Discovery and modification of cytochrome P450 for plant natural products biosynthesis

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ABSTRACT

Cytochrome P450s are widespread in nature and play key roles in the diversification and functional modification of plant natural products. Over the last few years, there has been remarkable progress in plant P450s identification with the rapid development of sequencing technology, "omics" analysis and synthetic biology. However, challenges still persist in respect of crystal structure, heterologous expression and enzyme engineering. Here, we reviewed several research hotspots of P450 enzymes involved in the biosynthesis of plant natural products, including P450 databases, gene mining, heterologous expression and protein engineering.

Backgrounds

Plant cytochrome (CYP) P450 gene family is the largest gene family in plants [1]. P450 enzymes (P450s) are widely involved in biosynthetic pathway of plant natural products (PNPs) due to the wide range of activities including hydroxylation, reduction, decarboxylation, sulfoxidation, N- and O-demethylation, epoxidation, deamination, and dehalogenation [2]. Since the first P450 gene was debuted to the world in 1958, until now, more than 300,000 P450 genes have been collected in databases [3]. Unfortunately, only about 0.1% of them has been functionally characterized in the biosynthetic pathway of natural products. With the increasing market demand of PNPs, microbial cell factories have been becoming more and more popular to produce PNPs [4]. However, the expression level and catalytic efficiency of P450s are limited in the heterogeneous microbial hosts [5,6]. Therefore, identifying new catalytic functions and enhancing the heterologous activities of plant P450s have been the major challenges in establishing efficient and high-yielding microbial production platforms for complex PNPs [5]. In this review, we summarized the recent progress of P450 enzyme databases, P450s mining, heterologous expression and enzyme engineering (Fig. 1).

Functional and structural databases of P450 enzymes

With the development of modern biotechnologies, more and more P450 genes have been well-characterized including their catalytic functions, crystal structures and the roles in biosynthetic pathway of secondary metabolites. In the near future, the number of P450 genes will reach to more than one million. It is impossible to obtain the desired P450s for scientific researches and industrial applications from so many genes without well-organized information. Therefore it is necessary to construct databases to collect the sequences, structures and functions of P450s.

Functional distribution of the known P450s

The plant P450s were named and classified into CYP71-CYP99 and CYP701-CYP999 gene families according to the standard CYP450 nomenclature sequences in Nelson's lab [3]. In general, P450s were grouped into a family when sharing more than 40 sequence identities [7]. To date, about 200 plant P450 genes, which are from 55 gene families, have been identified and characterized (Fig. 2). 135 secondary metabolites, including 86 terpenoids, 11 flavonoids, 18 alkaloids were modified by these P450 enzymes and the important reactions catalyzed

Databases of P450 enzymes

Fig. 1. Discovery and modification of cytochrome P450 for PNPs biosynthesis.
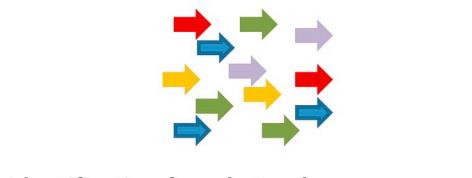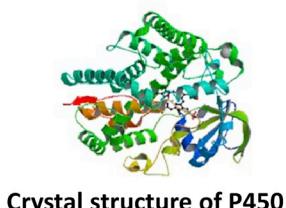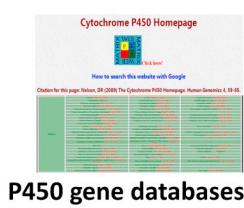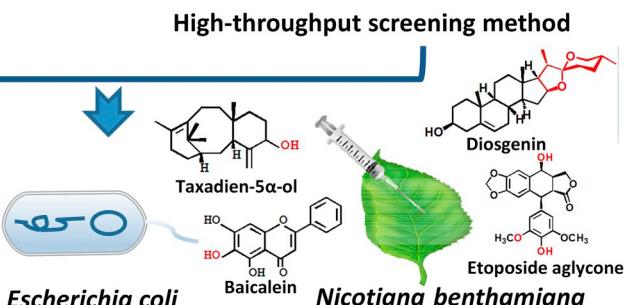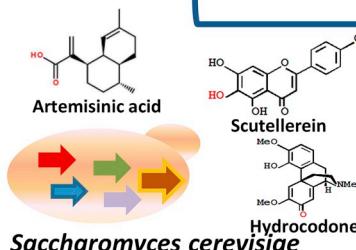
by plant P450s were summarized in Table 1. Among of them, CYP71 family is mainly involved in many sesquiterpenes biosynthesis, including capsidiol, solavetivone, gossypol, artemisinin, parthenolide, zerumbone, caryophyllene and so on. CYP76, CYP701, CYP725, CYP720 and CYP714 families are mainly involved in diterpenes biosynthesis, including tanshinone, oryzalexin, phytocassane, oryzalexin, gibberellin, taxol, abietate [8–11]. Except the CYP725 and CYP720 families, all families in CYP85 clan (CYP716, CYP90, CYP724, CYP87, CYP705, CYP708, CYP85 and CYP88) are involved in triterpenes biosynthesis, including glycyrrhizin, maslinic acid, ginsenoside, betulinate, zanhic acid, brassinolide, diosgenin, cucurbitacin, mogroside, thalianol and so on [12–14]. Besides, CYP51, CYP92, CYP710, CYP72 families also play important roles in many triterpenoid saponins biosynthesis [12,15]. However P450s for monoterpenes biosynthesis are sporadically distributed in different gene families, such as CYP71D13 for menthol biosynthesis, CYP750B1 for thujone biosynthesis and CYP72A1 for strictosidine biosynthesis [16–19].

