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Rational Design Strategies for Functional Reconstitution of Plant Cytochrome P450s in Microbial Systems

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Abstract

Plant natural products (NPs) are of pharmaceutical and agricultural significance, yet the low abundance is largely impeding the broad investigation and utilization. Microbial bioproduction is a promising alternative sourcing to plant NPs. Cytochrome P450s (CYPs) play an essential role in plant secondary metabolism, and functional reconstitution of plant CYPs in the microbial system is one of the major challenges in establishing efficient microbial plant NP bioproduction. In this review, we briefly summarized the recent progress in rational engineering strategies for enhanced activity of plant CYPs in *Escherichia coli* and *Saccharomyces cerevisiae*, two commonly used microbial hosts. We believe that in-depth foundational investigations on the native microenvironment of plant CYPs are necessary to adapt the microbial systems for more efficient functional reconstitution of plant CYPs.

Introduction

The successful microbial production of the antimalarial artemisinic acid shed lights on the potential of synthesizing plant specialized metabolites from microbial systems as an alternative sourcing to these scarce and expensive molecules [1]. This strategy is further highlighted with the recent engineering efforts on the microbial production of opiates [2,3], tropane alkaloids [4], cannabinoids [5], and precursors to Taxol [6], vinblastine [7], and many more [8,9]. Cytochrome P450s (CYPs) are important biocatalysts that exist in almost all the living organisms in nature, and plants are especially enriched in CYPs – more than half of the currently annotated CYPs are encoded by plants [10], with a significant portion involved in plant specialized metabolism. Plant CYPs belong to Class II and are mostly localized in the endoplasmic reticulum (ER) membrane, with a few exceptions in the membrane of plastid and other organelles [11]. In addition, another

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Declaration of interests

None.

membrane-bound redox partner, CYP reductase (CPR) that normally uses NADPH as the electron donor, is always required by plant CYPs [12]. Multiple choices are available for the heterologous expression of plant CYPs: tobacco-agroinfiltration transient expression system [13,14] and baculovirus-insect cell expression system [15] are highly reliable in respect of functional reconstitution, while it is faster, cheaper, and easier to use microbial host systems. It is generally challenging to functionally reconstitute plant CYPs in microbial hosts that exhibit different membrane infrastructure and/or microenvironment. Although various microorganisms such as *Lactococcus lactis* [16], cyanobacteria [17], and algae [18,19] have emerged as feasible microbial hosts for the functional expression of plant CYPs or membrane-bound proteins, *E. coli* and yeast (mainly *Saccharomyces cerevisiae*, while non-conventional yeast such as *Pichia* and *Yarrowia* are gaining more attention recently [20]) remain the most used microorganisms due to the very well established genetic modification tools and metabolic engineering techniques. Here, we reviewed recent engineering strategies on functional reconstitution of plant CYPs in *E. coli* and yeast, aiming to depict a roadmap to rationally engineer plant CYPs in these microbes (Figure 1). The following topics have been very well reviewed [11,21–24] elsewhere and are not covered here: improving the activity of P450s through directed evolution, expression level tuning, process engineering, and co-factor optimization.

E. coli or Yeast, which is better for functional reconstitution of plant CYPs?

First, a choice needs to be made on the microbial host for the reconstitutions of plant CYPs. Numerous plant CYPs have been successfully reconstituted in both prokaryotic (primarily *E. coli*) and eukaryotic (primarily *Saccharomyces cerevisiae*) microbial systems (Table 1). Many argue that *E. coli* is not feasible for the functional reconstitution of CYPs due to the lack of endomembrane systems such as ER. However, as shown in Table 1, numerous plant CYPs have been reconstituted in *E. coli* for the synthesis of diverse phytochemicals. Yeast system, on the side, contains the ER membrane, and has been generally considered a preferred system for the heterologous expression of plant membrane-bound proteins. From Table 1, the variety of CYPs successfully reconstituted in *S. cerevisiae* is more diverse than *E. coli*. However, there are also certain CYPs that have been functionally expressed in *E. coli* but failed to exhibit activity in yeast, e.g. CYP81A15 and CYP81A24 [25,26]. Since there are no specific clans of P450s or types of metabolites that cannot be reconstituted in *E. coli*, we believe that with the correct choice of engineering strategies, both *E. coli* and yeast systems are suitable hosts for plant CYPs, yet more engineering efforts are normally necessary in *E. coli*.

Rational design to enable the functional reconstitution of plant CYPs

To have plant CYPs functionally reconstituted in the microbial host, engineering efforts need to be applied to meet two criteria: 1) the heterologous CYPs and CPRs are correctly localized, folded, and stable; 2) there is sufficient and efficient electron transfer from CPR to CYPs.

N-terminal modification could enhance folding and localization.

Currently, the major strategy to enhance folding and localization is to engineer the N-terminus of these membrane-bound proteins from plants. The N-terminal segment of plant P450s is responsible for locating CYPs into ER membrane [27,28]. Additionally, according to the widely accepted cotranslational protein folding theory, the N-terminus as the starting point of the folding process is also essential for correct protein folding [29]. Thus, N-terminal modifications are believed to improve the correct localization and folding of heterologous CYPs, and have been successfully applied to increase the activity of CYPs from different kingdoms [30–33]. N-terminal modification is almost a "must" strategy when using the *E. coli* system to establish a plant pathway involving CYPs, generally through removing or replacing the transmembrane peptide with more hydrophilic peptides, transmembrane peptides, or peptides shown to enhance expression levels or solubility (Table 2). While it is unknown where the hydrophilic peptides and the expression-enhancing tags would target CYPs, the transmembrane peptide of *E. coli* membrane proteins was believed to help implement CYPs into the cell membrane [34,35]. Plant CYPs are exclusively membrane-bound enzymes, yet surprisingly in some studies, the transmembrane peptides did not outperform other types of N-terminus modifications (Table 2), e.g. SohB$^{1-48}$-CYP79A1$^{36-558}$ exhibited a lower activity than 28-codon tag-CYP79A1$^{1-558}$ [34]. *E. coli* membrane is different from plant ER membrane in respect to chemical composition and membrane-associated protein degradation machinery, which in turn may affect the stability of membrane-bound proteins and the access to the substrates depending on the hydrophobicity. Thus, the transmembrane peptide may not always be the best choice in the *E. coli* system. Interestingly, an enhanced expression level does not always positively correlate with an enhanced catalytic activity (Table 2).

Compared with prokaryotic systems, it is easier to functionally reconstitute plant CYPs in yeast due to the presence of similar membrane infrastructures. The N-terminal modifications successfully utilized in yeast include exchanging the transmembrane peptide of the target P450s with more hydrophilic peptides, and transmembrane peptides from CYPs of a subfamily close to the target CYP (Table 2). The choice of boundary for the N-terminus modification is also important. In the yeast-based synthesis of opioid, chimeras of salutaridine synthase (SalSyn, CYP719B subfamily) and cheilanthifoline synthase from *Eschscholzia californica* (EcCFS, CYP719A5) at different boundaries were tested and only yEcCFS$^{1-83}$-yPsSalSyn$^{95-505}$ exhibited enhanced activity compared to yPsSalSyn [2]. In the N-terminal modification of CYP82Y1, N-terminus truncation or replacement with the N-terminus of membrane-bound proteins not close to CYP82Y1 all abolished the catalytic activity (Table 2) [36]. On the other hand, removing the N-terminus of CYP90B1 from *Arabidopsis thaliana* decreased but did not abolish the C22-hydroxylation activity [37]. The distinct behaviors of the truncated CYP82Y1 and CYP90B1 in yeast are possibly due to the different levels of interactions with *A. thaliana* CPR (ATR1), which is correctly localized on ER and may help recruit the truncated P450s back to ER under a stronger protein-protein interaction. These results indicate that certain groups of CYPs are highly sensitive towards N-terminal modifications, and the activity can be completely lost upon the improper truncation or modification.

