 2020; Urban et al., 1994), the best enzymatic activity monitored in our experiment was achieved with a lower starting cell density at about 0.6 × 10^7 cells/mL.
2. The longer induction time increases cell density of yeast culture; but the monitored P450 enzymatic activity decreases.
3. The catalytic efficiency of C4H is relatively high. Therefore, a relatively shorter incubation time was applied after the supplement of substrate to ensure adequate amount of substrate remaining during the reaction thus obtaining a more precise max velocity.
4. The catalytic efficiency of F5H1 is about 10 times lower than that of C4H. To obtain detectable amount of reaction product of F5H1, increasing the yeast amount in the reaction mixture is a better choice than extending incubation time.

5. Quantification of reaction products by UHPLC-MS

The P450 catalytic products were resolved and determined via an Ultra High Performance Liquid Chromatography (UHPLC) hyphenated to a Q-Exactive mass spectrometer. Retention times, ultraviolet-visible spectra and mass spectra were referred to confirm the F5H1-catalyzed product 5-hydroxyconiferyl alcohol and the C4H-catalyzed product *p*-coumaric acid. UV absorbance peak areas were acquired to quantify product abundance.

5.1 Materials

1. Thermofisher Scientific Dionex Ultimate 3000 UHPLC hyphenated to a Q-Exactive Plus mass spectrometer
2. C18 column (Luna, 150×2.1 mm, 1.6 µm, 00F-4742-AN, Phenomenex)
3. Guard cartridge (AJ0-9502, Phenomenex)
4. Mobile phase A: 0.1% acetic acid in water
5. Mobile phase B: 0.1% acetic acid in acetonitrile
6. A series of diluted coniferyl alcohol, coniferaldehyde and *p*-coumaric acid authentic standard samples
7. Eppendorf 5424R centrifuge with FA-45-24-11 rotor

5.2 Procedure

1. Redissolve the dried extracts in 100 µL 80% methanol and transfer samples to vial inserts sitting in 2 mL vials. Load experimental sample and standard sample vials in the autosampler tray maintaining at 4 °C.
2. Set chromatographic program with a gradient of solvent B in solvent A as 5% B (0 min); 30% B (1 min); 80% B (19 min); 99% B (20 min); 99% B (22 min); 5% B (23 min) at flow rate of 0.1 mL/min. The mass spectrometer parameters are set as the follows: sheath gas flow rate 40 units (negative model), aux gas unit flow rate 10; capillary temperature 350 °C; aux gas heater temperature 250 °C; spray voltage 3.5 kV; S lens RF level 60. The UV-Vis diode array detector detection wavelengths are 265, 280, 310 and 330 nm. Set an acquisition sequence including an instrument method, a file name, a directory path, a vial position, and an injection volume of 1 µL. A blank sample (80% methanol) is loaded at the first position to equilibrate the column.
3. Start the sequence run and data acquisition.
4. Perform peak integration of authentic standard samples and define the individual peak areas for coniferyl alcohol and *p*-coumaric acid, respectively. Plot the peak areas *vs* injection amount of a series of diluted standard samples and obtain the linear regression standard curves (Fig. 3) (see note 1).

[Insert Fig. 3 here]

Fig. 3 Standard curves for quantifying P450 C4H and F5H reaction products.
(A) *p*-Coumaric acid. **(B)** Coniferyl alcohol. The x-axis defines the amount of *p*-coumaric acid (**A**) or coniferyl alcohol (**B**), and the y-axis defines the peak area at 280 nm for *p*-coumaric acid (**A**) or 265 nm for coniferyl alcohol (**B**). Formula $y = mx + b$ was used to calculate the linear regression standard curve, where m and b represent the slope and intercept, respectively. (**C**) and (**D**) UV spectra of coniferyl alcohol (**C**) and 5-hydroxyconiferyl alcohol (**D**).