In addition, many P450 genes associated with the biosynthesis of flavonoids and alkaloids also have been identified. P450 genes in CYP73A subfamily (like cinnamate 4-hydroxylase) and CYP93 family (flavone synthases) help to form the skeleton of flavonoids skeleton. Post-modifications of flavonoids at C6, C8, C3′ and C5′ positions usually are hydroxylated by P450 enzymes [20]. For example, CYP75A and CYP75B (C3′ and C5′ flavonoid hydroxylases), play the key roles in cyanidin biosynthesis. P450 genes in CYP706X and CYP82D subfamilies

(C6 and C8 flavonoid hydroxylases), are necessary for the biosynthesis of scutellarin, baicalin, wogonin and so on [4,21]. P450 enzymes from CYP80, CYP82 and CYP719 families usually are required for the biosynthesis of many alkaloids, such as magnoflorine, morphine, sanguinarine and noscapine. For example, CYP80B1 (the (S)–N-methylcoclaurine 3′-hydroxylase) plays an important role for biosynthesis of the key precursor of (S)-reticuline [22]; CYP719A1 helps to generate the skeleton of isoquinoline alkaloids [23]; P450 genes from CYP82N, CYP82P, CYP82X and CYP82Y subfamilies also contribute a lot on biosynthesis of the final product of alkaloids [23–27].

