Engineering medicinal plant-derived CYPs: a promising strategy for production of high-valued secondary metabolites

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Main conclusion Cytochrome P450s (CYPs) play a critical role in the catalysis of secondary metabolite biosynthetic pathways. For their commercial use, various strategies for metabolic pathway engineering using CYP as a potential target have been explored.

Abstract Plants produce a vast diversity of secondary metabolites which are being used to treat various ailments and diseases. Some of these metabolites are difficult to obtain in large quantities limiting their industrial use. Cytochrome P450 enzymes (CYPs) are important catalysts in the biosynthesis of highly valued secondary metabolites, and are found in all domains of life. With the development of high-throughput sequencing and high-resolution mass spectrometry, new biosynthetic pathways and associated CYPs are being identified. In this review, we present CYPs identified from medicinal plants as a potential game changer in the metabolic engineering of secondary metabolic pathways. We present the achievements made so far in enhancing the production of important bioactivities through pathway engineering, giving some popular examples. At last, current challenges and possible strategies to overcome the limitations associated with CYP engineering to enhance the biosynthesis of target secondary metabolites are also highlighted.

Keywords Cytochrome P450 · CYPs · Secondary metabolites · Medicinal plants · Metabolic pathway engineering

Introduction

Plants synthesize a rich diversity of heterogeneous secondary metabolites that affect their development and naturally provide protection against a variety of pests, pathogens, and unfavorable environment (Dhar et al. 2015). Medicinal plants produce a variety of secondary metabolites such as phenolics, terpenes, steroids, alkaloids, and flavonoids, and have been proved useful in Unani, Ayurveda, Siddha, and Chinese medicine since time immemorial (Malhotra et al. 2001). Even today, these plants and their extracts are being used as an alternative treatment for many ailments, including diabetes, fibrosis, oxidative stress, inflammation, cardiovascular disorders, COVID-19, Alzheimer’s disease, and cancer (Crozier et al. 2008; Shree et al. 2022). Along with this, secondary metabolites are known for their rejuvenating, health-promoting, and immune system boosting properties. As per WHO reports, over 80% of the world’s population dwells upon herbs and derived products for primary healthcare needs. The major benefit of herbal drugs is attributed to their multiple benefits with minimal side effects. Moreover, the application of secondary metabolites for developing semi-synthetic and synthetic drugs for treating inflammatory bowel diseases, malaria, cardiovascular disorders, cancer, and others is well evident (Seca and Pinto 2019). However, season dependence, low yield, and high extraction cost of therapeutic compounds pose the biggest challenge for the utilization of medicinal plants in herbal industries (Renault et al. 2014). Additionally, reckless harvesting and habitat destruction of medicinally important species have made several of them endangered. Strategies such as breeding to develop high yielding varieties take years to get a stable phenotype. Furthermore, due to their complex biosynthetic pathways, synthetic production of secondary metabolites is technically challenging and also costly.
Metabolic pathway engineering utilizes various strategies such as the introduction of a biosynthetic gene, transcription factor or precursor feeding, overexpression of rate-limiting enzymes, or silencing or deletion of alternate branch pathway genes to shift the metabolic flux toward the desired pathway to enhance the production of rare metabolites for commercial use (Kulkarni 2016). A Nobel prize-winning research on *Artemisia annua* is a great example of using metabolic engineering for the production of a pharmaceutically important compound, Artemisinin as an antimalarial drug. Several strategies such as overexpression, suppression, and global regulation of genes were used to get the desired Artemisinin yield (Lu et al. 2016).

CYPs belong to the monoxygenase superfamily of proteins found in all domains of life including plants, and are critical players in the production of some of the highly valued secondary metabolites. They contain a heme prosthetic group (Fe-protoporphyrin IX) with anionic, thiolate sulfur of a cysteine residue ligation to the heme iron (Fig. 1) (Lamb and Waterman 2013; Xu et al. 2015). The cofactor heme plays an important role in catalysis, and when bound to carbon monoxide produces a spectrum of wavelength at 450 nm; hence, CYPs are also called P450s. The thiolate bond associated with the formation of Fe (IV) oxo species (compound I), a highly reactive intermediate which is involved in oxidizing inactivated C–H bonds, is indispensable in the CYP catalytic cycle (Rittle and Green 2010). All CYPs share a common mechanism of reduction and oxidation, wherein these reductases are involved in the transfer of two electrons from NADPH to the substrate, and then the addition of one oxygen to the substrate and the other one yields water (Xu et al. 2015). Other than this, CYPs are also involved in hydroxylation, dealkylation, dimerization, and isomerization reactions. Due to their ability to catalyze diverse reactions, they are found to be involved in a variety of biological pathways, including antioxidant biosynthesis, fatty acid metabolism, defence, secondary metabolite biosynthesis, hormonal regulations, and metabolism of xenobiotic substances (Pandian et al. 2020). Next-generation sequencing has facilitated identification of over 30,000 CYPs in various domains of life and their functional role (Pandian et al. 2020). Due to their diverse catalytic functions (mentioned above), they have been used as a prime candidate for biotechnological interventions and metabolic engineering of secondary metabolite biosynthesis (Villa-Ruano et al. 2015; Rasool and Mohamed 2016). In this review, a detailed account on the emergence of CYPs as a game changer in metabolic engineering in medicinal plants along with the challenges and forthcoming possibilities are being discussed.