Can C-terminal modification enhance folding and activity?

Different from N-terminal modification, C-terminal modification is less frequently utilized. In our previous study on CYP82Y1, introduction of the C-terminal 3×HA tag decreased the activity by more than 80% in yeast (data not published), compared to the one unmodified. The presence of the 3×HA tag at the C-terminus might affect the electron transfer efficiency between CYP82Y1 and CPR, which led to a substantial decrease in the catalytic activity [38]. However, the 3×HA tag has been utilized extensively in many biochemical characterization studies of plant CYPs expressed from *S. cerevisiae*. In *E. coli*, fusion GFP to C-terminus of CYP was used as a convenient way to indicate whether the engineered CYP was correctly folded or not [34]. The effect of the C-terminal GFP fusion was examined on CYP79A1 with different N-terminal modifications, and the C-terminal GFP fusion generally led to 15%−75% decrease of the catalytic activity in comparison to the corresponding CYP79A1 variants without the GFP fusion. This is consistent with what we observed on the effect of 3×HA tag on CYP82Y1 in yeast. For now, there is not enough data to conclude whether a C-terminal modification can help the folding and activity of plant CYPs in microbial systems or not. However, if a C-terminal modification is needed, tags of smaller size should be considered over the ones of larger size.

Strategies other than N- or C-terminal modifications to enhance the folding.

Although some chaperons have been utilized to enhance the folding of plant CYPs in *E. coli*, plant chaperones have not been used for this purpose [24]. Both yeast and *E. coli* have been utilized to express and characterize plant chaperons [39–41], so introducing plant chaperons to the microbial system may help the folding of plant CYPs and CPRs. In addition, expression levels may also affect the folding of P450s and morphology of ER membranes. When EcCFS was expressed from a high-copy plasmid in *S. cerevisiae*, the stability of the plasmid much decreased (10.7% versus 90% maintained for low-copy plasmid) and the morphology of the ER changed with EcCFS highly concentrated in patches instead of averagely distributed across ER [34]. The overall activity of EcCFS was also lower when expressed from a high-copy plasmid. In *E. coli*, similar observations have also been reported that sometimes lower expression levels of P450s and CPRs would favor the overall catalytic performance of the bioproduction platform [34,42]. However, mechanistic investigations need to be conducted to clearly conclude how expression level affects the folding, localization, and activity in these cases.

Strategies to enhance the pairing efficiency between P450 and CPR.

Although *S. cerevisiae* has an endogenous CPR (ScCPR), the efficiency of ScCPR is substantially lower than plant CPRs when paring with plant CYPs in yeast. Pairing CFS with ATR1 led to > 50-fold enhancement in cheilanthifoline production than ScCPR in yeast [43]. The paring with different plant CPRs often makes a substantial difference in the activity of CYPs [43–45], as well. This is consistent with the recent observation that monolignol biosynthetic CYPs exhibit distinct levels of interactions with different CPRs (ATR1 and ATR2) in yeast [46]. The prokaryotic system, on the other hand, does not encode CPRs and thus a plant CPR is needed for the functional reconstitution of CYPs. Since plant CPRs are also membrane-bound, the engineering strategy is similar to plant CYPs,

i.e. N-terminal truncation and modification. CPRs were often fused to the C-terminus of plant P450s [47,48] to enhance the CYP-CPR interaction, and has been successfully utilized in yeast (both *S. cerevisiae* and *Yarrowia lipolytica*) [49–52]. However, in one example, CYP725A4-CPR fusion proteins exhibited less than half of the activity towards the synthesis of oxygenated taxanes in comparison to the standalone CYP725A4 and CPR [42]. In another example, the activity of SbF6H(CYP82D1.1) was substantially enhanced when paired with the full length ATR1, instead of the N-terminal-truncated ATR1 in *E. coli* [53]. Thus, whether N-terminus of CPR should be truncated, and CYPs should be fused with CPR as a single peptide remain inconclusive. Previous investigation also indicates that the optimal ratio between P450 and CPR was approximately 12:1 [42]. Thus, adjusting the expression level of both P450 and CPR seems critical to achieving higher P450 activities based on several recent reviews [11,23,24].

Future Prospect

To date, we engineer plant CYPs in microbes case by case, because the engineering strategies are not generally applicable. More generic engineering strategies may be developed in the future if there is better understanding of the natural microenvironment around the CYPs in plants. For example, recently, *Arabidopsis* membrane steroid-binding proteins (MSBPs) was discovered as scaffolds to organize monolignol monooxygenases (belonging to CYPs) [46]. This discovery may enable the establishment of a more sophisticated CYP-CPR complex in yeast to achieve better electron transfer efficiency. In addition to native metabolon [54], artificial enzyme scaffolding [55] may also serve as a solution to mimic the native protein-protein interactions. Yeast cells producing cholesterol has been shown as a better heterologous host for the mammalian membrane proteins [56], and phytosterol-producing microbes may also provide a favorable membrane infrastructure for the folding and localization of plant membrane-bound proteins including CYPs. To sustain the rapid development of microbial production of plant NPs, it is necessary to go back to plant for in-depth understanding of plant specialized metabolism (e.g., temporal and spatial localization of metabolites and enzymes, metabolons, folding and post-translational modifications of enzymes, interactions between CYPs/CPRs and the membranes, regulation of these processes). These new insights would help “plantize” the microbial factories for better production efficiency.

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Reference:

1. Paddon CJ, Keasling JD: Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. Nature Reviews Microbiology2014, 12:355–367. [PubMed: 24686413]

*2. Galanie S, Thodey K, Trenchard IJ, Interrante MF, Smolke CD: Complete biosynthesis of opioids in yeast. Science2015, 349:1095–1100. [PubMed: 26272907] The authors tested N-terminal modification of SalSyn with different tags at different boundaries. The best N-terminus

modification enhanced the activity of SalSyn by > 2-fold in comparison to the unmodified version.

