



Rational design strategies for functional reconstitution of plant cytochrome P450s in microbial systems

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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.

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Introduction

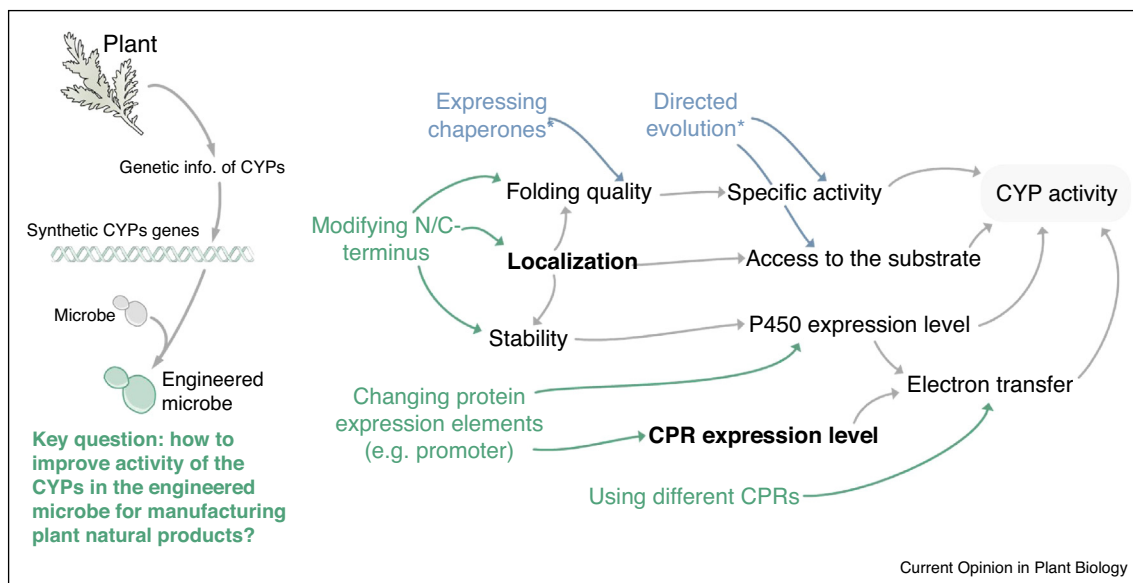
The successful microbial production of the antimalarial artemisinic acid shed lights on the potential of synthesizing plant specialized metabolites from microbial systems as an alternative sourcing to these scarce and expensive molecules [1]. This strategy is further highlighted with the recent engineering 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 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, *Escherichia 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 microorganism 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 [11,21,22,23,24] elsewhere and are not covered here: improving the activity of CYPs 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 reconstitution of plant CYPs. Numerous plant CYPs have been successfully reconstituted in both prokaryotic (primarily *E. coli*) and eukaryotic (primarily *S. 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

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.

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 had been functionally expressed in *E. coli* but failed to exhibit activity in yeast, for example, CYP81A15 and CYP81A24 [25**,26**]. Since there are no specific clans of CYPs 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 CPRs to CYPs.

N-terminal modification could enhance the folding and localization

Currently, the major strategy to enhance folding and localization is to engineer the N-termini of these membrane-bound proteins from plants. The N-terminal segment of plant CYPs 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 peptides of *E. coli* membrane proteins were 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-terminal modifications (Table 2), for example, SohB¹⁻⁴⁸-CYP79A1³⁶⁻⁵⁵⁸ exhibited a lower activity than 28-codon tag-CYP79A1¹⁻⁵⁵⁸ [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