**General mechanism of CYP catalysis**

CYPs are known to catalyze a wide variety of complex biochemical reactions such as aryl–aryl coupling, ring contractions and expansions, S-dealkylations, N-dealkylations, and O-dealkylations, decarboxylation, oxidative cyclization, alcohol and aldehyde oxidation, desaturation, sulfoxidation, nitrogen oxidation, epoxidation, C–C bond scission, decarbonylation, and nitration (Fig. 2) (McIntosh et al. 2014; Rasool and Mohamed 2016).

Mostly CYPs are found to be localized at the cytoplasmic surface of the endoplasmic reticulum along with the presence of cytochrome P450 reductase (CPR) for their proper functioning (Li et al. 2019a). They show high regio- and stereo-selectivity during catalysis steps in the biosynthesis of bioactive compounds (Rasool and Mohamed 2016). The presence of a nonpolar active site and high conformational flexibility, makes them a perfect catalyst in the biosynthesis of pharmaceutically important natural products (Jensen and Møller 2010). Reduced pyridine nucleotides (NAD(P)H) act as an electron source for its catalytic function which is assisted by auxiliary redox partner proteins. Generally, these redox partners can be majorly divided into three classes: Fe-containing ferredoxin (Fdx) and a flavin adenine dinucleotide (FAD)-containing ferredoxin reductase (FDR) involved in prokaryotic CYPs reduction; a membrane-bound flavin mononucleotide (FMN)/FAD with NADPH CYP oxidoreductase involved in eukaryotic CYPs; and adrenodoxin (Adx) and adrenodoxin reductase (ADR) involved in mitochondrial CYPs (Lamb and Waterman 2013). The basic mechanism of catalysis of CYP involves firstly binding of organic substrate (R) to the heme group of the enzyme (Fig. 3). This induces electron transfer from NADPH through FAD and FMN domains which leads to
Fig. 2 Some of the important reactions catalyzed by CYPs in medicinal plants. Adapted from Rasool and Mohamed (2016)

Fig. 3 A general mechanism of CYP catalysis. Adapted from Kalra (2007)
the reduction of then heme ferric iron (Fe$^{3+}$) to the ferrous state (Fe$^{2+}$). Then, ferrous CYPs get converted to ferrous CYP–dioxygen complex as a result of molecular oxygen binding which leads to the transfer of a second electron from CPR or any other associated reductase. This results in the formation of a short-lived peroxo complex that rapidly gets protonated twice, leading to the formation of one molecule of water and an iron–oxo complex. Lastly, the oxygen atom in the complex binds to the organic substrate (R) and forms the oxidized reaction product (RO) (Pandian et al. 2020).

The most important interacting partners for efficient catalysis by CYPs are reductases. Along with this, a conserved active site and axial cysteine ligating the heme iron are involved in the production of other key intermediates. The threonine (T268) promotes the scission of dioxygen bond by protonating the iron-peroxo and iron-hydroperoxy intermediates with the help of water. The thiol bond is also involved in the promotion of dioxygen bond scission of iron-hydroperoxy intermediates. It also makes ferric ion a poorer electron acceptor which prevents the catalytic cycle to start without the presence of a substrate (McIntosh et al. 2014).

**CYPs in the production of medicinally important secondary metabolites**

A large variety of natural products derived from medicinal plants have been used as traditional medicine since time immemorial (Dhar et al. 2015). For instance, vincristine, vinorelbine isolated from *Artemisia annua* (artemisinin) in malaria, *Papaver somniferum* (morphine, codeine, papaverine, noscapine) in pain, cough, and cramps, *Physostigma venenosum* (physostigmine) in Alzheimer’s disease, has been reported (Bhattacharjee 2000). Ayurveda, Siddha, Homeopathy, Unani, and Traditional Chinese Medicine (TCM) are the alternative system of medicine that make use of natural products to treat different ailments (Malhotra et al. 2001). Today, procedures like chromatography have made it possible to analyze and understand the secondary metabolite composition of herbal extracts (Singh 2003). These secondary metabolites comprises of majorly flavonoids, terpenoids, phenylpropanoids, alkaloids, steroids, cyanogenic glycosides, and glucosinolates are known to have a variety of biological and medicinal properties (Villa-Ruano et al. 2015; Xu et al. 2015). Due to their functionally diverse roles, CYPs are considered as the most versatile biological catalysts in nature (Rana et al. 2014). The role of various CYPs in plants used in medicines is given in Table 1.