Construction of P450 gene databases

In 1996, the first P450 database was constructed, which systematically named the existing 481 P450 genes [28]. In 2007, a tool for searching and predicting P450 protein family, CYPED (Cytochrome P450 Engineering Database) was developed [29]. CYPED included 8613 sequences and 47 protein structures, and integrated several tools such as multiple sequence alignment, phylogenetic tree and HMM (Hidden Markov Model) profile, which could be used to analyze sequences, structures, and their relationship to biochemical properties. Subsequently, Nelson's lab constructed the Cytochrome P450 Homepage [30], which manually organized and collected P450 genes with systematic nomenclature and sequence information. To date, the database have collected 300,000 P450 sequences and 41,000 named P450s [3].
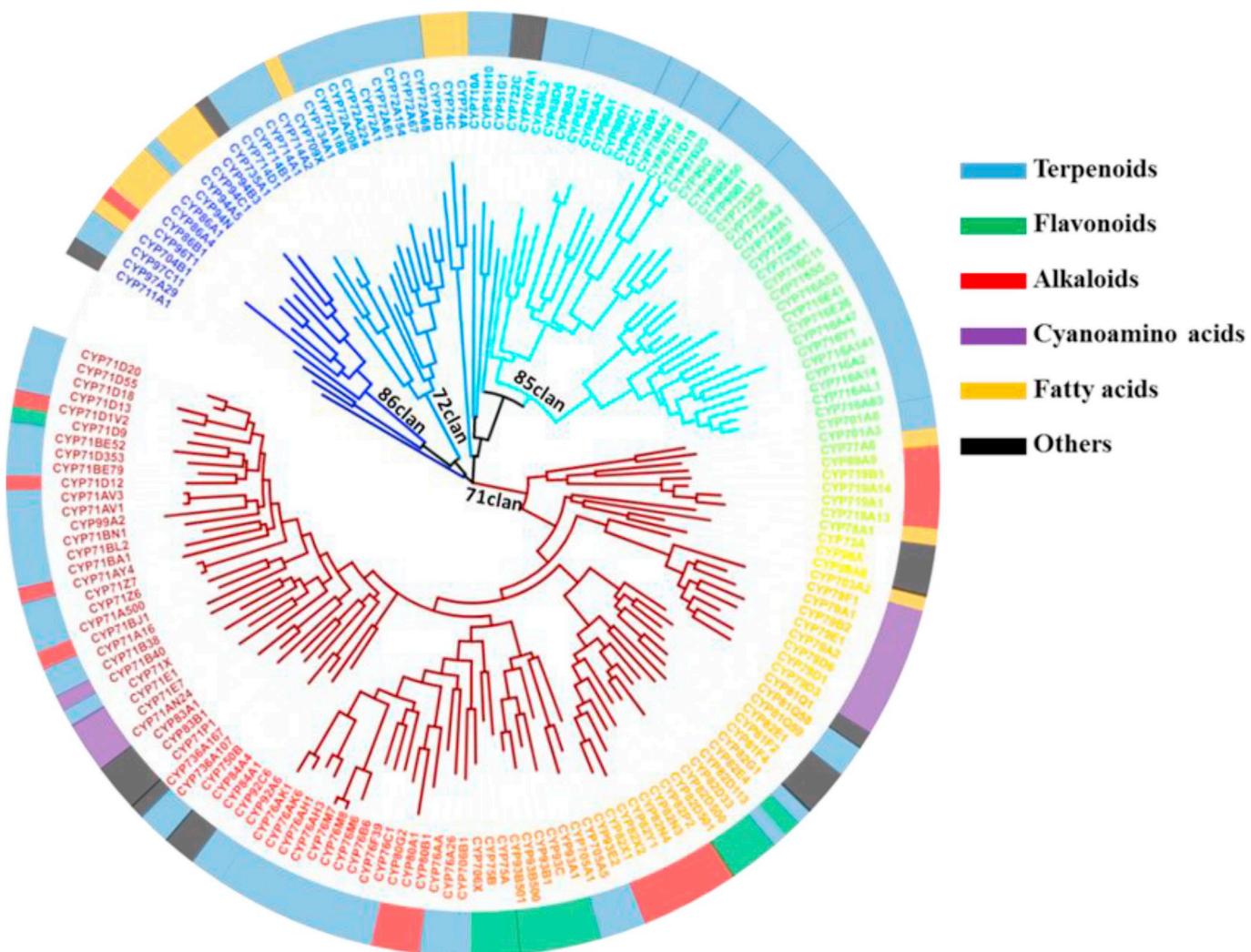
Fig. 2. Phylogenetic tree of 174 plant P450 genes with known functions.

Besides the collection of P450 sequences, a CYP-drug interactive analysis database (SuperCYP) was developed [31], which focused on the metabolic relationship between P450s and drugs. This database contained about 1170 drugs and 2785 CYP-drug interactions.

P450 gene databases had been developed for decades and have contributed a lot on gene mining, annotation and functional analysis. Taking the advantage of genome sequencing technology, more and more P450 genes have be annotated from the genome databases of some model species, such as http://www.p450.kvl.dk/ [32], http://www.arabidopsis.org/ [12] and http://www.phytozome.net/ [33], and also some plant genome sequencing projects such as the One thousand plants (1 KP) database [34], 10 KP project (https://db.cngb.org/10kp/), the JGI Open Green Genomes initiative, the PhytoMetaSyn project (www.phytometasyn.ca) and the Medicinal Plant Genome project [35]. It is necessary to construct more robust database of P450 genes according to the fast development of genome sequencing.