Table 1

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

Clan#	CYP#	Metabolite type	Functional in yeast? ^a	Functional in <i>E. coli</i> ? ^a	Native localization ^b
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 ^c
	SbCYP79A1	Cyanogenic glycoside	Y [60]	Y [71]	ER
	CYP79A2	Glucosinolate	Y [72]	Y [73]	ER/mitochondrion ^b
	CYP79F1	Glucosinolate	Y [74]	Y [75]	ER
	CYP79F2	Glucosinolate	Y [74]	N [74]	ER
	DRS (CYP80Y)-DRR	Benzylisoquinoline alkaloid	Y [2*]	N ^d [3]	ER
	CYP81A12/21	Herbicide detoxification	Y [76]	Y [26**]	ER
	CYP81A15/24	Herbicide detoxification	N [25**]	Y [26**]	ER
	SbF6H (CYP82D1.1)	Flavonoid	Y [77]	Y [53*]	ER
	CYP82D113	Sesquiterpene	Y [78]	–	ER
	AbCYP80F1/AbCYP82M3	Tropane alkaloid	Y [4]	–	ER
	CYP82J17	Triterpene	Y [79]	–	ER
	MSH (CYP82N)/P6H (CYP82N)	Benzylisoquinoline alkaloids	Y [43**]	–	ER
	CYP83A1	Glucosinolate	Y [80]	Y [75]	ER
	CYP83B1	Glucosinolate	Y [80]	Y [47]	ER
	SbFNSII-1 (CYP93B24)	Flavonoid	Y [81]	–	ER
	SbFNSII-2 (CYP93B25)	Flavonoid	Y [81]	Y [53*]	ER
	CYP93E1	Triterpene	Y [82]	–	ER
	CYP701A3	Diterpene	Y [83]	Y [83]	Chloroplast
	CYP701A8	Diterpene	Y [84]	N [84]	ER/chloroplast
	CYP705A1	Triterpene	Y [85]	–	ER
	CYP706B1	Sesquiterpene	Y [86]	Y [63]	ER
	CYP706M1	Sesquiterpene	Y [87]	–	ER
	EbF6H (CYP706X)	Flavonoid	Y [88]	–	ER
	CFS (CYP719A)/STS (CYP719A)	Benzylisoquinoline alkaloid	Y [43**]	–	ER [43**]
	SalSyn (CYP719B)	Benzylisoquinoline alkaloid	Y [2*]	Y [3]	ER
	CYP716A47	Triterpene	Y [89]	–	ER
	CYP716A49	Triterpene	Y [45]	–	ER
	JcC5OX1(CYP726A35)	Diterpene	Y [90]	–	Chloroplast [91]
	CYP750B1	Monoterpene	Y [92]	–	ER
72 clan	SLS (CYP72A1)	Monoterpene	Y [7]	–	ER
	CYP72A63	Triterpene	Y [44]	N [93]	ER
	CYP72A154	Triterpene	Y [94]	–	ER
	7-DLH (CYP72A224)	Monoterpene	Y [7]	–	ER
	CYP714A2	Diterpene	Y [95]	Y [48*]	ER
85 clan	CYP87D16	Triterpene	Y [96]	–	ER
	CYP88D6	Triterpene	Y [94]	–	ER
	CYP90A1/CYP90B1	Sterol	Y [37*]	–	ER
	CYP90B27	Sterol	Y [97]	–	ER
	CYP90G4/CYP90B50	Triterpene	Y [79]	–	ER
	CYP708A2	Triterpene	Y [85]	–	ER
	PPDS(CYP716A47)	Triterpene	Y [49]	–	ER
	CYP716AL1	Triterpene	Y [98]	–	ER
	CYP725A4	Diterpene	Y [99]	Y [100]	ER [101]
86 clan	CYP94D108	Triterpene	Y [79]	–	ER
97 clan	CYP97C27	Carotene	–	Y [102]	Chloroplast [103]

^a Y: can be functionally reconstituted; N: cannot be functionally reconstituted; -: no data reported.^b unless specified, predicted from Uniprot database or DeepLoc [104].^c predicted from the Arabidopsis Information Resource (TAIR).^d enhanced compared to native DRS-DRR, but with only trace activity detected.