5. Perform peak integration of experimental samples and define absorptive peak areas for 5-hydroxyconiferyl alcohol (at 265 nm), *p*-coumaric acid (at 280 nm) and the internal standard chrysin (at 265 nm or 280 nm).

6. Calculate the catalytic efficiencies of F5H1 and C4H according to the equation below. Here, *A1* is the peak area of the product (5-hydroxyconiferyl alcohol or *p*-coumaric acid), *A2* is the peak area of the internal standard (chrysin). *c* is the y-intercept of peak area, and *m* is the slope of linear regression equition of standard curve. *V1* is the injection volume, *V2* is total sample volume, *t* is reaction time (min) and *n* is yeast cell number. *B1* is the total amount of ethyl acetate added to sample and *B2* is the amount of ethyl acetate taken from the partitioning.

$$\text{Catalytic efficiency (pmol. min}^{-1}\text{. cells}^{-1}) = \frac{(A1/(A2/\overline{A2}) - c) \times V2 \times B1}{m \times V1 \times t \times n \times B2}$$

7. Examples of the calculated F5H1 and C4H catalytic efficiencies from whole-cell biocatalytic assay in *cyb5* strain are shown in Fig. 4.

[Insert Fig. 4 here]

Fig. 4 The catalytic efficiencies of Arabidopsis C4H and F5H1 in *cyb5* strain determined via the whole cell biocatalytic assay.
(**A**) Catalytic activities of C4H with different electron transfer components. (**B**) Catalytic activities of F5H1 with different electron transfer components. Yeast strain deficient in its endogenous cytochrome b_5 (*cyb5 strain*) were used as the host. *Trans*-cinnamic acid or coniferyl alcohol were supplied as the substrate for C4H or F5H1, respectively. After 0.5- or 3- hours incubation at 28 °C, ethyl acetate extracts were monitored by UHPLC. Data are presented as means ± s. d. of three biological replicates from one set of representative experiment.

5.3 Notes

1. Because of the commercial unavailability of 5-hydroxyconiferyl alcohol authentic compound, the standard curve of coniferyl alcohol was used instead to quantify F5H enzymatic product. This is to take advantage of both compounds sharing similar UV spectra at the wavelength of 264~268 nm (Fig. 3C and D).

6. Yeast induction and microsomal protein preparation

6.1 Materials

1. Yeast strains containing different expression cassettes: *pYeDP60-F5H1-2muCB5D-C4H, pYeDP60-F5H1-CB5D-C4H, pYeDP60-F5H1-2muCB5D-C4H-CBR1, pYeDP60-F5H1-CB5D-C4H-CBR1, pYeDP60-F5H1-2muCB5D-C4H-ATR1, pYeDP60-F5H1-CB5D-C4H-ATR1, pYeDP60-F5H1-2muCB5D-C4H-ATR2* and *pYeDP60-F5H1-CB5D-C4H-ATR2*
2. Buffer A: 50 mM Tris/HCl (pH 7.4), 2 mM EDTA, 100mM KCl
3. Buffer B: 50 mM Tris/HCl (pH7.4), 2 mM EDTA, 0.6 M sorbitol
4. Buffer C: 50 mM Sodium Pipes (pH 7.0), 4 mM EDTA, 20% glycerol
5. 0.5 mm glass mill beads (BioSpec Products, # 11079105)
6. 100 µm cell strainers (Corning, # 07-201-432)
7. WHEATON® Tenbroeck Tissue Grinder, 2 mL
8. Eppendorf 5424R centrifuge with FA-45-24-11 rotor
9. GENESYS™ 10S UV-Visible Spectrophotometers (Thermo Scientific)
10. Beckman GS-6 centrifuge with G.H. 3.8 rotor
11. Sorvall RC 5C Plus centrifuge with SS-34 rotor