Crystal structure of P450 enzymes

Despite the advances of P450 gene discovery, only a limited number of P450s have been successfully crystallized [36]. The first crystal structure of cytochrome P450 was published in 1985 (CYP101A1) [37]. Until now, 963 P450 crystal structures (X-Ray resolution ≤ 3 Å) have been deposited in the Protein Data Bank (PDB). However, only five crystal structures of P450 are from plants (https://www.rcsb.org/). The

first two crystal structures of P450 in plants are two CYP74s [38,39]. Due to their atypical mechanism, which does not require molecule oxygen and NADPH reductase, it is not suitable for modeling other typical plant P450s based on these two structures. Fortunately, the X-ray crystal structure of CYP76AH1 containing inhibitor 4-phenylimmidazole (4-PI), which is a typical membrane-associated cytochrome P450 enzyme and plays a critical role in the biosynthetic pathway of tanshinones, was determined at the resolution of 2.6 Å [36]. In recent years, the *Arabidopsis campesterol* 22-hydroxylase CYP90B1 involved in brassinosteroids biosynthesis and carotenoid-ring hydroxylase CYP97A3 (PDB Number: 6J94) involved in lutein biosynthesis had been respectively crystallized by Shingo Nagano's lab and Zhao's lab [40]. The area of crystal structure analysis of plant P450 still has a long way to go.

Identification of P450 genes from plants

Identifying P450s is the key step to establish the original biosynthetic mechanisms of PNPs. In general, P450s are screened and identified from the plant by genomic or transcriptomic sequencing. For some plants without sequencing data, it is also possible to dig an alternative isozyme from database, which would catalyze the same biological reaction to realize PNPs biosynthesis. In this section, both strategies of P450s discovery will be discussed.

Table 1
Summary of the important reactions catalyzed by plant P450s.

Reactions	Substrates	Products	Enzymes	Sources
Hydroxylation	Apigenin	Scutellein		Erigeron breviscapus [4]
	L-Tyrosine	L-DOPA		Beta vulgaris and Mirabilis jalapa [71]
	Taxa-4(5),11(12)-diene	Taxa-4(20),11(12)-dien-5a-ol		Taxus species [83]
	Coronaridine	10-Hydroxycoronaridine		Tabernantheiboga [84]
	Dihydrochelerythrine	10-Hydroxydihydrochelerythrine		California poppy [23]
	Baicalein			Scutellaria baicalensis [21]
	C-2α hydroxylated triterpenoids			Crataegus pinnatifida and Centella asiatica [48]
	Chrysin			
	Miltiradiene	Ferruginol		Salvia miltiorrhiza [85]
	Ferruginol			
	(−)-5′-Desmethoxyyatein	(−)-5′-Desmethylyatein		Podophyllum hexandrum (mayapple) [67]
	(−)-5′-Desmethylyatein			

Table 1 (*continued*)

Reactions	Substrates	Products	Enzymes	Sources
7-keto-δ-cadinene	8-hydroxy-7-keto-δ-cadinene, 8,11-dihydroxy-7-keto-δ-cadinene		*Gossypium hirsutum* [86]	
Decarboxylation	L-Tyrosine		*Taxus baccata* [87]	
L-Valine or L-isoleucine	(Z)-2-methylpropanal oxime (Z)-2-methylbutanal oxime		Cassava (*Manihot esculenta Crantz*) [88]	
Epoxidation	β-amyrin		Oat (*Avena strigose*) [89,90]	
	Lycosantalene		*Solanum lycopersicum* [91]	
GA12	16α, 17-epoxy GA12		*Oryza sativa* [92]	

Table 1 (*continued*)