3. Nakagawa A, Matsumura E, Koyanagi T, Katayama T, Kawano N, Yoshimatsu K, Yamamoto K, Kumagai H, Sato F, Minami H: Total biosynthesis of opiates by stepwise fermentation using engineered Escherichia coli. Nature communications2016, 7:1–8.
4. Srinivasan P, Smolke CD: Biosynthesis of medicinal tropane alkaloids in yeast. Nature2020, 585:614–619. [PubMed: 32879484]
5. Luo XZ, Reiter MA, d'Espaux L, Wong J, Denby CM, Lechner A, Zhang YF, Grzybowski AT, Harth S, Lin WY, et al.: Complete biosynthesis of cannabinoids and their unnatural analogues in yeast. Nature2019, 567:123-+. [PubMed: 30814733]
6. Ajikumar PK, Xiao WH, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G: Isoprenoid Pathway Optimization for Taxol Precursor Overproduction in Escherichia coli. Science2010, 330:70–74. [PubMed: 20929806]
7. Brown S, Clastre M, Courdavault V, O'Connor SE: De novo production of the plant-derived alkaloid strictosidine in yeast. Proceedings of the National Academy of Sciences2015, 112:3205–3210.
8. Cravens A, Payne J, Smolke CD: Synthetic biology strategies for microbial biosynthesis of plant natural products. Nat Commun2019, 10:2142. [PubMed: 31086174]
9. Li S, Li Y, Smolke CD: Strategies for microbial synthesis of high-value phytochemicals. Nat Chem2018, 10:395–404. [PubMed: 29568052]
10. Nelson DR: Cytochrome P450 diversity in the tree of life. Biochim Biophys Acta Proteins Proteom2018, 1866:141–154. [PubMed: 28502748]

**11. Li Z, Jiang Y, Guengerich FP, Ma L, Li S, Zhang W: Engineering cytochrome P450 enzyme systems for biomedical and biotechnological applications. J Biol Chem2020, 295:833–849. [PubMed: 31811088] This review provides an up to date comprehensive overview on the engineering strategies of cytochrome P450 in different organisms, and covered topics not discussed here - directed evolution and protein engineering for enhanced and expanded activity of CYPs.

12. Mitchell AJ, Weng JK: Unleashing the Synthetic Power of Plant Oxygenases: From Mechanism to Application. Plant Physiol2019, 179:813–829. [PubMed: 30670605]
13. Reed J, Osbourn A: Engineering terpenoid production through transient expression in Nicotiana benthamiana. Plant Cell Rep2018, 37:1431–1441. [PubMed: 29786761]
14. Reed J, Stephenson MJ, Miettinen K, Brouwer B, Leveau A, Brett P, Goss RJM, Goossens A, O'Connell MA, Osbourn A: A translational synthetic biology platform for rapid access to gram-scale quantities of novel drug-like molecules. Metab Eng2017, 42:185–193. [PubMed: 28687337]
15. Duan H, Schuler MA: Heterologous expression and strategies for encapsulation of membrane-localized plant P450s. Phytochemistry Reviews2006, 5:507–523.
16. Frelet-Barrand A, Boutigny S, Moyet L, Deniaud A, Seigneurin-Berny D, Salvi D, Bernaudat F, Richaud P, Pebay-Peyroula E, Joyard J, et al.: Lactococcus lactis, an alternative system for functional expression of peripheral and intrinsic Arabidopsis membrane proteins. PLoS One2010, 5:e8746. [PubMed: 20098692]
17. Wlodarczyk A, Gnanasekaran T, Nielsen AZ, Zulu NN, Mellor SB, Luckner M, Thofner JFB, Olsen CE, Mottawie MS, Burow M, et al.: Metabolic engineering of light-driven cytochrome P450 dependent pathways into Synechocystis sp. PCC 6803. Metab Eng2016, 33:1–11. [PubMed: 26548317]
18. Gangl D, Zedler JA, Wlodarczyk A, Jensen PE, Purton S, Robinson C: Expression and membrane-targeting of an active plant cytochrome P450 in the chloroplast of the green alga Chlamydomonas reinhardtii. Phytochemistry2015, 110:22–28. [PubMed: 25556316]
19. D'Adamo S, Schiano di Visconte G, Lowe G, Szaub-Newton J, Beacham T, Landels A, Allen MJ, Spicer A, Matthijs M: Engineering the unicellular alga Phaeodactylum tricornutum for high-value plant triterpenoid production. Plant Biotechnol J2019, 17:75–87. [PubMed: 29754445]
20. Jiang L, Huang L, Cai J, Xu Z, Lian J: Functional expression of eukaryotic cytochrome P450s in yeast. Biotechnol Bioeng2020.

21. Liu X, Zhu X, Wang H, Liu T, Cheng J, Jiang H: Discovery and modification of cytochrome P450 for plant natural products biosynthesis. Synth Syst Biotechnol2020, 5:187–199. [PubMed: 32637672]

*22. Urlacher VB, Girhard M: Cytochrome P450 Monooxygenases in Biotechnology and Synthetic Biology. Trends Biotechnol2019, 37:882–897. [PubMed: 30739814] This recent review summarizes the types of chemistry CYPs may catalyze and their uses in biotechnology.

23. Xu LH, Du YL: Rational and semi-rational engineering of cytochrome P450s for biotechnological applications. Synth Syst Biotechnol2018, 3:283–290. [PubMed: 30533540]

**24. Hausjell J, Halbwirth H, Spadiut O: Recombinant production of eukaryotic cytochrome P450s in microbial cell factories. Biosci Rep2018, 38.This review provides a comprehensive overview on the strategies utilized to ensure heterologous expression of CYPs in the microbial systems not limited to *E. coli* and yeast. The authors have carefully discussed topics not covered here: harnessing chaperones and process engineering to enhance the folding of CYPs including plant CYPs.

**25. Guo F, Iwakami S, Yamaguchi T, Uchino A, Sunohara Y, Matsumoto H: Role of CYP81A cytochrome P450s in clomazone metabolism in Echinochloa phyllopogon. Plant Sci2019, 283:321–328. [PubMed: 31128703] The author claimed that they failed to detect the activity of CYP81A15 and CYP81A24 expressed in yeast using the same strategy that have functionally reconstituted CYP81A12 and CYP81A21 before.

**26. Dimaano NG, Yamaguchi T, Fukunishi K, Tominaga T, Iwakami S: Functional characterization of cytochrome P450 CYP81A subfamily to disclose the pattern of cross-resistance in Echinochloa phyllopogon. Plant Mol Biol2020, 102:403–416. [PubMed: 31898147] The activity of CYP81A15 and CYP81A24 was detected when co-expressed with ATR2 in *E. coli* (both N-terminal truncated).

27. Sakaguchi M, Mihara K, Sato R: A short amino-terminal segment of microsomal cytochrome P-450 functions both as an insertion signal and as a stop-transfer sequence. EMBO J1987, 6:2425–2431. [PubMed: 2822391]

28. Ruan KH, So SP, Zheng W, Wu J, Li D, Kung J: Solution structure and topology of the N-terminal membrane anchor domain of a microsomal cytochrome P450: prostaglandin I2 synthase. Biochem J2002, 368:721–728. [PubMed: 12193162]

29. Fedorov AN, Baldwin TO: Cotranslational protein folding. J Biol Chem1997, 272:32715–32718. [PubMed: 9407040]

30. Doray B, Chen CD, Kemper B: N-terminal deletions and His-tag fusions dramatically affect expression of cytochrome p450 2C2 in bacteria. Arch Biochem Biophys2001, 393:143–153. [PubMed: 11516171]

31. vonWachenfeldt C, Richardson TH, Cosme J, Johnson EF: Microsomal P450 2C3 is expressed as a soluble dimer in Escherichia coli following modifications of its N-terminus. Archives of Biochemistry and Biophysics1997, 339:107–114. [PubMed: 9056240]

32. Mao WF, Berenbaum MR, Schuler MA: Modifications in the N-terminus of an insect cytochrome P450 enhance production of catalytically active protein in baculovirus-Sf9 cell expression systems. Insect Biochemistry and Molecular Biology2008, 38:66–75. [PubMed: 18070666]

33. Leonard E, Koffas MA: Engineering of artificial plant cytochrome P450 enzymes for synthesis of isoflavones by Escherichia coli. Appl Environ Microbiol2007, 73:7246–7251. [PubMed: 17905887]

*34. Christensen U, Vazquez-Albacete D, Sogaard KM, Hobel T, Nielsen MT, Harrison SJ, Hansen AH, Moller BL, Seppala S, Norholm MHH: De-bugging and maximizing plant cytochrome P450 production in Escherichia coli with C-terminal GFP fusions. Appl Microbiol Biotechnol2017, 101:4103–4113. [PubMed: 28204885] This work used C-terminal GFP fusion as a quick screening strategy to indicate the expression level and folding of P450s with different N-terminal modifications. The CYP activity substantially decreased with the addition of the C-terminal GFP. Different bacterial transmembrane peptides, 8RP, and 28-codon tags all increased the expression level of CYPs tested, but only the CYP chimera with 28-codon tag and transmembrane tag from SohB exhibited enhanced activity. The 28-codon tag outperforms SohB N-terminus in respect of enhancing the activity of CYP79A1.