**CYP: a promising target for metabolic engineering**

Previous studies imply that CYPs are highly evolvable and adaptable to the mutations in their active site that makes them accept novel substrates readily (Jung et al. 2011). New specificities of CYPs are exhibited by the forceful molding of protein sequences which in turn are filtered by natural selection to favor the formation of certain intermediates (McIntosh et al. 2014). This enables the CYPs to generate valuable natural and non-natural products, and the synthesize the precursors of important metabolites from medicinal plants (Jung et al. 2011; Li et al. 2020). The application of CYPs in metabolic engineering has greatly facilitated the field of synthetic biology.

CYPs are considered to be the prime candidate for silencing of competitive pathways, optimizing the metabolic fluxes toward desired pathway, heterologous expression of desired metabolite, increasing enzyme–host compatibility and others (Li et al. 2020; Liu et al. 2020). There are several approaches used for CYP engineering such as gene overexpression, suppression, functional and structural manipulation by integration of computational designing of protein, evolutionary information related to that protein followed by optimization of the experimental data for alteration of the specificity of the substrate, and also integrated protein, substrate, and cofactor engineering (Li et al. 2019a). Some traditional methods of metabolic engineering using CYPs in plants for the discovery and regulation of pharmaceutically important metabolites and genes involved in their production (Lu et al. 2016) are discussed below:

(i) Overexpression of pathway genes: Overexpression of a single gene or co-expression of multiple genes of the target pathway is the most commonly used technique for enhancing the production of the desired metabolite. For instance, overexpression of CYP716A52v2, a key gene in the ginsenoside biosynthesis pathway showed enhanced production of oleanane-type ginsenoside in *Panax ginseng* plants (Han et al. 2013). Combined overexpression of genes involved in artemisinin biosynthesis *ADS* (Amorpha-4,11-diene synthase gene), CYP71AV1 and CPR promoted the accumulation of artemisinin in *A. annua* up to 2.4-fold higher than the controlled plant (Lu et al. 2013, 2016).

(ii) Downregulating competing metabolic pathways: Cassava (*Manihot esculenta*) is one of the main staple foods in some countries but it contains cyanogenic glucosides in its tuber which makes it toxic. CYP79D1 and CYP79D2 are two genes which are responsible for catalyzing the first few committed reactions in the biosynthesis of lotauastralin and linamarin. In the transgenic cassava, the downregulation of these enzymes leads to a significant decrease (by up to 92%) in cyanogenic glucoside production in its tuber (Jörgensen et al. 2005).
(iii) Global pathway regulation by regulating transcription factors, endogenous phytohormones, and primary metabolism: Overexpression of a transcription factor, i.e., AabZIP1 which belongs to the basic leucine zipper family leads to the upregulation of ADS and CYP71AV1. It also increases the artemisinin content by 1.5-fold in transgenic A. annua plants (Zhang et al. 2015). Jasmonic acid is also important in artemisinin biosynthesis. Overexpression of the allene oxide cyclase gene from A. annua leads to increase levels of endogenous jasmonate which further leads to upregulation of FDS, CYP71AV1 and DBR2, which resulted in a significantly increased production of artemisinin, DHAA and artemisinic acid (Lu et al. 2014, 2016).

(iv) Heterologous expression of genes: Heterologous expression of a gene can be observed in plant or a microbial host. CYP76B1 from Helianthus tuberosus catalyzes rapid oxidative dealkylation of various phenyl urea herbicides. Ectopic constitutive expression of CYP76B1 in tobacco (Nicotiana tabacum) and Arabidopsis showed a high tolerance against herbicides (Didierjean et al. 2002). Microbial systems can also be considered as a good heterologous host due to several factors such as their fast-doubling time, easy scalability, easy extraction of product, and cost-effectiveness. For example, the expression of CYP716A12 from M. truncatula, together with the bAS and CPR from Lotus japonicus, resulted in the production of oleanolic acid in yeast (Fukushima et al. 2011; Moses et al. 2013). When