Reactions	Substrates	Products	Enzymes	Sources
Cyclization	Tetrahydrocolumbamine	Canadine	*Papaver somniferum* [44]	
	(−) - matairesinol	(−) - pluviatolide	*Podophyllum hexandrum* (mayapple) [67]	
(S)-Scoulerine	(S)-Cheilanthifoline	(S)-Cheilanthifoline	California poppy [23]	
N-or O-demethylation	Obtusifoliol	4 alpha-methyl-5 alpha-ergosta-8, 14,24(28)-trien-3 beta-ol	*Sorghum Bicolor* (L.) [93]	
	Nicotine	Nornicotine	*Nicotiana Tabacum* [94]	
(−)- Deoxy-podophyllotoxin	(−)-4′-Desmethyl-deoxy-podophyllotoxin	(−)-4′-Desmethyl-deoxy-podophyllotoxin	*Podophyllum hexandrum* (mayapple) [67]	
Carbon-carbon double bond	Narigenin	Apigenin	*Chrysanthemum indicum* L [95].	
24-*epi*-Campestero	Brassicasterol	Brassicasterol	*Arabidopsis thaliana* [96]	
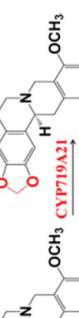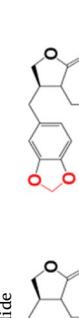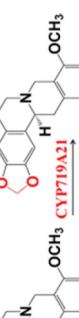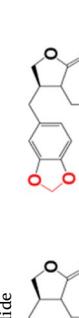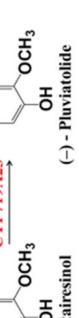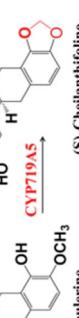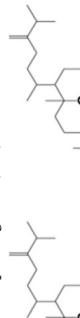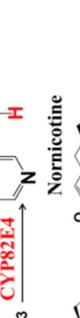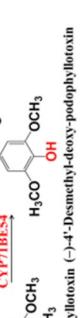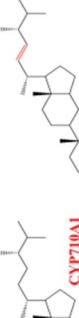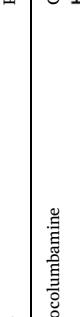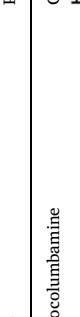
Table 1 (*continued*)

Reactions	Substrates	Products	Enzymes	Sources
Multistep reactions	7-Deoxyloganetic acid	Loganic acid and Secologanic acid	*Camptotheca acuminate* [97]	
	Cucurbitadienol	11-hydroxy-24,25-epoxy-cucurbitadienol	*Siraitia grosvenorii* [98]	
	Miltiradiene	8,12-Abietadienoic acid/Dehydroabietic acid	*Tripterygium wilfordii* [43]	
	Cucurbitadienol	11C–20H-Cuol	Cucumber (*Cucumis sativus*) [99]	

Table 1 (*continued*)

Reactions	Substrates	Products	Enzymes	Sources
Cucurbitadienol	A variety of hydroxylation products		Bitter melon (*Momordica charantia*) [100]	
Oleanolic acid	Gypsogenic acid		*Centella asiatica* [46]	
Lupeol	Betulinic acid (BA)		*Rosmarinus officinalis* (rosemary) [101]	

P450 enzyme discovery by next-generation sequencing

Genome sequencing not only provides the most comprehensive genetic information for P450 gene mining, which guarantees to contain the desired P450s, but also provides more opportunities to study the mechanisms of PNPs biosynthesis by comparative genomic analysis. For example, comparative genomic analysis of cucumber (*Cucumis sativus*) uncovered several P450s which involved in cucurbitacins biosynthesis, such as CYP88L2 for cucurbitadienol C19 hydroxylation, CYP81Q58 for C25 hydroxylation [41], CYP87D20 for C20 hydroxylation [42]. A whole-genome sequencing of *Erigeron breviscapus* leaded to the discovery of P450 enzyme in breviscapine biosynthesis with a strategy of combining evolutionary genomics and synthetic biology [4]. Two P450 genes CYP82P2 and CYP82P3 were confirmed to function as 10-hydroxylase in dihydrobenzophenanthridine alkaloid biosynthesis by using a draft genome of *California poppy* [23]. The reference-grade genome of *Tripterygium wilfordii* and the integrated genomic, transcriptomic, and metabolomics analysis resulted in the identification of CYP728B70, which catalyzes three steps of oxidation of a methyl to the acid moiety of dehydroabietic acid in triptolide biosynthesis [43].

Comparative transcriptomic analysis could also be used to identify candidate P450 enzymes by studying expression profiles between the producing and non-producing plant species or high-yield and low-yield plant varieties or even different tissues in the same plant. For example, a 10-gene cluster involved in noscapine biosynthesis was discovered through comparative transcriptomic profiling of noscapine-producing and non-producing poppy varieties [44]. *De novo* assembly of tissue-specific transcriptomes of *Paris polyphylla* and *Trigonella foenum--graecum* demonstrated that both species employ independently P450 genes to catalyze oxidative 5,6-spiroketalization of cholesterol to yield diosgenin [45]. Based on identification of unique sequences in the transcriptome of *Centella asiatica* and heterologous expression in yeast cells, *Kim* et al. discovered a novel multifunctional C-23 oxidase, CYP714E19, which involves in asiaticoside biosynthesis [46].