35. Vazquez-Albacete D, Montefiori M, Kol S, Motawia MS, Moller BL, Olsen L, Norholm MH: The CYP79A1 catalyzed conversion of tyrosine to (E)-p-hydroxyphenylacetaldoxime unravelled using an improved method for homology modeling. Phytochemistry2017, 135:8–17. [PubMed: 28088302]

*36. Li Y, Smolke CD: Engineering biosynthesis of the anticancer alkaloid noscapine in yeast. Nat Commun2016, 7:12137. [PubMed: 27378283] In this work, N-terminus truncation or improper N-terminus modifications completely abolished the activity of CYP82Y1 in yeast coexpresing ATR1.

*37. Xu S, Chen C, Li Y: Engineering of Phytosterol-Producing Yeast Platforms for Functional Reconstitution of Downstream Biosynthetic Pathways. ACS Synth Biol2020.In this work, N-terminus truncation slightly decreased but retained the activity of CYP90B1 in yeast coexpressing ATR1.

38. Guengerich FP: Mechanisms of Cytochrome P450-Catalyzed Oxidations. ACS Catal2018, 8:10964–10976. [PubMed: 31105987]

39. Zhang ZM, Sullivan W, Felts SJ, Prasad BD, Toft DO, Krishna P: Characterization of plant p23-like proteins for their co-chaperone activities. Cell Stress & Chaperones2010, 15:703–715. [PubMed: 20349287]

40. Cloney LP, Bekkaoui DR, Wood MG, Hemmingsen SM: Assessment of Plant Chaperonin-60 Gene-Function in Escherichia-Coli. Journal of Biological Chemistry1992, 267:23333–23336.

41. Aigner H, Wilson RH, Bracher A, Calisse L, Bhat JY, Hartl FU, Hayer-Hartl M: Plant RuBisCo assembly in E. coli with five chloroplast chaperones including BSD2. Science2017, 358:1272-+. [PubMed: 29217567]

**42. Biggs BW, Lim CG, Sagliani K, Shankar S, Stephanopoulos G, De Mey M, Ajikumar PK: Overcoming heterologous protein interdependency to optimize P450-mediated Taxol precursor synthesis in Escherichia coli. Proc Natl Acad Sci U S A2016, 113:3209–3214. [PubMed: 26951651] Investigations on the effect of N-terminus modification, CPR fusion, and expression level have been conducted on CYP725A4 in *E. coli*. The results indicate that CYP/CPR fusion is not always a better option than the stand-alone CYP and CPR, lower expression level may instead help the overall activity of CYPs, tags that enhance the expression level do not always enhance the activity.

**43. Trenchard IJ, Smolke CD: Engineering strategies for the fermentative production of plant alkaloids in yeast. Metab Eng2015, 30:96–104. [PubMed: 25981946] Investigations on the effect of expression method, CPR pairing, and expression level have been conducted on EcCFS in yeast. The results indicate that plasmid of different copy number showed different stability, CYP expressing from a high-copy number plasmid change ER morphology and the localization, pairing with different CPRs can lead to substantial difference in CYP activity, and ScCPR is far less active in facilitating the activity of CYP in comparison to plant CPRs.

44. Zhu M, Wang C, Sun W, Zhou A, Wang Y, Zhang G, Zhou X, Huo Y, Li C: Boosting 11-oxo-β-amyrin and glycyrrhetinic acid synthesis in Saccharomyces cerevisiae via pairing novel oxidation and reduction system from legume plants. Metabolic engineering2018, 45:43–50. [PubMed: 29196123]

45. Dai ZB, Liu Y, Sun ZT, Wang D, Qu G, Ma XL, Fan FY, Zhang LL, Li SL, Zhang XL: Identification of a novel cytochrome P450 enzyme that catalyzes the C-2 alpha hydroxylation of pentacyclic triterpenoids and its application in yeast cell factories. Metabolic Engineering2019, 51:70–78. [PubMed: 30339834]

46. Gou M, Ran X, Martin DW, Liu CJ: The scaffold proteins of lignin biosynthetic cytochrome P450 enzymes. Nat Plants2018, 4:299–310. [PubMed: 29725099]

47. Liu F, Yang H, Wang L, Yu B: Biosynthesis of the High-Value Plant Secondary Product Benzyl Isothiocyanate via Functional Expression of Multiple Heterologous Enzymes in Escherichia coli. ACS Synth Biol2016, 5:1557–1565. [PubMed: 27389525]

*48. Moon JH, Lee K, Lee JH, Lee PC: Redesign and reconstruction of a steviolbiosynthetic pathway for enhanced production of steviol in Escherichia coli. Microb Cell Fact2020, 19:20. [PubMed: 32013995] In this work, N-terminus truncated CYP714A2 and ATR2 fusion connected with linkers of different lengths (1×, 2×, and 3×GGGGS) were examined towards the synthesis of stevinol in *E. coli*. In comparison to the stand-alone truncated CYP714A2 and ATR2, all the three

CYP714A2-ATR2 fusions exhibited higher stevinol production, which is positively correlated with the length of the linker

