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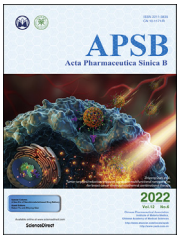

REVIEW

Cytochrome P450s in algae: Bioactive natural product biosynthesis and light-driven bioproduction

Shanmin Zheng[a,b,†], Jiawei Guo[b,†], Fangyuan Cheng[b], Zhengquan Gao[c], Lei Du[b], Chunxiao Meng[a,*], Shengying Li[b,d,*], Xingwang Zhang[b,*]

[a]*School of Life Sciences, Shandong University of Technology, Zibo 255000, China*
[b]*State Key Laboratory of Microbial Technology, Shandong University, Qingdao 266237, China*
[c]*School of Pharmacy, Binzhou Medical University, Yantai 264003, China*
[d]*Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China*

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Abstract Algae are a large group of photosynthetic organisms responsible for approximately half of the earth's total photosynthesis. In addition to their fundamental ecological roles as oxygen producers and as the food base for almost all aquatic life, algae are also a rich source of bioactive natural products, including several clinical drugs. Cytochrome P450 enzymes (P450s) are a superfamily of biocatalysts that are extensively involved in natural product biosynthesis by mediating various types of reactions. In the post-genome era, a growing number of P450 genes have been discovered from algae, indicating their important roles in algal life-cycle. However, the functional studies of algal P450s remain limited. Benefitting from the recent technical advances in algae cultivation and genetic manipulation, the researches on P450s in algal natural product biosynthesis have been approaching to a new stage. Moreover, some photoautotrophic algae have been developed into "photo-bioreactors" for heterologous P450s to produce high-value added pharmaceuticals and chemicals in a carbon-neutral or carbon-negative manner. Here, we comprehensively review these advances of P450 studies in algae from 2000 to 2021.

*Corresponding authors. Tel./fax: +86 532 58632496.
E-mail addresses: mengchunxiao@126.com (Chunxiao Meng), lishengying@sdu.edu.cn (Shengying Li), zhangxingwang@sdu.edu.cn (Xingwang Zhang).
[†]These authors made equal contributions to this work.
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1. Introduction

Cytochrome P450 enzymes (P450s) are a superfamily of heme (iron protoporphyrin) proteins that are broadly distributed in human, animals, plants, algae, fungi, bacteria, archaea and even viruses[1−4]. The name of "P450" is given owing to their distinctive spectroscopic absorption maximum at 450 nm upon its reduced heme-iron center bound with carbon monoxide[5]. Along with the explosion of genome sequencing data, a fast increasing number of P450-encoding genes, up to now more than 300,000, have been discovered from various organisms, which have greatly advanced our understanding on the important evolutionary, physiological and catalytic roles of the P450 superfamily[6,7]. It is now well known that P450s are extensively involved in both primary and secondary metabolisms[8−13]. For instance, many prokaryotic and eukaryotic P450s undertake essential physiological functions such as participating in the synthesis of membrane sterols, phytohormones and signaling molecules[14]. Meanwhile, the P450s from human or other higher animals are capable of mediating the degradation of drugs, toxins and other xenobiotics[11]. Moreover, a tremendous number of P450s from plants and microorganisms (both bacteria and fungi) are frequently found to play essential roles in natural product biosynthesis[3,4].

During the biosynthesis of natural products, generally, P450s implement their functions by catalyzing the common monooxygenation (*e.g.*, hydroxylation and epoxidation) reactions to decorate the core structure, improve the aqueous solubility of products, and provide chemical handles for further modifications[15]. Intriguingly, in an increasing number of cases, P450s have been found capable of mediating diverse "uncommon" reactions for skeleton construction, including C−X (X = C/N/S) bond formation/scission, ring formation/expansion/contraction and even more vagarious reactions, which dramatically expand the chemical space of natural products[16−20]. As a result, the P450-mediated reactions greatly increase the diversity of both the chemical structures and biological activities of natural products. Thus, the ubiquitous and versatile P450 enzymes have been recognized as one of the most significant biocatalysts in natural product biosynthesis and pharmaceutical bioproduction[21].