Identification of P450 isozymes from heterologous sources

Although the rapid development of next-generation sequencing, only several hundreds of plant genomes could be acquired in NCBI (National Center for Biotechnology Information) database until now. More than ten thousands of genomes and millions of genes from other species have been deposited in NCBI. Hence, an alternative strategy of identifying the isozymes or alternative enzymes from heterologous sources would be possible to realize the biosynthesis of some important PNPs without genomic sequencing data. One of the most successful cases was the complete biosynthesis of opioids in the yeast by combining with the successful expression of 21 enzymes derived from multiple species [47]. In addition, P450 isozymes could also be identified from other plants, where the same PNPs biosynthetic pathway independently evolved. For example, *Erigeron breviscapus* and *Scutellaria baicalensis* Georgi independently evolved a flavonoid 6-hydroxylase to catalyze apigenin into scutellarein from P450 subfamily CYP706X and CYP82D, respectively [4,21]. Both of a monocot medicinal plant himalayan paris and a eudicot culinary herb plant fenugreek independently recruited a pairs of P450s to convert the oxidative 5,6-spiroketalization of cholesterol into diosgenin [45]. It was reported that both *Crataegus pinnatifida* and *Centella asiatica* had C-2α hydroxylation enzyme. Interestingly, the C-2α hydroxylation enzyme from *C. asiatica* could produce 233% higher product than that from *C. pinnatifida* in yeast cell factories [48].

Optimization of P450 genes expression

Nowadays, with the rapid development of synthetic biology, researchers are making efforts to use various biological systems to functionally express the P450 genes and produce plant-derived natural molecules with complex structure and high-valued. In this part, P450-related metabolic engineering works in yeast, *E. coli* or tobacco cells will be discussed in detail.

Heterologous expression of P450 in yeast

Due to the excellent eukaryotic properties, varieties of P450s have been heterologously expressed in the yeast for the biosynthesis of many PNPs, such as artemisinic acid [49], triptolide [43], ganoderic acid [50], maslinic [48], various plant triterpenoids [51], vindoline [52], strictosidine [53], tropane alkaloids [54] have been successfully produced in yeast cell factories. Several means for optimization of P450s expression in yeast was established including optimization of P450 enzyme expression, enhancing the coupling between P450s and the cytochrome P450 reductase, increasement of reduction power supply, expansion of the endoplasmic reticulum (ER) and so on. For example, to overcome the poor coupling between *Panax ginseng* P450 enzyme PPDS (protopanaxadiol synthetase) and the *Arabidopsis thaliana* cytochrome P450 reductase ATR1, the transmembrane domain truncation and self-sufficient PPDS-ATR1 fusion were constructed, which resulted in approximately 4.5-fold improvement of P450 catalytic activity [55]. In another study, the naringenin to eriodictyol and taxifolin conversion ratio reached 90.5% and 56.8% by screening three CPRs (cytochrome reductases) and optimizing the ratio of F3′H−CPR to 1:2 [56]. Through increasing precursor supply, engineering rate-limiting pathway enzymes, optimizing enzyme expression levels and introducing modifications to the endogenous yeast metabolism to enhance NADPH supply, the biosynthesis of benzyl isoquinoline alkaloids in the yeast had been improved over 18,000-fold [47,57,58]. The disruption of the phosphatidic acid phosphatase (PAH1) resulted in a dramatic expansion of the ER, which led to the 8-fold increase of medicagenic acid by co-expressing three P450s [59]. Furthermore, over-expressing a key ER size regulatory factor (INO2) also improved the production of squalene and P450-mediated protopanaxadiol by 71-fold and 8-fold [60].