### Table 1 A few examples of CYPs involved in the secondary metabolic pathway of plants used in medicine

| CYP    | Role                                                                 | Plant species                     | References               |
|--------|----------------------------------------------------------------------|----------------------------------|--------------------------|
| CYP88D6| Licorice β-amyrin 11-oxidase involved in the biosynthesis of the triterpene sweetener glycyrrhizin | Glycyrrhiza spp.                 | Seki et al. (2008)       |
| CYP71A87| Marrubiin biosynthesis                                             | Marrubium vulgare                | Karunanithi et al. (2019) |
| CYP76B6| Iridoid monoterpenoids biosynthesis                                 | Catharanthus roseus              | Collu et al. (2001)      |
| CYP72A1| Converts loganin into secologanin                                   | Catharanthus roseus              | Irmler et al. (2000)     |
| CYP706X| Hydroxylation of Apiigenin for the production of Scutellene         | Erigeron brevisscapus            | Liu et al. (2018)        |
| CYP76AD1/6| Hydroxylation of Tyrosin for the production of L-DOPA, the precursor of dopamine | Beta vulgaris and Mirabilis jalapa | Polturak et al. (2016) |
| CYP73A | Encodes Cinnamic acid 4-hydroxylase (CA4H) which is involved in phenylpropanoid biosynthesis | Populus kitakamiensis           | Kawai et al. (1996)      |
| CYP75  | A hydroxylation enzyme involved in flavonoid biosynthesis          | Solanum melongena               | Toguri et al. (1993)     |
| CYP82D | Flavone-6-hydroxylase and 7-demethylase involved in flavonoid metabolism | Ocimum basilicum                | Berim and Gang (2013)    |
| CYP93B1| Involved in hydroxylation of flavones at C-2 position              | Glycyrrhiza echinata             | Akashi et al. (1998)     |
| CYP71D | Encodes flavonoid 6-hydroxylase that catalyzes the conversion of flavanones | Glycine max                     | Latunde-Dada et al. (2001) |
| CYP719 | Biosynthesis of berberine                                          | Coptis japonica                 | Ikezawa et al. (2003)    |
| CYP80F1| Rearrangement of (R)-littorine to (S)-hyoscyamine,                | Hyoscyamus niger                | Li et al. (2006)         |
| CYP716A| Hydroxylation of steroidal saponin precursor cycloartenol         | Aquilegia coerulea               | Miettinen et al. (2017)  |
| CYP706C55| Conversion of phenyl acetaldoxime into phenyl acetonitrile in prunasin biosynthesis | Eucalyptus cladocalyx            | Hansen et al. (2018)     |
| CYP71E1| Catalyze the conversion of p-hydroxyphenylacetaldoxime to p-hydroxymandelonitrile | Sorghum bicolor                | Kahn et al. (1999)       |
| CYP71D18| Encodes spearmint limonene 6-hydroxylase that hydroxylates (4S)-limonene to (-)-trans-carveol | Mentha spicata                 | Wust et al. (2001)       |
| CYP725A3| Encodes for taxoid 14 beta-hydroxylase involved in the biosynthesis of taxoid | Taxus cuspidata                | Jennnewein et al. (2003) |
| CYP76AH1| Catalyzes the conversion of mitiradiene to tanshinones             | Salvia miltiorrhiza             | Guo et al. (2013)        |
| CYP84A1| Involved in the biosynthesis of phenylpropanoid by transforms coniferaldehyde and coniferyl alcohol into 5-hydroxylated derivatives | Dendrobium officinale           | Humphreys et al. (1999)  |
| CYP716A244| Involved in oleanane-type saponin biosynthesis from 2,3-oxidosqualene | Eleatherococcus senticosus       | Jo et al. (2017)         |
| CYP72A154| Involved in C-30 oxidation in the glycyrrhizin biosynthesis pathway | Glycyrrhiza glabra              | Seki et al. (2011)       |
| CYP722C| Catalyzes the conversion of carlactonoic acid to 5-deoxystrigol     | Gossypium arboreum              | Wakabayashi et al. (2020) |
| CYP87D18| Involved in C-11 oxidation of cucurbitadienol                      | Siraitia grosvenorii            | Zhang et al. (2016)      |
expressed in a heterologous host, for the enhancement of CYPs catalytic activity, the membrane-bound domains can also be abridged (Xiao et al. 2019).

Successful metabolic engineering of CYP utilizes one or more of these techniques for obtaining high productivity of desired metabolites. For instance, two opioid drugs were completely biosynthesized in a heterologous host (S. cerevisiae) using CYP SalSyn from Papaver somniferum. This engineered SalSyn showing overactivity of salutaridine, when co-expressed with 21/23 heterologous enzymes and two native enzymes with one native yeast gene deleted, resulted in the production of thebaine/hydrocodone (Galanie et al. 2015). A CYP protopanaxadiol synthase from Panax ginseng was designed in an engineered S. cerevisiae strain. When it was co-expressed with AtCPR, it resulted in the production of 1400 mg/liter of protopanaxadiol (Li et al. 2020; Wei et al. 2018; Zhao et al. 2016).