12. New Brunswick Innova 42 incubator shaker

6.2 Procedure

1. Streak the yeast strain stock on a SD-Ura + 200 µg/mL G418 agar plate. Incubate the plate at 28 °C for 2-3 days until single colonies are observed.
2. Inoculate about 10 yeast colonies into 10 mL SD-Ura + 2% glucose selective medium. Incubate yeast starting culture at 28 °C with 250 rpm shaking for overnight.
3. Inoculate the 10 mL yeast starting culture into 40 mL SD-Ura + 2% glucose selective medium. Incubate the secondary culture at 28 °C with 250 rpm shaking for about 8 hours (see note 1).
4. Transfer the secondary culture to 50 mL centrifuge tubes and rinse the flasks with sterilized water once. Pellet yeast cells by centrifugation at 2,000 g (Beckman GS-6 centrifuge) for 5 min. Resuspend the yeast cell pellets with 2 mL SD-Ura + 2% galactose inductive medium.
5. Inoculate appropriate resuspended yeast cells into 500 mL SD-Ura + 2% galactose inductive medium to a cell density about 0.6×10^7 cells/mL. Incubate at 28 °C with 250 rpm shaking for about 14 hours.
6. Pellet yeast cells at 2,000 g (Beckman GS-6 centrifuge) for 5 min and wash once with buffer A.
7. Resuspend yeast pellet with appropriate cold buffer B (0.3-0.5 g wet cell / ml) and move yeast cells to 50 mL centrifuge tubes.
8. Add same volume of glass beads.
9. Disrupt yeast cell walls mechanically by handshaking (30 s of shaking and 30 s interval, 10 cycles) in cold room. Otherwise, if the procedure is done at room temperature, keep yeast cells on ice during interval time (see note 2).
10. Place a cell strainer on a 50 mL centrifuge tube and pass the crude extracts.
11. Wash the glass beads for four times with 6 mL of buffer B each time and pass the strainer.
12. Centrifuge at 7,650 g (Sorvall RC 5C Plus centrifuge) at 4 °C for 10 min and recover the supernatant.
13. Repeat step 12 once and recover the supernatant (see note 3). Pool supernatant in step 12 and 13 together.
14. Add polyethyleneglyco1 4000 (at the final concentration of 0.1 g/mL) and NaCl (at final concentration of 8.8 mg/mL) to the supernatant.
15. Incubate the extracts on ice with periodic mixing or rotating in cold room for 1 hour.
16. Centrifuge at 9,682 g (Sorvall RC 5C Plus centrifuge) for 20 min to collect the microsomal protein fractions. Discard the supernatant. Wash the pellet twice with 10 mL buffer B.
17. Add 1.5 mL buffer C to the microsomal pellet and briefly resuspend the pellet. Transfer the microsomal pellet to a 2 mL Cole-Parmer Essentials PTFE tissue grinder. Add 0.5 mL buffer C to rinse the centrifuge tube to transfer all the pellet.
18. Homogenize the pellet gently with about 10 strokes.
19. Quantify the microsomal protein concentration using Bradford reagent. Adjust concentration to 10 mg/mL.
20. 200 µL aliquots are snap-frozen in liquid nitrogen and stored at -80°C until further use.

6.3 Notes

1. The purpose of this protocol is to compare the catalytic efficiencies of P450s coupling with different electron transfer components. Therefore, it is critical to keep all the experimental strains under the same condition and treatment.
2. Yeast cells are tough to lyse. The handshaking should be done vigorously.
3. When transferring the supernatants to a new tube after centrifugation, do not try to pour out all the upper layer liquid. Otherwise, it will introduce yeast cell debris contamination.

7. In vitro enzymatic assays with yeast microsomal preparation

NADH and NADPH are the reductant cofactors for CBR and CPR, respectively. Using the isolated microsomal proteins containing monolignol P450 enzymes and particular redox proteins, the preferences for NADH and NADPH in augmenting C4H- or F5H1-catalyzed reaction can be assessed.