Heterologous expression of P450 in E. coli

Although the intrinsic P450 expression system is default, there were still many successful cases for metabolic engineering of P450 enzyme-mediated biosynthesis of PNPs in *E. coli*. The significant advances of P450 expression in bacterial expression systems, including N-terminal modifications, chaperonin coexpression, reduction of secondary mRNA structure, bacterial codon usage, selection of vector and host strain, as well as varying external growth conditions, have been comprehensively summarized by *Zelasko* et al. [61]. The N-terminal modifications of P450 enzymes for biosynthesis of PNPs were listed in Table 2. By optimizing P450 expression, reductase partner interactions, and N-terminal modifications, the highest titer of oxygenated taxanes achieved to ~570 ± 45 mg/L in *E. coli* [62]. It has been well documented that cellular redox balance and co-factor availability could significantly influence the yield of metabolites in microorganisms. For example, the disruption of glutamate dehydrogenase (gdhA) could increase 10% of NADPH/NADP$^+$ ratio and also improved the product ent-kaurenoic acid about 22.5% [63]. The expression of a fusion protein, containing P450 gene CYP714A2 and its reductase AtCPR2 from *A. thaliana,* improved the yield of steviol to 38.4 mg/L in batch fermentation [64]. Co-culture both *E. coli* and *S. cerevisiae* would be a good strategy to adequately take advantages of both species. For example, benzylisoquinoline alkaloids (BIAs) were successfully synthesized in an *E. coli* and *S. cerevisiae* co-culture system. *E. coli* was engineered for biosynthesis of the branchpoint intermediate (S)-reticuline, and *S. cerevisiae* was engineered to express membrane-bound P450 enzymes. The final yields of magnoflorine and scoulerine were reached to 7.2 mg/L and 8.3 mg/L [65]. Similarly, by integrating the rapid production of taxadiene in *E.*

Table 2
N-terminal modifications of plant P450 enzymes.

N-terminus modifications	P450 enzymes Plant Origin	Products	Hosts	References
LLLAVFL	CYP706B1 *Artemisia annua*	8-Hydroxycadinene artemisinic acid	*E. coli*	[102]
LLLAVFL	Isoflavone synthase *Glycine max*	Genistein	*E. coli*	[103]
LLLAVFL	CYP701A3 *Arabidopsis thaliana*	*Ent*-kaurenoic acid	*E. coli*	[64]
LLLAVFL	*CYP389C16* *Tetranychus cinnabarinus*	Acaricide decomposition product	*E. coli*	[104]
LLLAVFL	CYP79A2 and CYP83B1 *Arabidopsis thaliana*	Phenylacetaldoxime, Phenylacetonitrile oxide	*E. coli*	[105]
LLLAVFL	CYP71AV1 *Artemisia annua*	Artemisinic alcohol Artemisinic aldehyde Dihydroartemisinic acid Artemisinic acid	Yeast	[106]
AKKTSS	AmI2′H *Astragalus membranaceus*	Medicarpin malonyl glucoside	*E. coli*	[107]
2B1	CYP701A3 *Arabidopsis thaliana*	*Ent*-kaur-16-en-19-ol *Ent*-kaur-16-en-19-al	Yeast	[108]
2B1	CYP725A4 *Taxus cuspidata*	Taxadien-5a-ol	*E. coli*	[62]
2B1	CYP99A3 *Oryza sativa*	Syn-pimaradien-19-ol syn-pimaradien-19-al syn-pimaradien-19-oic acid	Sf21insect cells	[109]
2B1	CYP76M5,6,7,8 *Oryza sativa*	C11-hydroxy-ent-cassadiene hydroxylated *ent*-sandaracopimaradiene(M6) M5	Sf21 insect cells	[110]
2B1	CYP701A8 *Oryza sativa*	C3α hydroxylation of ent-sandaracopimar-adiene ent-cassadiene ent-kaurene	*E. coli* Yeast	[9]
2B1	CYP71Z6 & 7 *Oryza sativa*	Ent-isokaurene C2-hydroxylase	*E. coli,*	[111]
2B1	CYP701A8,CYP71Z6 & 7 *Oryza sativa*	3β-Hydroxylation of syn-pimaradiene(701A8)2α,3α-dihydroxy-ent-isokaurene(71Z6) 3α-hydroxy-ent-cassadien-2-one(71Z7)	*E. coli*	[112]
2B1	CYP82D1.1 *Scutellaria baicalensis*	Baicalein and scutellarein	*E. coli*	[113]
PD1	CYP73A1 *Helianthus tuberosus*	Trans-cinnamic acid hydroxylation product	Yeast	[114]
PD1; OMPA	CYP706B1 *Gossypium arboreum*	8-Hydroxycadinene	*E. coli*	[102]
MBP tag	CYP82D1.1 *Scutellaria baicalensis*	Baicalein and scutellarein	*E. coli*	[113]

coli and the efficient expression of P450 gene to hydroxylate taxadiene in *S. cerevisiae*, the synthetic consortium produced the highest yield of oxygenated taxanes with 33 mg/L [66].

