

Exploring the molecular basis for selective C-H functionalization in plant P450s

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Highly prevalent in nature, cytochrome P450 enzymes are powerful biocatalysts for selective C-H functionalization.¹ These heme-containing enzymes activate inert C-H bonds within complex molecules for a plethora of transformations with exquisite regio-, chemo- and stereoselectivity. Many plant P450s are of industrial relevance in the production of pharmaceuticals, fragrances, pesticides and vitamins, yet few have been employed commercially.² Key examples include the P450 CYP71AV1 used for large-scale production of the antimalarial drug artemisinin, and CYP75 enzymes exploited for their differential hydroxylation abilities to manipulate color patterns in top-selling flowers.³ These cases illustrate a minuscule portion of the synthetic potential of P450s.

Unveiling the molecular basis for selective C-H oxidation in plant-derived P450s can facilitate their engineering towards increased commercial applications. However, despite their widespread occurrence,^{1,2} little is known about the basis for their late-stage selectivity given the limited number of crystal structures available.⁴ Contrary to bacterial P450s, which are generally soluble and expressed in high levels,^{1,3} most plant P450s are found in low yields in native tissues complicating their isolation. Moreover, they are typically membrane-bound through the N-terminus in the endoplasmic reticulum (Fig. 1A), and insoluble when produced in bacterial expression systems such as *Escherichia coli*.² Other challenges include the need for co-expression of native reductase partners, proper incorporation of the heme co-factor, differences in codon preference and genetic instabilities when large or multiple plasmids are employed.⁵ Eukaryotic-based heterologous hosts exist, including *Saccharomyces cerevisiae* and *Pichia pastoris*, yet similar challenges remain.

In their recent *ACS Catalysis* article,⁶ authors Chun Li and co-workers highlight an interesting avenue towards the rational engineering of plant P450s that are difficult to express *in vitro*. This work deepens our understanding of the selectivity and iterative mechanism involved in the biosynthesis of medicinally relevant molecules.

Elucidating the role P450s play in functionalizing plant natural products is fundamental for guiding engineering efforts towards improving the performance of these enzymes. This may include redirecting the selectivity of the biocatalyst, designing self-sufficient P450-redox partner chimeric fusions, allowing the use of light-driven cofactor regeneration processes, or enabling new-to-nature chemical reactions.⁷

The work of Chun Li et al.⁶ is focused on decoding and fine-tuning the molecular factors controlling selectivity and iterative oxidation in the CYP72A63 from *Medicago truncatula* towards

the synthesis of high-value, bioactive licorice triterpenoids. When efforts towards expressing CYP72A63 in *E. coli* failed, Chun Li et al. engineered a yeast strain to produce 11-oxo- β -amyrin *in vivo* by introducing the genes responsible for its biosynthesis along with CYP72A63, which was previously determined to synthesize glycyrrhetic acid from 11-oxo- β -amyrin. Although efficient, the P450 does not catalyze formation of glycyrrhetic acid selectively as other intermediates and by products, including glycyrrhetol, glycyrrhetaldehyde and 29-OH-11-oxo- β -amyrin, are also generated. Based on this catalytic promiscuity, Chun Li et al. postulated that key residues in the P450 active site may govern selectivity.

Although recombinantly producing plant P450s is challenging, there is an increasing number of strategies to do so (Fig. 1B). Truncating the membrane-anchoring segment can increase the solubility of a P450 as exemplified by Nagano et al. in their work to crystallize the plant CYP90B1.⁴ If this deletion approach adversely impacts the ability of the P450 to couple with CYP reductase for electron transfer, modifying the transmembrane helix is an alternative option.⁵ In some cases, the need of P450s for NADPH, which is provided by the reductase, can stress an organism leading to metabolic imbalances. Although often low yielding, this can be resolved by co-overexpressing the reductase or creating the corresponding P450 fusion protein. Co-expressing chaperones to facilitate protein folding can also promote protein expression. Additional approaches include media supplementation and optimization of cultivation parameters such as pH, temperature and dissolved oxygen concentrations.⁵ Generally, the *S. cerevisiae* expression system leads to higher yields of P450s in comparison to *E. coli* and *P. pastoris*, although the use of novel photosynthetic hosts such as cyanobacteria is becoming more prevalent.⁸ It is important to consider that prior to rigorously characterizing P450s *in vitro*, transient expression systems can provide insightful, rapid information into the biochemical function of these enzymes.⁹