49. Zhao F, Bai P, Liu T, Li D, Zhang X, Lu W, Yuan Y: Optimization of a cytochrome P450 oxidation system for enhancing protopanaxadiol production in Saccharomyces cerevisiae. Biotechnol Bioeng2016, 113:1787–1795. [PubMed: 26757342]
50. Guo X, Sun J, Li D, Lu W: Heterologous biosynthesis of (+)-nootkatone in unconventional yeast Yarrowia lipolytica. Biochemical Engineering Journal2018, 137:125–131.
51. Sun J, Zhang C, Nan W, Li D, Ke D, Lu W: Glycerol improves heterologous biosynthesis of betulinic acid in engineered Yarrowia lipolytica. Chemical Engineering Science2019, 196:82–90.
52. Li D, Wu Y, Zhang C, Sun J, Zhou Z, Lu W: Production of Triterpene Ginsenoside Compound K in the Non-conventional Yeast Yarrowia lipolytica. J Agric Food Chem2019, 67:2581–2588. [PubMed: 30757901]
*53. Li J, Tian C, Xia Y, Mutanda I, Wang K, Wang Y: Production of plant-specific flavones baicalein and scutellarein in an engineered E. coli from available phenylalanine and tyrosine. Metabolic engineering2019, 52:124–133. [PubMed: 30496827] The authors tested the activity of a set of N-terminal modified F6H in the presence of N-terminus truncated CPR or full-length CPR. Surprisingly, when paired with the N-terminus truncated CPR, there is no difference in the activity of the N-terminus truncated or modified F6H; while the activity of F6H mutant substantially enhanced when assayed with full length CPR, with the best using the more hydrophilic 2B1 peptide.
54. Obata T: Metabolons in plant primary and secondary metabolism. Phytochemistry Reviews2019, 18:1483–1507.
55. Wheeldon I, Minteer SD, Banta S, Barton SC, Atanassov P, Sigman M: Substrate channelling as an approach to cascade reactions. Nat Chem2016, 8:299–309. [PubMed: 27001725]
56. Hirz M, Richter G, Leitner E, Wriessnegger T, Pichler H: A novel cholesterol-producing Pichia pastoris strain is an ideal host for functional expression of human Na,K-ATPase alpha3beta1 isoform. Appl Microbiol Biotechnol2013, 97:9465–9478. [PubMed: 23955473]
57. Kunii M, Kitahama Y, Fukushima EO, Seki H, Muranaka T, Yoshida Y, Aoyama Y: β-Amyrin oxidation by oat CYP51H10 expressed heterologously in yeast cells: the first example of CYP51-dependent metabolism other than the 14-demethylation of sterol precursors. Biological and Pharmaceutical Bulletin2012, 35:801–804. [PubMed: 22687421]
58. Gavira C, Hofer R, Lesot A, Lambert F, Zucca J, Werck-Reichhart D: Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by Saccharomyces cerevisiae. Metab Eng2013, 18:25–35. [PubMed: 23518241]
59. Meng X, Liu H, Xu W, Zhang W, Wang Z, Liu W: Metabolic engineering Saccharomyces cerevisiae for de novo production of the sesquiterpenoid (+)-nootkatone. Microbial cell factories2020, 19:21. [PubMed: 32013959]
60. Kotopka BJ, Smolke CD: Production of the cyanogenic glycoside dhurrin in yeast. Metab Eng Commun2019, 9:e00092. [PubMed: 31110942]
61. Bak S, Kahn RA, Nielsen HL, Moller BL, Halkier BA: Cloning of three A-type cytochromes P450, CYP71E1, CYP98, and CYP99 from Sorghum bicolor (L.) Moench by a PCR approach and identification by expression in Escherichia coli of CYP71E1 as a multifunctional cytochrome P450 in the biosynthesis of the cyanogenic glucoside dhurrin. Plant Mol Biol1998, 36:393–405. [PubMed: 9484480]
62. Ro DK, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, et al.: Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature2006, 440:940–943. [PubMed: 16612385]
63. Chang MC, Eachus RA, Trieu W, Ro DK, Keasling JD: Engineering Escherichia coli for production of functionalized terpenoids using plant P450s. Nat Chem Biol2007, 3:274–277. [PubMed: 17438551]
64. Hodgson H, De La Pena R, Stephenson MJ, Thimmappa R, Vincent JL, Sattely ES, Osbourn A: Identification of key enzymes responsible for protolimonoid biosynthesis in plants: Opening the door to azadirachtin production. Proc Natl Acad Sci U S A2019, 116:17096–17104. [PubMed: 31371503]

65. Zhang C, Liu J, Zhao F, Lu C, Zhao G-R, Lu W: Production of sesquiterpenoid zerumbone from metabolic engineered Saccharomyces cerevisiae. Metabolic engineering2018, 49:28–35. [PubMed: 30031850]

66. Yu F, Okamoto S, Harada H, Yamasaki K, Misawa N, Utsumi R: Zingiber zerumbet CYP71BA1 catalyzes the conversion of alpha-humulene to 8-hydroxy-alpha-humulene in zerumbone biosynthesis. Cell Mol Life Sci2011, 68:1033–1040. [PubMed: 20730551]

67. Schoch GA, Attias R, Belghazi M, Dansette PM, Werck-Reichhart D: Engineering of a water-soluble plant cytochrome P450, CYP73A1, and NMR-based orientation of natural and alternate substrates in the active site. Plant Physiol2003, 133:1198–1208. [PubMed: 14576280]

68. Zhou K, Qiao KJ, Edgar S, Stephanopoulos G: Distributing a metabolic pathway among a microbial consortium enhances production of natural products. Nature Biotechnology2015, 33:377–U157.

69. Gu MY, Wang MZ, Guo J, Shi C, Deng J, Huang LQ, Huang LX, Chang ZZ: Crystal structure of CYP76AH1 in 4-PI-bound state from Salvia miltiorrhiza. Biochemical and Biophysical Research Communications2019, 511:813–819. [PubMed: 30837155]

*70. Forman V, Bjerg-Jensen N, Dyekjær JD, Møller BL, Pateraki I: Engineering of CYP76AH15 can improve activity and specificity towards forskolin biosynthesis in yeast. Microbial cell factories2018, 17:1–17. [PubMed: 29306327] This work highlights the potential of using site-directed mutagenesis of the substrate recognition sites to enhance the activity of plant CYPs: the best CYP76AH15 mutant (A99I) exhibited a 5.6-fold increase in the synthesis of desired product in comparison to the wildtype CYP76AH15.

71. Vazquez-Albacete D, Cavaleiro AM, Christensen U, Seppala S, Moller BL, Norholm MH: An expression tag toolbox for microbial production of membrane bound plant cytochromes P450. Biotechnol Bioeng2017, 114:751–760. [PubMed: 27748524]

72. Wang C, Crocoll C, Nodvig CS, Mortensen UH, Clemmensen SE, Halkier BA: Production of benzylglucosinolate by engineering and optimizing the biosynthetic pathway in Saccharomyces cerevisiae. bioRxiv2020.

73. Petersen A, Crocoll C, Halkier BA: De novo production of benzyl glucosinolate in Escherichia coli. Metab Eng2019, 54:24–34. [PubMed: 30831267]

74. Chen S, Glawischnig E, Jorgensen K, Naur P, Jorgensen B, Olsen CE, Hansen CH, Rasmussen H, Pickett JA, Halkier BA: CYP79F1 and CYP79F2 have distinct functions in the biosynthesis of aliphatic glucosinolates in Arabidopsis. Plant J2003, 33:923–937. [PubMed: 12609033]

75. Yang H, Liu F, Li Y, Yu B: Reconstructing Biosynthetic Pathway of the Plant-Derived Cancer Chemopreventive-Precursor Glucoraphanin in Escherichia coli. ACS Synth Biol2018, 7:121–131. [PubMed: 29149798]

76. Nakagawa A, Matsumura E, Koyanagi T, Katayama T, Kawano N, Yoshimatsu K, Yamamoto K, Kumagai H, Sato F, Minami H: Total biosynthesis of opiates by stepwise fermentation using engineered Escherichia coli. Nat Commun2016, 7:10390. [PubMed: 26847395]

77. Iwakami S, Kamidate Y, Yamaguchi T, Ishizaka M, Endo M, Suda H, Nagai K, Sunohara Y, Toki S, Uchino A, et al.: CYP81A P450s are involved in concomitant cross-resistance to acetolactate synthase and acetyl-CoA carboxylase herbicides in Echinochloa phyllopogon. New Phytol2019, 221:2112–2122. [PubMed: 30347444]