7.1 Materials

1. Coniferaldehyde (Sigma, #382051), 2 mM solution is prepared in the same way as above.
2. *t*-Cinnamic acid (Sigma, #C80857), 2 mM solution is prepared in the same way as above.
3. Assay buffer: 50 mM Sodium Pipes (pH 7.0), 4 mM EDTA, 20% glycerol
4. 50 mM $NaHCO_3$ (pH 9)
5. NADH (Sigma, #10128023001), 100 mM solution was prepared in 50 mM $NaHCO_3$ (pH 9)
6. NADPH (Sigma, # 1010782400), 100 mM solution was prepared in 50 mM $NaHCO_3$ (pH 9)
7. Water saturated ethyl acetate containing 0.1 mM chrysin
8. Centrivap benchtop vacuum concentrator (Labconco)
9. Eppendorf 5424R centrifuge with FA-45-24-11 rotor
10. Beckman GS-6 centrifuge with G.H. 3.8 rotor
11. New Brunswick Innova 42 incubator shaker

7.2 Procedure:

1. Label two rows of nine 1.5 mL centrifuge tubes with A1 to A9 for F5H assays and B1 to B9 for C4H assays, respectively.
2. Add 1 µL of 50 mM $NaHCO_3$ (as control), 100 mM NADH, and 100 mM NADPH, respectively, to each of three tubes, until to all nine tubes, thus obtaining triplicates.
3. For F5H enzymatic assays, mix 95 µL 2 mM coniferaldehyde and 190 µL microsomal protein (at the concentration of 10 mg/mL) in 6.65 mL assay buffer; then aliquot 100 uL of the mixture to each tube of the row A. The final concentration of coniferaldehyde is 0.2 mM and microsomal protein is 200 µg per reaction (see note 1).
4. For C4H enzymatic assays, mix 95 uL 2 mM *t*-cinnamic acid and 47.5 µL microsomal protein (10 mg/mL) in 8.07 mL assay buffer; then aliquot 100 µL of the mixture to each tube of the row B. The final concentration of *t*-cinnamic acid is 0.2 mM and microsomal protein is 50 µg per reaction (see note 2).
5. Incubate the reactions at 28 °C for 1 hour.
6. Add 0.2 mL water saturated ethyl acetate containing 0.1 mM chrysin to each tube and vigorously vortex to stop reactions. Partition the reaction products by centrifugation at 20,000

g (Eppendorf 5424R centrifuge) for 5 min. 150 µL extracts are vacuum-dried and stored in freezer.

7. The reaction products are resuspended with 60 µL 80% methanol and quantified by UHPLC-MS following the same procedure as described for the whole cell biocatalytic assays (Section 6).
8. In LC-MS chromatographic profile, two product peaks can be observed in the F5H1-catalyzed in vitro reaction with coniferaldehyde as substrate. They are the expected reaction product 5-hydroxconiferaldehyde and the non-specific product ferulic acid (Fig. 5B). Presumably, the endogenous aldehyde dehydrogenase activity in yeast microsome might cause the formation of ferulic acid. Note that 5-hydroxyconiferaldehyde and ferulic acid have the same molecular weight but different MS2 spectra, which enable to distinguish them from each other (Fig. 5E to H).

[Insert Fig. 5 here]

Fig. 5 Characterization of C4H- and F5H1-catalysed reactions.