The newer methodologies are focusing more on protein engineering for enhanced metabolite flux which includes techniques such as directed evolution, rational structurally guided mutation, and random approaches like error-prone PCR, terminal modification, integrated approach combining computational protein design, evolutionary information, and experimental data-driven optimization (Caswell et al. 2013; Li et al. 2019a; Xiao et al. 2019). Directed evolution is a method which is used for the optimization of enzyme properties as per application demands (Xiao et al. 2019). It involves error-prone PCR, saturation mutagenesis, and gene recognition. This technique prerequisites a deep knowledge and understanding of the relationship of sequence, structure, and function of a protein. An integrated database of CYPs, using extensive sequence analysis within members of protein family, serves as a tool for the identification of functionally related and amino acid residues determining the selectivity of the proteins (Moses et al. 2013). In this method, a catalytically improved and completely functional CYP CinA-ADD-CinC fusion protein variant (KB8) is generated for bio-electrocatalysis (an alternative for the replacement of NADPH in cell-free CYP-catalyzed reactions). This variant is found to be more suitable in several synthetic and electrochemical approaches by replacing the reconstituted CYP in the system. It will be a step further to use multi-component CYP systems in different bioelectrochemical applications (Belsare et al. 2017). Random design involves the engineering of enzyme by improving CYP performance with a recognized crystal structure or an existing homology model (Xiao et al. 2019). In a study, a combination of random site-directed and saturation mutagenesis techniques were used which led to a significant change in the regioselectivity of progesterone hydroxylation from the 15β- to the 11α-position which is dependent on CYP106A2 (Nguyen et al. 2012). Terminal modification involves modification of the membrane anchor region of CYP as they are bound to ER membrane which can make it insoluble, unstable, or lead to total loss of activity in heterologous CYP expression (Xiao et al. 2019). For instance, a gene from Jerusalem artichoke (Helianthus tuberosus), i.e., CYP73A1 is known for hydroxylation of cinnamic acid for the synthesis of lignin monomers and many phenolic compounds in higher plants. For the purpose of simple purification from recombinant yeast, improved solubility and stability of this gene in the absence of detergent, it was engineered. The hydrophobic N-terminal was replaced by the peptitenger amphipathic sequence PD1 (Schoch et al. 2003a).

Directed evolution, random mutagenesis and structure-guided protein designing are time consuming and complex. This might be due to the absence of efficient high-throughput screening and crystal structure of the protein. De novo computational protein design is a method which is used for the introduction of new structures and functions to the enzymes, guided by the physical principles that underlie protein folding (Huang et al. 2016; Li et al. 2019a). There are several online databases and sites available which are related to the discovery and engineering of CYP (Box 1). Rosetta is a popularly used method for macromolecular modeling, docking, and designing of proteins (Leman et al. 2020). Along with Rosetta, GERMLIN is used to compute co-evolving amino acids which assists in generating high-resolution protein structure and ligands docking. This integrated approach can be followed where information related to protein structure, its folding ability, binding and assembly is used with computational all-energy atom functions (Kellogg et al. 2011; Shang and Huang 2020). For instance, in 2019, Li and his team members have successfully engineered CYP87D20 which is involved in the cucurbitacin C biosynthetic pathway and was transformed into single specific hydroxylation performing CYP at the C-11 position of cucurbitadienol. Then, this approach was used to fashion a de novo pathway for the production of mogrol, a natural sweeter mogroside’s precursor (Li et al. 2019a). Different approaches for CYP engineering and factors limiting them have been summarized in Fig. 4. Successful usage of these techniques is explained in the later sections of the review.

### Box 1: Popular tools and databases related to CYP discovery and engineering

- **CYPED**: A collection of tools for classification and analysis of CYPs (Sirim et al. 2009).
- **Cytochrome P450** ([https://drnelson.uthsc.edu/](https://drnelson.uthsc.edu/)): A database with organized and systematic collection of CYP genes (Nelson et al. 2009).
- **The Plant Cytochrome P450 Database** ([erda.dk/public/vgrid/PlantP450/](https://erda.dk/public/vgrid/PlantP450/)): An online compiled data of functionally characterized Plant CYPs (Hansen et al. 2021).
• LICRED: A Versatile Drop-In Vector for Rapid Generation of Redox-Self-Sufficient Cytochrome P450s (Sabbadin et al. 2010).

• PCPD: Plant cytochrome P450 database and web-based tools for structural construction and ligand docking (Wang et al. 2021).

• CYPminer: An automated cytochrome P450 identification, classification, and data analysis tool for genome data sets across kingdoms (Kweon et al. 2020).

• CYPsI: A structure-based interface for CYPs and ligands in Arabidopsis thaliana (Zhang et al. 2012).

• Arabidopsis Cytochrome P450 and TAIR (http://www.p450.kvl.dk/p450.shtml and https://www.arabidopsis.org/): CYP genes annotated from the genome database of Arabidopsis (Rhee et al. 2003).

• Phytozome (http://www.phytozome.net/)—Database for accurate and insightful comparative genomics studies having large datasets of plant genomes (Goodstein et al. 2012).

• Phytometasyn project (https://www.bioinformatics.tugraz.at/phytometasyn/)—Assembled transcriptome databases of non-model plants (Xiao et al. 2013).