78. Zhao Q, Cui MY, Levsh O, Yang D, Liu J, Li J, Hill L, Yang L, Hu Y, Weng JK, et al.: Two CYP82D Enzymes Function as Flavone Hydroxylases in the Biosynthesis of Root-Specific 4'-Deoxyflavones in Scutellaria baicalensis. Mol Plant2018, 11:135–148. [PubMed: 28842248]

79. Tian X, Ruan JX, Huang JQ, Yang CQ, Fang X, Chen ZW, Hong H, Wang LJ, Mao YB, Lu S, et al.: Characterization of gossypol biosynthetic pathway. Proc Natl Acad Sci U S A2018, 115:E5410–E5418. [PubMed: 29784821]

80. Christ B, Xu C, Xu M, Li FS, Wada N, Mitchell AJ, Han XL, Wen ML, Fujita M, Weng JK: Repeated evolution of cytochrome P450-mediated spiroketal steroid biosynthesis in plants. Nat Commun2019, 10:3206. [PubMed: 31324795]

81. Naur P, Petersen BL, Mikkelsen MD, Bak S, Rasmussen H, Olsen CE, Halkier BA: CYP83A1 and CYP83B1, two nonredundant cytochrome P450 enzymes metabolizing oximes in the biosynthesis of glucosinolates in Arabidopsis. Plant Physiol2003, 133:63–72. [PubMed: 12970475]

82. Zhao Q, Zhang Y, Wang G, Hill L, Weng JK, Chen XY, Xue H, Martin C: A specialized flavone biosynthetic pathway has evolved in the medicinal plant, Scutellaria baicalensis. Sci Adv2016, 2:e1501780. [PubMed: 27152350]
83. Shibuya M, Hoshino M, Katsube Y, Hayashi H, Kushiro T, Ebizuka Y: Identification of β-amyrin and sophoradiol 24-hydroxylase by expressed sequence tag mining and functional expression assay. The FEBS journal2006, 273:948–959. [PubMed: 16478469]
84. Morrone D, Chen X, Coates RM, Peters RJ: Characterization of the kaurene oxidase CYP701A3, a multifunctional cytochrome P450 from gibberellin biosynthesis. Biochem J2010, 431:337–344. [PubMed: 20698828]
85. Wang Q, Hillwig ML, Wu Y, Peters RJ: CYP701A8: a rice ent-kaurene oxidase paralog diverted to more specialized diterpenoid metabolism. Plant Physiol2012, 158:1418–1425. [PubMed: 22247270]
86. Castillo DA, Kolesnikova MD, Matsuda SP: An effective strategy for exploring unknown metabolic pathways by genome mining. Journal of the American Chemical Society2013, 135:5885–5894. [PubMed: 23570231]
87. Luo P, Wang YH, Wang GD, Essenberg M, Chen XY: Molecular cloning and functional identification of (+)-delta-cadinene-8-hydroxylase, a cytochrome P450 monooxygenase (CYP706B1) of cotton sesquiterpene biosynthesis. Plant J2001, 28:95–104. [PubMed: 11696190]
88. Cankar K, van Houwelingen A, Goedbloed M, Renirie R, de Jong RM, Bouwmeester H, Bosch D, Sonke T, Beekwilder J: Valencene oxidase CYP706M1 from Alaska cedar (Callitropsis nootkatensis). FEBS letters2014, 588:1001–1007. [PubMed: 24530525]
89. Liu X, Cheng J, Zhang G, Ding W, Duan L, Yang J, Kui L, Cheng X, Ruan J, Fan W: Engineering yeast for the production of breviscapine by genomic analysis and synthetic biology approaches. Nature communications2018, 9:1–10.
90. Yan X, Fan Y, Wei W, Wang P, Liu Q, Wei Y, Zhang L, Zhao G, Yue J, Zhou Z: Production of bioactive ginsenoside compound K in metabolically engineered yeast. Cell Res2014, 24:770–773. [PubMed: 24603359]
91. Wong J, de Rond T, d'Espaux L, van der Horst C, Dev I, Rios-Solis L, Kirby J, Scheller H, Keasling J: High-titer production of lathyrane diterpenoids from sugar by engineered Saccharomyces cerevisiae. Metab Eng2018, 45:142–148. [PubMed: 29247866]
92. King AJ, Brown GD, Gilday AD, Forestier E, Larson TR, Graham IA: A Cytochrome P450-Mediated Intramolecular Carbon-Carbon Ring Closure in the Biosynthesis of Multidrug-Resistance-Reversing Lathyrane Diterpenoids. Chembiochem2016, 17:1593–1597. [PubMed: 27272333]
93. Ignea C, Raadam MH, Motawia MS, Makris AM, Vickers CE, Kampranis SC: Orthogonal monoterpenoid biosynthesis in yeast constructed on an isomeric substrate. Nature communications2019, 10:1–15.
94. Xue HJ, Liu H, Lv B, Yu Y, Wang Y, Huang ML, Li C: Controlling Chemo- and Regioselectivity of a Plant P450 in Yeast Cell toward Rare Licorice Triterpenoid Biosynthesis. Acs Catalysis2020, 10:4253–4260.
95. Wang C, Su X, Sun M, Zhang M, Wu J, Xing J, Wang Y, Xue J, Liu X, Sun W: Efficient production of glycyrrhetinic acid in metabolically engineered Saccharomyces cerevisiae via an integrated strategy. Microbial cell factories2019, 18:95. [PubMed: 31138208]
96. Nomura T, Magome H, Hanada A, Takeda-Kamiya N, Mander LN, Kamiya Y, Yamaguchi S: Functional Analysis of Arabidopsis CYP714A1 and CYP714A2 Reveals That They are Distinct Gibberellin Modification Enzymes. Plant and Cell Physiology2013, 54:1837–1851. [PubMed: 24009336]
97. Moses T, Pollier J, Faizal A, Apers S, Pieters L, Thevelein JM, Geelen D, Goossens A: Unraveling the triterpenoid saponin biosynthesis of the African shrub Maesa lanceolata. Molecular plant2015, 8:122–135. [PubMed: 25578277]
98. Yin Y, Gao L, Zhang X, Gao W: A cytochrome P450 monooxygenase responsible for the C-22 hydroxylation step in the Paris polyphylla steroidal saponin biosynthesis pathway. Phytochemistry2018, 156:116–123. [PubMed: 30268044]

99. Huang L, Li J, Ye H, Li C, Wang H, Liu B, Zhang Y: Molecular characterization of the pentacyclic triterpenoid biosynthetic pathway in Catharanthus roseus. Planta2012, 236:1571–1581. [PubMed: 22837051]

100. Walls LE, Malci K, Nowrouzi B, Li RA, d'Espaux L, Wong J, Dennis JA, Semiao AJC, Wallace S, Martinez JL, et al.: Optimizing the biosynthesis of oxygenated and acetylated Taxol precursors inSaccharomyces cerevisiaeusing advanced bioprocessing strategies. Biotechnology and Bioengineering2020.

101. Sagwan-Barkdoll L, Anterola AM: Taxadiene-5α-ol is a minor product of CYP725A4 when expressed in Escherichia coli. Biotechnology and applied biochemistry2018, 65:294–305. [PubMed: 28876471]

102. Li J, Mutanda I, Wang K, Yang L, Wang J, Wang Y: Chloroplastic metabolic engineering coupled with isoprenoid pool enhancement for committed taxanes biosynthesis in Nicotiana benthamiana. Nature communications2019, 10:1–12.