(**A**) and **(B)** the portion of UHPLC-UV profiles of the C4H (**A**) and F5H1 (**B**) -catalysed reactions. Microsomal proteins were extracted from the engineered *cyb5* yeast co-expressing C4H, F5H1, CBR1 and CB5D. The arrows in (**A**) and (**B**) indicate *p*-coumaric acid and 5-hydroxyconiferaldehyde products, respectively. The asterisk in (**B**) indicates the by-product ferulic acid. (**C** and **D**) Mass spectrum of *p*-coumaric acid (**C**) and MS2 spectrum of the molecular ion 163.0384 *m/z* (**D**). (**E** and **F**) Mass spectrum of 5-hydroxyconifer aldehyde product (**E**) and MS2 spectrum of the molecular ion 193.0491 *m/z* (**F**). (**G** and **H**) Mass spectrum of ferulic acid by-product (**G**) and MS2 spectrum of the molecular ion 193.0492 *m/z* (**H**). *Trans*-cinnamic acid and coniferaldehyde were used as the substrates for C4H (**A**) and F5H1 (**B**) respectively. NADH or NADPH were added to the reactions as reductants and a reaction without reductant (wo) was conducted as the control. After 1 hour incubation at 28 °C, ethyl acetate extracts were monitored by UHPLC-MS.

7.3 Notes

1. Both coniferaldehyde and coniferyl alcohol can be used as substrates for the in vitro enzymatic assays of F5H1 activity, but coniferaldehyde appears to be a more preferred substrate for the enzyme.
2. The catalytic efficiency of C4H is much higher than that of F5H1, which requires less microsomal proteins to obtain the detectable activity. Attention should be paid to ensure enough substrate in the reaction to obtain the precise max velocity.

8. Summary

Plant P450 systems (monooxygenases and the related redox partners) are the ER-localized membrane-bound proteins, which makes their characterization challenging. The heterologous yeast expression system is conventionally the preferential choice to produce P450 proteins and to determine their activities. P450 monooxygenase is insufficient for its activity, and the redox components are necessarily required for delivering reducing power to support the P450-catalyzed oxidative reaction. The method described here employs polycistronic gene expression strategy to integrate monolignol biosynthetic P450 enzymes with different electron transfer components in yeast mutant cells. This strategy combines genes encoding P450 enzymes and redox partners into a same operon, thus ensuring the synchronized gene expression and protein production. With the developed whole cell biocatalytic assays and the in vitro enzymatic activity confirmation, the described method offers a relatively time- and labour-efficient procedure to examine and compare the functional associations of

monolignol P450 enzymes with their preferred electron transfer systems. The described procedure can be applied to evaluate any given P450 monooxygenase-electron transport chain system.

Acknowledgment

This work was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under contract number DE-SC0012704 - specifically through the Physical Biosciences program of the Chemical Sciences, Geosciences and Biosciences Division (to C.-J.L.).

Author Contributions

X. Z. and C.-J. L. designed the experiments. X.Z. conducted experiments. X.Z. and C.-J. L. analyzed the data and wrote the manuscript.

Competing interests

The authors declare that they have no competing interests.

Data and materials availability

All data and materials needed to evaluate the conclusions in the paper are present in the paper. Additional data and materials related to this paper may be requested from C.J.L.

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Table 1. The primers used in this study

Primer name	Sequence (5' to 3')
F5H1-F	aatacacacactaaattacc**ggatcc**atggagtcttctatatcacaaac
F5H1-R	ctcctccacgtcaccgcatgttagaagacttcctctgccctcaagagcacagatgaggcg
CB5D-F	ctaacatgcggtgacgtggaggagaatcccggccctatgggcggagacggaaaag
CB5D-R	ctccagcttgcttaagaagagaaaaattagtagctccagatccagaagaaggagccttgg
C4H-F	tcttcttaagcaagctggagatgttgaagaaaatcctggacccatggacctcctcttgct
C4H-R	ctccacgtcaccgcatgttagaagacttcctctgccctcacagttccttggtttcataa
C4H-Rs*	agacatgggagatcccccgc**gaattc**ttaacagttccttggtttcataa
CBR1-F	ctaacatgcggtgacgtggaggagaatcccggccctatggataccgagtttctc
CBR1-R	agacatgggagatcccccgc**gaattc**tcagaactggaattgcatc
ATR1-F	ctaacatgcggtgacgtggaggagaatcccggccctatgacttctgctttgtatgcttc
ATR1-R	agacatgggagatcccccgc**gaattc**tcaccagacatctctgaggtatc
ATR2-F	ctaacatgcggtgacgtggaggagaatcccggccctatgtcctcttcttcttcttc
ATR2-R	agacatgggagatcccccgc**gaattc**ttaccatacatctctaagatatc

*C4H-Rs contains a stop codon which is designed for F5H1-CB5D-C4H and F5H1-2muCB5D-C4H tricistrons. The bold letters indicated *BamHI* and *EcoRI* restrictive enzyme sites.