Achievements in metabolic engineering of CYPs

Several cytochromes involved in the production of important secondary metabolites have been identified in medicinal plants. A few of them have been engineered in heterologous host or utilized for (semi) synthetic production. A classic example of important metabolite synthesis from a medicinal plant is the biosynthesis of taxol, an anti-cancer drug produced by engineering of a CYP (CYP725A4) involved in selective oxygenation. Optimization of the expression of CYP, interaction of reductase partner, and N-terminus modifications lead to the highest yield of oxygenated taxanes in E. coli (Biggs et al. 2016). Cellular redox balance and availability of cofactors are playing a significant role in influencing the yield of metabolites (Liu et al. 2020). Another major achievement with CYP engineering was semi-synthetic production of Artemisinin which is a sesquiterpene lactone having antimalarial properties, produced by the plant Artemisia annua (Paddon et al. 2013). It was difficult to produce it in its natural form in bulk because of its unstable nature, which often resulted in drug shortage and price fluctuation.

CYP71AV1 was found to be involved in catalyzing three successive oxidations steps converting amorpha-4,11-diene to artemisinic acid (Renault et al. 2014). Overexpression of CYP71AV1 in plants or heterologous system provides an opportunity to enhance artemisinin through semi-synthesis (Teoh et al. 2006). Other examples of CYP engineering include the co-expression of Glycyrrhiza uralensis CYP88 and CYP72A154 in Saccharomyces cerevisiae in combination with Arabidopsis thaliana beta-amyrin synthase (β-AS) and AtNADPH-CPR leads to the accumulation of...
glycyrhetinic acid (GA) and a small amount of β-amyrin. GA production is further enhanced by Glycyrrhiza uralensis cytochrome b5 (GcUYB5) (Wang et al. 2019). For the biosynthesis of morphine in Papaver somnifera, a critical bioconversion of (S)-reticuline into (R)-reticuline was required. It was achieved by fusion of CYP82Y2-like P450 from an aldo–keto reductase (AKR) leading to this epimerization of reticuline via 1,2-dehydroreticuline (Farrow et al. 2015; Galanie et al. 2015). A comprehensive list of CYPs identified in medicinal plants and engineered in heterologous host is compiled in Table 2.

Challenges in CYP engineering and biotechnological intervention

For engineering any metabolic pathway, it is a prerequisite to have complete information of its biosynthetic pathway. But sequencing and characterization of genes remain broadly limited to crop and horticulturally important plants. Lack of sequence and secondary metabolic pathway information of medicinal plants has been the biggest challenge in the identification and characterization of CYPs which show huge sequence and functional diversities. Expression of these enzymes varies with tissue, developmental stage, and environmental conditions, which makes their regulation very complex.

As CYPs are the largest family of enzymes, they are shown to be involved in the regulation of multiple biosynthetic pathways. They are flexible enough to recognize multiple substrates and produce a spectrum of products. Metabolic engineering of CYPs to produce desired product may lead to silent metabolism and/or hidden crosstalk among various pathways, giving rise to unintended metabolic outcomes (Lynch et al. 2021). For example, overexpression of CYP71BE79 during partial reconstitution of gossypol biosynthetic pathway in tobacco leaves leads to unintended production of glycylated product 8,11-dihydroxy-7-keto-δ-cadinene instead of 11-dihydroxy-7-keto-δ-cadinene itself (Tian et al. 2018). Great versatility for multiple substrates confers low catalytic efficiencies to the CYPs (Bar-Even and Salah Tawfik 2013; Bernhardt and Urlacher 2014; Shang and Huang 2020). Missense mutation in AtCYP83B1 is also a great example of this. The mutation shows reduced levels of indole glucosinolate, and accumulation of indole-3-acetaldoxime, while surprisingly limiting the phenylpropanoid metabolism (Kim et al. 2015; Lynch et al. 2021). Further, the catalytic activity of CYPs relies on transfer of electrons from reductases (CPR). An extrication between the formation of product and availability of NAD(P)H to provide electrons may significantly affect the catalytic activity and stability of CYP (Lundemo and Woodley 2015).

CYP shows varied expression in heterologous systems which makes the determination of CYP function difficult. Renault and his team concluded that almost 40% of plant CYPs are poorly expressed in yeast. This makes it difficult to predict the protein expression and its application in industrial production (Nørholm et al. 2013; Renault et al. 2014). Heterologous production of bioactive compounds can also be toxic for the system. For instance, a CYP71D51v2 from tobacco expressed in recombinant yeast and a CYP reductase from Arabidopsis were used for the optimization of valencene conversion into nootkatol and nootkatone. But because of the toxicity of the bioproduct, i.e., β-nootkatol in yeast system, it did not match the requirements for implementation of an industrial process (Gavira et al. 2013).