103. Sintupachee S, Ngamrojanavanich N, Sitthithaworn W, De-Eknamkul W: Molecular cloning, bacterial expression and functional characterisation of cytochrome P450 monooxygenase, CYP97C27, and NADPH-cytochrome P450 reductase, CPR I, from Croton stellatopilosus Ohba. Plant Sci2014, 229:131–141. [PubMed: 25443840]

104. Quinlan RF, Shumskaya M, Bradbury LMT, Beltran J, Ma CH, Kennelly EJ, Wurtzel ET: Synergistic Interactions between Carotene Ring Hydroxylases Drive Lutein Formation in Plant Carotenoid Biosynthesis. Plant Physiology2012, 160:204–214. [PubMed: 22786888]

105. Almagro Armenteros JJ, Sonderby CK, Sonderby SK, Nielsen H, Winther O: DeepLoc: prediction of protein subcellular localization using deep learning. Bioinformatics2017, 33:3387–3395. [PubMed: 29036616]

106. Chen X, Zhang C, Too H-P: Multienzyme biosynthesis of dihydroartemisinic acid. Molecules2017, 22:1422.

107. Kudla G, Murray AW, Tollervey D, Plotkin JB: Coding-sequence determinants of gene expression in Escherichia coli. Science2009, 324:255–258. [PubMed: 19359587]

Highlights

- Cytochrome P450s (CYPs) play an essential role in plant specialized metabolism
- Plant CYPs are generally challenging to be functionally reconstituted in microbial systems
- Engineering strategies to functionally reconstitute plant CYPs are limiting
- Current strategies focus on adapting plant CYPs to microbial microenvironment
- In-depth *in planta* studies are needed to adapt microbes for the functional reconstitution of CYPs

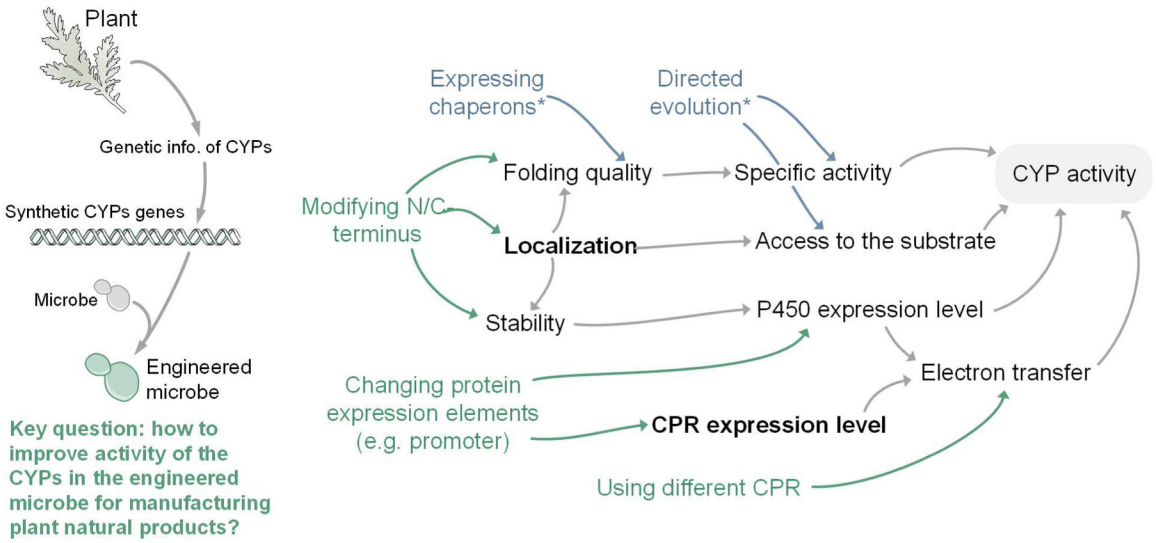

Figure 1.

Functional reconstitution of plant cytochrome P450s (CYPs) for producing phytochemicals in microbes. Many strategies have been developed to improve the activity of recombinant CYPs in microbes. Some working mechanisms of these strategies have been proposed and further study is needed to validate them. *These strategies are reviewed elsewhere [11,24]. Process conditions (temperature, pH, etc.) affect CYP activity through multiple mechanisms and are reviewed elsewhere [24]. CPR: CYP reductase.

Table 1

Examples of plant cytochrome P450s reconstituted in Yeast and *E. coli*

clan#	CYP#	Metabolite type	Functional in yeast?*	Functional *in E. coli*?*	Native localization**
51 clan	CYP51H10	triterpene	Y [57]	Y [34]	ER
71 clan	CYP71D51v2	sesquiterpene	Y [58]	-	ER
	HPO(CYP71D55)	sesquiterpene	Y[59]	-	ER
	CYP71E1	cyanogenic glycoside	Y [60]	Y [61]	ER
	CYP71AV1	sesquiterpene	Y [62]	Y [63]	ER
	MaCYP71CD2/ MaCYP71BQ5/ CsCYP71CD1/CsCYP71BQ4	triterpene	Y [64]	-	ER
	CYP71BA1	sesquiterpene	Y [65]	Y [66]	ER
	CYP73A1	flavonoid	Y [67]	-	ER
	IO (CYP76A26)	monoterpene	Y [7]	-	ER
	G8H (CYP76B6)	monoterpene	Y [7]	-	ER
	CYP76AH1	diterpene	Y [68]	Y [69]	ER
	CYP76AH15	diterpene	Y [70]	-	ER
	AtCYP79A1	cyanogenic glycoside	-	Y [34]	ER/mitochondrion***
	SbCYP79A1	cyanogenic glycoside	Y [60]	Y [71]	ER
	CYP79A2	glucosinolate	Y [72]	Y [73]	ER/mitochondrion**
	CYP79F1	glucosinolate	Y [74]	Y [75]	ER
	CYP79F2	glucosinolate	Y [74]	N [74]	ER
	DRS (CYP80Y)-DRR	benzylisoquinoline alkaloid	Y [2]	N**** [76]	ER
	CYP81A12/21	herbicide detoxification	Y [77]	Y [26]	ER
	CYP81A15/24	herbicide detoxification	N [25]	Y [26]	ER
	SbF6H (CYP82D1.1)	flavonoid	Y [78]	Y [53]	ER
	CYP82D113	sesquiterpene	Y [79]	-	ER
	AbCYP80F1/AbCYP82M3	tropane alkaloid	Y [4]	-	ER
	CYP82J17	triterpene	Y [80]	-	ER
	MSH (CYP82N)/P6H (CYP82N)	benzylisoquinoline alkaloids	Y [43]	-	ER
	CYP83A1	glucosinolate	Y [81]	Y [75]	ER
	CYP83B1	glucosinolate	Y [81]	Y [47]	ER
	SbFNSII-1 (CYP93B24)	flavonoid	Y [82]	-	ER
	SbFNSII-2 (CYP93B25)	flavonoid	Y [82]	Y [53]	ER
	CYP93E1	triterpene	Y [83]	-	ER
	CYP701A3	diterpene	Y [84]	Y [84]	chloroplast
	CYP701A8	diterpene	Y [85]	N [85]	ER/chloroplast
	CYP705A1	triterpene	Y [86]	-	ER