Figure legends

Fig. 1 Electron transfer systems supplying reducing equivalence to microsomal cytochrome P450.

Fig. 2 The schema of T/P2A-based polycistronic expression cassettes for heterologous yeast protein production.

(**A**) F5H1-CB5D/2muCB5D-C4H. (**B**) F5H1-CB5D/2muCB5D-C4H-CBR1. (**C**) F5H1-CB5D/2muCB5D-C4H-ATR1. (**D**) F5H1-CB5D/2muCB5D-C4H-ATR2. GAL10-CYC1 promoter and PGK terminator were located at the pYeDP60 vector. *BamHI* and *EcoRI* recognition sites were used to insert PCR fragments of expressing cassettes.

Fig. 3 Standard curves for quantifying P450 C4H and F5H reaction products.

(A) *p*-Coumaric acid. **(B)** Coniferyl alcohol. The x-axis defines the amount of *p*-coumaric acid (**A**) or coniferyl alcohol (**B**), and the y-axis defines the peak area at 280 nm for *p*-coumaric acid (**A**) or 265 nm for coniferyl alcohol (**B**). Formula $y = mx + b$ was used to calculate the linear regression standard curve, where *m* and *b* represent the slope and intercept, respectively. (**C**) and (**D**) UV spectra of coniferyl alcohol (**C**) and 5-hydroxyconiferyl alcohol (**D**).

Fig. 4 The catalytic efficiencies of Arabidopsis C4H and F5H1 in *cyb5* strain determined via the whole cell biocatalytic assay.

(**A**) Catalytic activities of C4H with different electron transfer components. (**B**) Catalytic activities of F5H1 with different electron transfer components. Yeast strain deficient in its endogenous cytochrome b_5 (*cyb5 strain*) were used as the host. *Trans*-cinnamic acid or coniferyl alcohol were supplied as the substrate for C4H or F5H1, respectively. After 0.5- or 3- hours incubation at 28 °C, ethyl acetate extracts were monitored by UHPLC. Data are presented as means ± s. d. of three biological replicates from one set of representative experiment.

Fig. 5 Characterization of C4H- and F5H1-catalysed reactions.

(**A**) and **(B)** the portion of UHPLC-UV profiles of the C4H (**A**) and F5H1 (**B**) -catalysed reactions. Microsomal proteins were extracted from the engineered *cyb5* yeast co-expressing C4H, F5H1, CBR1 and CB5D. The arrows in (**A**) and (**B**) indicate *p*-coumaric acid and 5-hydroxyconiferaldehyde products, respectively. The asterisk in (**B**) indicates the by-product ferulic acid. (**C** and **D**) Mass spectrum of *p*-coumaric acid (**C**) and MS2 spectrum of the molecular ion 163.0384 *m/z* (**D**). (**E** and **F**) Mass spectrum of 5-hydroxyconifer aldehyde product (**E**) and MS2 spectrum of the molecular ion 193.0491 *m/z* (**F**). (**G** and **H**) Mass spectrum of ferulic acid by-product (**G**) and MS2 spectrum of the molecular ion 193.0492 *m/z* (**H**). *Trans*-cinnamic acid and coniferaldehyde were used as the substrates for C4H (**A**) and F5H1 (**B**) respectively. NADH or NADPH were added to the reactions as reductants and a reaction without reductant (wo) was conducted as the control. After 1 hour incubation at 28 °C, ethyl acetate extracts were monitored by UHPLC-MS.