Plants provide an outstanding platform for core metabolism to produce desired products by grafting specialized pathways but alongside this, due to the activity of host plant enzymes, they tend to convert those desired products further into conjugates or other derivatives. According to a study, it was shown that CYP76B6, a multifunctional enzyme from Catharanthus roseus is involved in sequential oxidation steps leading to the formation of 8-oxogeraniol from geraniol. In planta, it was observed that the first step of geraniol hydroxylation was very efficient and fast. However, when expressed in leaf tissues, in the absence of the next enzyme of the secoiridoid pathway, 8-oxogeraniol was converted into further oxidized and/or reduced compounds (Höfer et al. 2013). Therefore, it is very important to channel the desired metabolic intermediate to downstream product rapidly (Renault et al. 2014). In summary, instability, insolubility, low activity, poor specificities, incompatibility with non-native hosts, protein designing difficulties due to inadequate amount of substrate bound 3D structure, cofactor requirement, inefficiency of electron transfer as a result of either the lack of sufficient NAD(P)H levels, or poor interactions between CYPs and CPRs are among the major challenges to successful metabolic engineering (Armstrong et al. 2013; Bernhardt & Urlacher 2014; Shang & Huang 2020).

Circumventing the limitations of CYP for pathway engineering

Researchers have been trying to find ways to overcome the challenges in using this versatile multifunctional enzyme for the production of high-value metabolites at large scale through biotechnological interventions. The main challenge of lack of knowledge of genome sequence and mechanisms of silent metabolism was because of the limited ability of a systematic generation of large metabolites datasets and analyzing them computationally. But, recent advances in technology lead to the usage of omics approaches which include transcriptomics, proteomics, genomics and metabolomics that can alleviate...
| CYP         | Organism                  | Enzyme/reaction                                      | Engineering method                                                                 | Objective/modulations after engineering                                                                 | References                                                                                     |
|------------|---------------------------|------------------------------------------------------|-----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| CYP88D     | Glycyrrhiza uralensis     | β-amyrin 11-oxidase                                  | Codon optimisation of CYP88D6 and CYP72A154 with co-expression of β-amyrin synthase encoding gene and AtNADPH+ GucYB5 expression in S. cerevisiae | Glycyrrhizin accumulation                                                                                   | Seki et al. (2008), Wang et al. (2019)                                                          |
| CYP71AV1   | Artemisia annua           | Amorpha-4,11-diene C-12 oxidase                      | Expressed in conjunction with CPR and N-terminus modification, high copy number plasmid and a strong inducible promoter in S. cerevisiae | Anti-malaria-drug precursor artemisinic acid, artemisinic alcohol, artemisinic aldehyde and dihydroartemisinic aldehyde production | Chen et al. (2017), Teoh et al. (2006)                                                          |
| CYP76B1    | Helianthus tuberosus      | 7-ethoxycoumarin O-deethylase involved in double N-dealkylation of phenylurea | Ectopic constitutive expression in Nicotiana tabacum and Arabidopsis               | Increase in tolerance for several phenylurea herbicides                                                   | Didierjean et al. (2002)                                                                        |
| CYP87D20   | Cucurbitaceae family      | C11 carbonylase and C20 hydroxylase                 | Structural and data-driven approach using Rosetta software and GREMLIN              | Creating a de novo pathway to produce mogrol                                                              | Li et al. (2019a)                                                                              |
| CYP706B1   | Gossypium arboreum       | Cadinene-8-hydroxylase                              | N-terminal modification in E. coli                                                | 8-Hydroxyccadinene production                                                                            | Chang et al. (2007)                                                                            |
| CYP75A     | Camellia sinensis        | Flavonoid 3,5-hydroxylase                           | Gene overexpression in conjunction with CPR in tobacco                             | Accumulation of catechin                                                                                   | Wang et al. (2014)                                                                            |
| CYP73A1    | Helianthus tuberosus      | Trans-cinnamate 4-hydroxylase                       | N-terminus modification in S. cerevisiae                                          | Trans-cinnamic acid hydroxylation product                                                                   | Schoch, et al. (2003b)                                                                         |
| P450 isoavone synthase 1 | Glycine max          | Isoflavone synthase                                 | Electron flux optimisation by fusion of CPR in E. coli                              | Genistein accumulation                                                                                     | Leonard & Koffas (2007)                                                                       |
| CYP82D1.1  | Scutellaria baicalensis   | Flavone hydroxylases                                | N-terminal modification and truncation in E. coli                                 | Improved yield of baikalein and scutellain                                                               | Li et al. (2019b)                                                                              |
| CYP716A47  | Panax ginseng             | Encodes protopanaxadiol synthase                    | Fusion with AtCPR in S. cerevisiae                                                | Increase in protopanaxadiol production                                                                  | Zhao et al. (2016)                                                                            |
| CYP76AH15  | Coleus forskohlii        | Hydroformylation (converts 13R-manoyl oxide to 11-oxo-13R-manoyl oxide) | SRSs (Substrate Recognition Sites) engineering in S. cerevisiae                    | Increased production of forskolin                                                                        | Forman et al. (2018)                                                                          |
| CYP76AH1   | Salvia miltiorrhiza       | Ferruginol synthase                                 | In-vivo optimization of redox partners in S. cerevisiae                            | Ferruginol production                                                                                     | Guo et al. (2013)                                                                             |
| CYP725A4   | Taxus cuspidata           | Taxadiene-5α-Hydroxylase                            | Optimizing expression of CYP, interaction with reduction partner and N-terminal modification in E. coli | Increased oxygenated taxanes production                                                                 | Biggs et al. (2016)                                                                           |
| Aml2/H/CYP81E42 | Astragalus membranaceus | Isoflavone 2′-hydroxylase                           | N-terminal and ORF modification in E. coli                                         | Medicarpin malonyl glucoside accumulation                                                                  | Chen et al. (2015)                                                                            |
this problem. Synthetic biology in combination with computational tools can also be used to overcome these challenges (Jacobowitz and Weng 2020; Lynch et al. 2021). It also gives a deep insight into the plant metabolic networks and potential hidden silent constrictions (Erb and Kliebenstein 2020). Metabolic flux can be controlled by overexpressing the upstream enzymes or by silencing the side-branch pathways. It can also be done by a more appropriate genetic background. Co-expression of optimal protein sets or reconstitution of modular pathways can also be used to control metabolic flux (Renault et al. 2014). Optimization of codon partially or reduction of RNA secondary structure at the N-terminal can be used to solve the problem of heterologous expression of plant CYPs in yeast (Goodman et al. 2013). Toxicity of substrate/intermediates/product can be controlled by a biphasic bioreactor, trapping the products by anion exchange, conjugation of products, compartmentation of products, or boosting the flux to final non-toxic products (Renault et al. 2014). Directed evolution is used to improve the tolerability of CYPs toward organic solvent which is required for substrate molecules’ solubility. Immobilization and directed evolution have shown to increase the shelf life of these multifunctional enzymes (Armstrong et al. 2013). Implying all these techniques with appropriate analysis tools will help to resolve many problems such as the distribution of carbon flux within the networks of metabolic pathways. This will help in unveiling the channeling of metabolites and inactivated metabolic pools (Lynch et al. 2021; Shih & Morgan 2020). Further to solve the problem of cofactor requirement in CYP, scientists have used—(1) Regenerating NADPH cofactor by coupling of oxidation reaction with reductase generating NADPH; (2) Electrochemical reduction of CYP via either by mediators’ actions (indirect approach) or enzyme’s direct attachment to an electrode. Cofactor can also be reduced electrochemically; (3) Photoinduction methods can also be used for electron transfer; (4) Using cheaper cofactors for the reduction of CYP, for instance directed evolution can be used to produce efficient cofactors; (5) Peroxide shunt can also be used, but many CYPs are not efficient in the usage of this pathway, so researchers are attempting to increase their efficiency and lastly, by fusing the reductase with the CYP (Armstrong et al. 2013).