Heterologous expression of P450 genes in tobacco

Nicotiana benthamiana, a wild relative of tobacco, has been increasingly used as a system for reconstituting biosynthetic pathway of PNPs [67,68]. *N. benthamiana* is able to be operated under *Agrobacterium* mediated transient expression which enables expression of multi-gene pathways or combinatorial biosynthetic libraries, without building multi-gene constructs. Moreover, many metabolic precursors for PNPs biosynthesis often could be present with sufficient quantities in *N. benthamiana* [67–69]. So far, several pathways for different types of natural products have been successfully reconstituted in *N. benthamiana,* including terpenoid [70], alkaloids [68], etoposide aglycone [67] or betalains [71]. For example, using a chloroplastic compartmentalized metabolic engineering strategy, Yong Wang's team introduced taxadiene synthase, taxadiene-5α-hydroxylase, and a cytochrome P450 reductase into tabacco and demonstrated that tabacco has the potential as an alternative platform for taxol production [69]. Jing-Ke Weng's team revealed that instantaneous expression of P450s in tabocco is sufficient to elicit diosgenin production in *N. benthamiana* [45].

P450 protein engineering

Although P450 enzymes possess powerful catalytic function with irreplaceable regioselectivity and stereoselectivity, the natural P450 enzymes are still very limited because of low activity, poor stability and narrow substrate scope, which could not satisfy the requirements of rapid development of industrial biotechnology. The natural P450s have been engineered by many approaches such as directed evolution, random mutation, rational design and so on [72,73]. Here, we will discussed the two most useful methods for protein engineering of P450s.

A lot of efforts has been put into protein engineering, which not only improved the activity and stability of P450s, but also performed novel reactions [73], which was pioneered by the Nobel Laureate *Frances H. Arnold* [74–76]. Directed evolution has been widely applied to engineer the well-characterized P450s, such as P450BM3 and P450cam [77]. These extensive works of directed evolution and site-directed mutagenesis have been summarized up by *Whitehouse* et al. [78] and *Li* et al. [73]. However, there is very few research being done on plant derived P450s. *Brühlmann* et al. applied directed evolution technology to engineer a 13-hydroperoxide lyase (CYP74B) from guava. A mutant with 15-fold increased product yield was generated by an iterative cycle of *in vitro* DNA recombination using L-ShufflingTM and rational random mutagenesis using EvoSightTM [79]. By engineering the SRSs (Substrate Recognition Sites) of CYP76AH15, which is responsible for the first putative oxygenation step of the forskolin pathway, about 6 fold improvement of catalytic activity was observed with the variant A99I [80]. Although many successful examples of P450s engineering have been done, it is still restricted by high-throughput screening method.

Establishment of a high-throughput screening method is very important for P450 enzyme engineering. A fluorescence-based screen was established for CYP76AD1, which exhibits tyrosine hydroxylase activity based on the further conversion of the product levodopa (L-DOPA) to

betaxanthin by a plant DOPA dioxygenase. However, the CYP76AD1 mutants with higher activity of tyrosine hydroxylase also conducted DOPA oxidase activity, which could branch the metabolic flux toward undesired synthesis of L-dopaquinone [81]. Depending on a C-terminal GFP (Green fluorescent protein) fusions, a high-throughput screening platform was constructed. Several highly expressing an robustly performing chimeric designs of cytochrome P450s were identified. This results provided a framework for the broad application of N-terminal tags in the design of microbial cell factories [81].

Conclusions and future prospects

Cytochrome P450 enzymes are one of the dominating driving forces of plant natural products diversity. Currently, P450s in most biosynthetic pathways of PNPs remind unclear. Fortunately, the advances in genomics, bioinformatics and synthetic biology speed up the elucidation of PNPs biosynthetic pathways. We have seen notable progress in gene mining, heterologous expression and protein engineering. The collection of P450 genes in databases also provided a huge improvement for P450 studies [82]. However, post-modification by P450 is still a speed limited step for the biosynthesis of PNPs. At present, the directed evolution and semi-rational design have been applied to improve the performance of P450 enzymes. In the future, desired modifications of the target PNPs would be realized by *de novo* design of P450 genes.

CRediT authorship contribution statement

Xiaonan Liu: Writing - original draft. **Xiaoxi Zhu:** Writing - original draft. **Hui Wang:** Data curation. **Tian Liu:** Data curation. **Jian Cheng:** Writing - review & editing. **Huifeng Jiang:** Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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