clan#	CYP#	Metabolite type	Functional in yeast?*	Functional *in E. coli*?*	Native localization**
	CYP706B1	sesquiterpene	Y [87]	Y [63]	ER
	CYP706M1	sesquiterpene	Y [88]	-	ER
	EbF6H (CYP706X)	flavonoid	Y [89]	-	ER
	CFS (CYP719A)/STS (CYP719A)	benzylisoquinoline alkaloid	Y [43]	-	ER [43]
	SalSyn (CYP719B)	benzylisoquinoline alkaloid	Y [2]	Y [76]	ER
	CYP716A47	triterpene	Y [90]	-	ER
	CYP716A49	triterpene	Y [45]	-	ER
	JcC5OX1(CYP726A35)	diterpene	Y [91]	-	chloroplast [92]
	CYP750B1	monoterpene	Y [93]	-	ER
72 clan	SLS (CYP72A1)	monoterpene	Y [7]	-	ER
	CYP72A63	triterpene	Y [44]	N [94]	ER
	CYP72A154	triterpene	Y [95]	-	ER
	7-DLH (CYP72A224)	monoterpene	Y [7]	-	ER
	CYP714A2	diterpene	Y [96]	Y [48]	ER
85 clan	CYP87D16	triterpene	Y [97]	-	ER
	CYP88D6	triterpene	Y [95]	-	ER
	CYP90A1/CYP90B1	sterol	Y [37]	-	ER
	CYP90B27	sterol	Y [98]	-	ER
	CYP90G4/CYP90B50	triterpene	Y [80]	-	ER
	CYP708A2	triterpene	Y [86]	-	ER
	PPDS(CYP716A47)	triterpene	Y [49]	-	ER
	CYP716AL1	triterpene	Y [99]	-	ER
	CYP725A4	diterpene	Y [100]	Y [101]	ER [102]
86 clan	CYP94D108	triterpene	Y [80]	-	ER
97 clan	CYP97C27	carotene	-	Y [103]	chloroplast [104]

*Y: can be functionally reconstituted; N: cannot be functionally reconstituted; -: no data reported.

**unless specified, predicted from Uniprot database or DeepLoc [105]

***predicted from the Arabidopsis Information Resource (TAIR)

****enhanced compared to native DRS-DRR, but with only trace activity detected

Table 2

Examples of N-terminus modifications on plant cytochrome P450s

Host	CYP#	N-terminus modification*	Type**	Expression***	Activity***	Reference
E. coli	CYP79A1	CYP79A1$^{36-558}$	T	-	Y	[34]
		28aa-CYP79A1$^{36-558}$	E	Y+	Y	
		8RP-CYP79A1$^{1-558}$	H	Y	-	
		SohB$^{1-48}$-CYP79A1$^{36-558}$	TM	Y+	Y	
		YafU$^{1-88}$- CYP79A1$^{36-558}$	TM	N+	N+	
		LepB$^{1-288}$- CYP79A1$^{1-558}$	TM	Y	N+	
	SbCYP79A1	28aa-SbCYP79A1	E	Y+	Y	[71]
		SohB$^{1-48}$-SbCYP79A1	TM	Y+	-	
		OmpA-SbCYP79A1	TM	Y	Y+	
		DsbA-SbCYP79A1	TM	Y	Y	
	SbF6H **** (CYP82D1.1)	MBP-SbF6H$^{25-517}$	E	Y	N−	[53]
		2B1-SbF6H$^{25-517}$	H	Y	Y+	
	CYP701A3	8RP-CYP701A3$^{3-509}$	H	-	Y	[84]
		8RP-CYP701A3$^{9-509}$	H	-	Y	
		8RP-CYP701A3$^{18-509}$	H	-	Y	
		8RP-CYP701A3$^{25-509}$	H	-	Y	
		2B1-CYP701A3$^{51-509}$	H	-	Y+	
	SalSyn (CYP719B1)	tCYP719B1	T	Y	Y	[3]
		tDRS (CYP80Y)-DRR	T	Y	N+	
	CYP725A4	8RP-CYP725A4$^{25-297}$	H	Y	Y	[42]
		MA-CYP725A4$^{43-297}$	T	Y	-	
		2B1-CYP725A4$^{60-297}$	H	Y	-	
		28aa-CYP79A1$^{36-558}$	E	Y+	Y	
		8RP-CYP79A1$^{1-558}$	H	Y	-	
		SohB$^{1-48}$-CYP79A1$^{36-558}$	TM	Y+	Y	
		YafU$^{1-88}$-CYP79A1$^{36-558}$	TM	N+	N+	
		LepB$^{1-288}$-CYP79A1$^{1-558}$	TM	Y	N+	
Yeast	CYP71AV1	8RP-CYP71AV1^{25}	H	-	Y	[106]
	CYP73A1	PD1-CYP73A1$^{21-505}$ **	E	N−	NN	[67]
	CYP82Y1	MSH$^{1-44}$ – CYP82Y1$^{58-556}$	A	-	Y	[36]
		L14D$^{1-56}$ – CYP82Y1$^{58-556}$	B	-	X	
		CmCPR$^{1-41}$ – CYP82Y1$^{58-556}$	C	-	NN	
		EcCFS$^{1-32}$ – CYP82Y1$^{58-556}$	B	-	X	
		CYP82Y1$^{58-556}$	T	-	X	
	PsSalSyn (CYP719B1)	yEcCFS$^{1-66}$-yPsSalSyn$^{78-505}$	A	-	N−	[2]

Host	CYP#	N-terminus modification*	Type**	Expression***	Activity***	Reference
		yEcCFS$^{1-90}$-yPsSalSyn$^{102-505}$	A	-	N−	
		yEcCFS$^{1-23}$-yPsSalSyn$^{33-505}$	A	-	N−	
		yEcCFS$^{1-26}$-yPsSalSyn$^{36-505}$	A	-	N−	
		yEcCFS$^{1-83}$-yPsSalSyn$^{95-505}$	A	-	Y	
	PbSalSyn (CYP719B)	yEcCFS$^{1-26}$-yPbSalSyn$^{33-504}$	A	-	N+	
		yEcCFS$^{1-23}$-yPbSalSyn$^{30-504}$	A	-	N+	
		yEcCFS$^{1-83}$-yPbSalSyn$^{92-504}$	A	-	Y	

*t: N-terminus truncated; y: yeast codon optimized; MA: Met-Ala; 8RP: MALLLAVP; 2B1: MAKKTSSKGKLPPGPS; 28aa: 28 codon tag with weak mRNA structure shown to enhance expression levels [107]; Sumo: S. cerevisiae small ubiquitin-like modifier; MBP: maltose binding protein; PD1: MEELLKQALQQAQQLLQQAQELAKK, an amphipathic tag to enhance solubility of membrane proteins [67].

**T, N-terminus truncation. Types of the N-terminus modification peptide sequences: H, hydrophilic or relatively hydrophilic peptide; E, peptide to enhance expression level or solubility; TM, transmembrane peptide from bacterial membrane proteins; A, transmembrane peptide from analogous P450s belonging to the same subfamily; B, transmembrane peptide from P450s belonging to different subfamilies; C, transmembrane peptide from yeast membrane proteins.

***Y: enhanced; Y+: enhanced more than other N-terminus modifications; N−: slightly lower, statistically not significant; N+: slightly higher, statistically not significant; NN: decreased much; X: abolished; -: no data reported.

****when paired with full length ATR1, no enhancement across the N-terminus modifications when paired with N-terminus truncated ATR1.