Conclusion and future perspectives

Medicinal plants are critical bioresources, synthesizing a wide array of secondary metabolites that have been found to play important role in treating different ailments in various system of medicines. However, low bioaccumulation of some of the high-valued secondary metabolites is the major bottleneck for their utilization for commercial application. Metabolic pathway engineering is a promising approach to alter the genes and their expression in a way to enhance the

| CYP | Organism | Enzyme/reaction | Engineering method | Objective/modulations after engineering | References |
|-----|-----------|----------------|-------------------|----------------------------------------|------------|
| CYP82Y2-like | Papaver bracteatum | 1,2-dihydromorphine synthase | Gene mining, protein mutagenesis, codon optimization, and heterologous expression in yeast | Bioconversion of (S)-reticuline to (R)-reticuline for morphine biosynthesis | Farrow et al. (2015), Galanie et al. (2015) |
| P450 SalSyn | Papaver somniferum | Salutaridine synthase | Co-expression of heterologous proteins and deletion of one host gene in yeast | Production of thebaine/hydrocodone | Galanie et al. (2015) |
| CYP716a155 | Rosmarinus officinalis | SA-specific lupeol C28 oxidase | Cognate expression of CYP CPR in S. cerevisiae | A potent anti-cancer agent, betulinic acid accumulation | An et al. (2020), Huang et al. (2019) |

Table 2 (continued)
accumulation of desired product. CYPs are known to catalyze the synthesis of a wide variety of useful metabolites, and often are the target of choice for metabolic engineering. In a short span of time, a lot of progress has been made in the discovery and modification of plant CYPs to produce natural, semi-synthetic, and synthetic products. However, incomplete pathway information of secondary metabolites, multiple reactions catalyzed by versatile CYPs producing undesired products, and lack of structural information for substrate and cofactor binding limit their application at industrial level. With the evolution of more affordable high-throughput sequencing, computational modeling for structure prediction and possible substrate binding, and development of superior bioreactors, a lot of unexplored possibilities for the production of valuable medicinal products through precise metabolic engineering of CYPs are underway.

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**Declarations**

**Conflict of interest** The authors declare no known conflict of interests or personal relationships.

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