In the absence of crystallographic data, indirect methods such as molecular modeling serve to investigate substrate docking in a P450 active site.^{7,9,10} To enable structure-based engineering of CYP72A63, Chun Li et al.⁶ created homology models of the enzyme for docking with 11-oxo- β -amyrin and glycyrrhetol. This led to the identification of nine active site residues, of which two had been previously reported,¹⁰ hypothesized to impact the differential oxidation patterns observed. Using site-directed and site-saturation mutagenesis, the investigators gained valuable information on the structure-function relationship of the P450 and engineered variants that fine-tune catalytic activity, regio- and chemoselectivity. In comparison to wild type CYP72A63, the T338S mutant increases the distance between the C-29 hydrogen and the heme iron, resulting in complete regioselectivity for the C-30 position and enhanced iterative abilities, producing glycyrrhetic acid at a 7-fold higher level. Reversing regioselectivity to favor C-29 oxidation, the L398I mutant facilitates a 180° rotation of the substrate leading to exclusive formation of 29-OH-11-oxo- β -amyrin. With increased hydrophobicity, the CYP72A63 mutant L509I changes substrate accessibility into the active site, altering the chemical output and selectively producing glycyrrhetol in a 90.6% yield. Given that efforts to design a variant that selectively generates glycyrrhetaldehyde were not fruitful, the researchers screened heterologous CYP reductases and determined that co-overexpressing GuCPR2 from *Glycyrrhiza uralensis* with CYP72A63 selectively produces the aldehyde in an 82.3% yield.⁶ By exploring the versatile catalytic potential of CYP72A63, the authors accentuate the value of plant P450s as essential tools for late-stage

oxidation of triterpenoid molecules, providing an excellent starting point for additional engineering of related enzymes.

Increasing technologies for the *in silico*, *in vitro* and *in planta* characterization of plant-derived P450s^{2,5,8,9} will continue to unveil the molecular factors governing their selectivity in C-H functionalization. The article of Chun Li et al.⁶ demonstrates the potential of plant P450s for protein engineering and expanding the synthetic abilities of these biocatalysts. Future work will undoubtedly reveal their increasingly important role for introducing late-stage structural diversity in complex chemical scaffolds.