Table 2

Examples of N-terminal modifications on plant cytochrome P450s

Host	CYP#	N-terminal modification ^a	Type ^b	Expression ^c	Activity ^c	Reference
<i>E. coli</i>	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 ^d (CYP82D1.1)	MBP-SbF6H ²⁵⁻⁵¹⁷	E	Y	N–	[53*]
		2B1-SbF6H ²⁵⁻⁵¹⁷	H	Y	Y+	
		8RP-CYP701A3 ³⁻⁵⁰⁹	H	–	Y	
		8RP-CYP701A3 ⁹⁻⁵⁰⁹	H	–	Y	
	CYP701A3	8RP-CYP701A3 ¹⁸⁻⁵⁰⁹	H	–	Y	[83]
		8RP-CYP701A3 ²⁵⁻⁵⁰⁹	H	–	Y	
		2B1-CYP701A3 ⁵¹⁻⁵⁰⁹	H	–	Y+	
		tCYP719B1	T	Y	Y	
	SalSyn (CYP719B1)	tDRS (CYP80Y)-DRR	T	Y	N+	[3]
		8RP-CYP725A4 ²⁵⁻²⁹⁷	H	Y	Y	
		MA-CYP725A4 ⁴³⁻²⁹⁷	T	Y	–	
		2B1-CYP725A4 ⁶⁰⁻²⁹⁷	H	Y	–	
Yeast	CYP71AV1	8RP-CYP71AV1 ²⁵	H	–	Y	[105]
		PD1-CYP73A1 ^{21-505b}	E	N–	NN	
	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	
		yEcCFS ¹⁻⁶⁶ -yPsSalSyn ⁷⁸⁻⁵⁰⁵	A	–	N–	
	PsSalSyn (CYP719B1)	yEcCFS ¹⁻⁹⁰ -yPsSalSyn ¹⁰²⁻⁵⁰⁵	A	–	N–	[2*]
		yEcCFS ¹⁻²³ -yPsSalSyn ³³⁻⁵⁰⁵	A	–	N–	
		yEcCFS ¹⁻²⁶ -yPsSalSyn ³⁶⁻⁵⁰⁵	A	–	N–	
		yEcCFS ¹⁻⁸³ -yPsSalSyn ⁹⁵⁻⁵⁰⁵	A	–	Y	
		yEcCFS ¹⁻²⁶ -yPbSalSyn ³³⁻⁵⁰⁴	A	–	N+	
		yEcCFS ¹⁻²³ -yPbSalSyn ³⁰⁻⁵⁰⁴	A	–	N+	
	PbSalSyn (CYP719B)	yEcCFS ¹⁻⁸³ -yPbSalSyn ⁹²⁻⁵⁰⁴	A	–	Y	

^a t: N-terminus truncated; y: yeast codon optimized; MA: Met-Ala; 8RP: MALLLAVP; 2B1: MAKKTSSKGKLPGPS; 28aa: 28-codon tag with weak mRNA structure shown to enhance expression levels [106]; Sumo: *S. cerevisiae* small ubiquitin-like modifier; MBP: maltose binding protein; PD1: MEELLKQALQQAQQLQQAQELAKK, an amphipathic tag to enhance solubility of membrane proteins [67].

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

^c Y: enhanced; Y+: enhanced more than other N-terminal modifications; N–: slightly lower, statistically not significant; N+: slightly higher, statistically not significant; NN: decreased much; X: abolished; –: no data reported.

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

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 CYPs 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-terminal 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 yEcCFS¹⁻⁸³-yPsSalSyn⁹⁵⁻⁵⁰⁵ exhibited the enhanced activity compared to yPsSalSyn [2*]. In the N-terminal modification of CYP82Y1, N-terminal truncation or replacement with the N-termini 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 CYPs 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 the 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 the 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-terminal or C-terminal modifications to enhance the folding

Although some chaperones 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 chaperones [39–41], so introducing plant chaperones to the microbial system may help the folding of plant CYPs and CPRs. In addition, expression levels may also affect the folding of CYPs 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 was 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 CYPs 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 CYP and CPR

Although *S. cerevisiae* has an endogenous CPR (ScCPR), the efficiency of ScCPR is substantially lower than plant CPRs when pairing with plant CYPs in yeast. Pairing CFS with ATR1 led to >50-fold enhancement in cheilanthifoline production than ScCPR in yeast [43^{••}]. The pairing with different plant CPRs often makes a substantial difference in the activity of CYPs [43^{••},44,45], as well. This is consistent with the recent observation that monolignol biosynthetic CYPs exhibit distinct levels of interactions with different plant 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, that is, N-terminal truncation and modification. CPRs were often fused to the C-termini of plant CYPs [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-terminus-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 CYP and CPR was approximately 12:1 [42^{••}]. Thus, adjusting the expression level of both CYP and CPR seems critical to achieving higher CYP 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) were 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 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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- of special interest
- of outstanding interest

Declaration of Competing Interest

The authors report no declarations of interest.

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