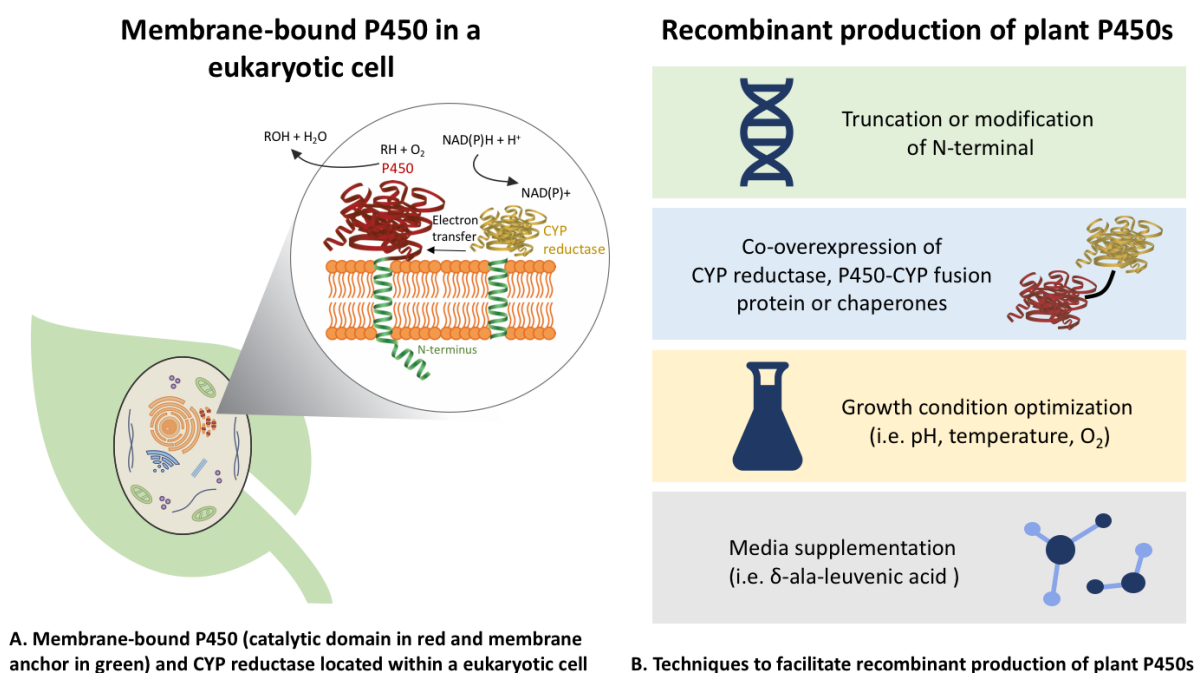


Figure 1

References

1 Guengerich, F.P.

Mechanisms of cytochrome P450-catalyzed oxidations

ACS Catal, 8(12) (2018), pp.10964-10976, 10.1021/acscatal.8b03401

2 Shang, Y. and Huang, S.

Engineering plant cytochrome P450s for enhanced synthesis of natural products: past achievements and future perspectives

Plant Comm, 1 (13) (2019), p.100012, 10.1016/j.xplc.2019.100012

3 Renault, H., Bassard, J.E., Hamberger, B. and Werck-Reichhart, D.

Cytochrome P450-mediated metabolic engineering: current progress and future challenges

Curr Opin Plant Biol, 19 (2014), pp.27-34, 10.1016/j.pbi.2014.03.004

4 Fujiyama, K., Hino, T., Kanadani, M., Watanabe, B., Lee, H.J., Mizutani, M. and Nagano, S.

Structural insights into a key step of brassinosteroid biosynthesis and its inhibition

Nat Plants, 5(6) (2019), pp.589-594, 10.1038/s41477-019-0436-6

5 Hausjell, J., Halbwirth, H. and Spadiut, O.

Recombinant production of eukaryotic cytochrome P450s in microbial cell factories

Biosci Rep, 38(2) (2018), 10.1042/BSR20171290

6 Sun, W., Xue, H., Liu, H., Lv, B., Yu, Y., Wang, Y., Huang, M. and Li, C.

Controlling Chemo-and Regio-selectivity of a plant P450 in yeast cell towards rare licorice triterpenoids biosynthesis

ACS Catal, 10(7) (2020), pp. 4253-4260, 10.1021/acscatal.0c00128

7 Chen, K. and Arnold, F.H.

Engineering new catalytic activities in enzymes

Nat Catal, 3 (2020), pp. 203-213, 10.1038/s41929-019-0385-5

8 Mellor, S.B., Nielsen, A.Z., Burow, M., Motawia, M.S., Jakubauskas, D., Møller, B.L. and Jensen, P.E.

Fusion of ferredoxin and cytochrome P450 enables direct light-driven biosynthesis

ACS Chem Biol, 11(7) (2016), pp.1862-1869, 10.1021/acschembio.6b00190

9 Dang, T.T.T., Franke, J., Carqueijeiro, I.S.T., Langley, C., Courdavault, V. and O'Connor, S.E.

Sarpagan bridge enzyme has substrate-controlled cyclization and aromatization modes

Nat Chem Biol, 14(8) (2018), pp.760-763, 10.1038/s41589-018-0078-4

10 Fanani, M.Z., Fukushima, E.O., Sawai, S., Tang, J., Ishimori, M., Sudo, H., Ohyama, K., Seki, H., Saito, K. and Muranaka, T.

Molecular basis of C-30 product regioselectivity of legume oxidases involved in high-value triterpenoid biosynthesis