Algae are a polyphyletic group of aquatic organisms that include species from the microscopic unicellular cyanobacteria to the giant kelps up to tens of meters in length[22,23]. Most of the algal species are photosynthetic organisms, which are responsible for roughly 50% of the total photosynthesis on the earth[5,24]. Meanwhile, algae play their fundamental ecological roles by acting as oxygen producers and the food base for almost all aquatic life. Also, upon decades of natural organic chemistry studies, algae have been recognized as a rich source of high-value bioactive natural products[25−27]. Recent genome and transcriptome analyses have discovered numerous P450 genes involved in the basic life cycle of both cyanobacteria and eukaryotic algae. However, compared with other organisms, the knowledge on the functions of algal P450s still remains limited[28]. Fortunately, recent technical advances in algal cultivation and genetic manipulation have led to more and more functional characterization of algal P450s[29,30].

Furthermore, with the rapid development of synthetic biology, the photoautotrophic algae are expected to be used as "photobioreactors" for heterologous P450s to produce high-value added products in a carbon-neutral or carbon-negative manner[31]. Specifically, for the reducing power-driven P450 reactions, in theory, the algal photosystem derived electrons can be directly employed to drive their catalytic cycle, thus circumventing the requirement of the complex redox partner systems and the precious reducing force NAD(P)H. Such solar-energy-driven P450-reaction systems have been successfully constructed in several algal species, in which the biosynthesis of target compounds can be fueled by the excess excitation energy during photosynthesis.

Herein, we will not only comprehensively review the important advances of algal P450 studies on natural product biosynthesis and photo-driven P450 reactions designed in algae, but also analyze the current questions/challenges and the future prospects of P450 studies in algae, aiming to inspire more cost-effective, eco-friendly and sustainable algae-based bioproduction of chemicals and pharmaceuticals in the future.

2. Algal P450 catalytic processes

Generally, the P450-mediated monooxygenation reactions follow a common equation of $R{-}H + O_2 + 2H^+ + 2e^- \rightarrow R{-}OH + H_2O$[32]. To initiate the reaction, two electrons originated from NAD(P)H need to be delivered to the heme-iron reactive center of P450 with the aid of redox partner protein(s) (Fig. 1)[33]. Therefore, according to the importance of redox partner(s), the algal P450 catalytic systems can be classified into two major types (types I and II; Fig. 1). The prokaryotic algae (*i.e.*, cyanobacteria) adopt the three-component type I P450 catalytic system, in which the redox partners include a FAD-containing ferredoxin reductase (FdR) and a small iron-sulfur ([Fe−S]) protein ferredoxin (Fdx). By contrast, the eukaryotic algae mainly possess the Type II P450 catalytic system, wherein the P450 enzyme employs a single FAD/FMN-containing flavoprotein, referred to as cytochrome P450 reductase (CPR), to transfer the reducing equivalents. Compared with the Type I system that all the elements are cytosolically soluble proteins, the type II algal P450s and their cognate CPRs are usually endomembrane-associated proteins (Fig. 1)[34]. This difference has led to thriving functional studies of prokaryotic P450s, while greatly limited the corresponding researches on higher algal P450s due to the technical difficulties associated with the heterologous overexpression of membrane-bound proteins[35].

Thereafter, the P450 sequentially accepts the two electrons shuttled from NAD(P)H by redox partner(s) to activate the inert O_2 at the heme-iron reactive center and inserts a single "O" atom to the bound substrate (R−H) to yield R−OH (Fig. 1). The detailed P450 catalytic cycle has been described in many recent reviews[3,4,12,36]. Briefly, the intact catalytic process could be divided into two halves: in the first half cycle, the substrate-bound heme-iron center accepts two electrons, a O_2 molecule, and a pair of protons to generate the highly reactive species compound I (CpdI); and in the second half cycle, CpdI triggers the important "hydrogen-abstraction" process against the substrate R−H to

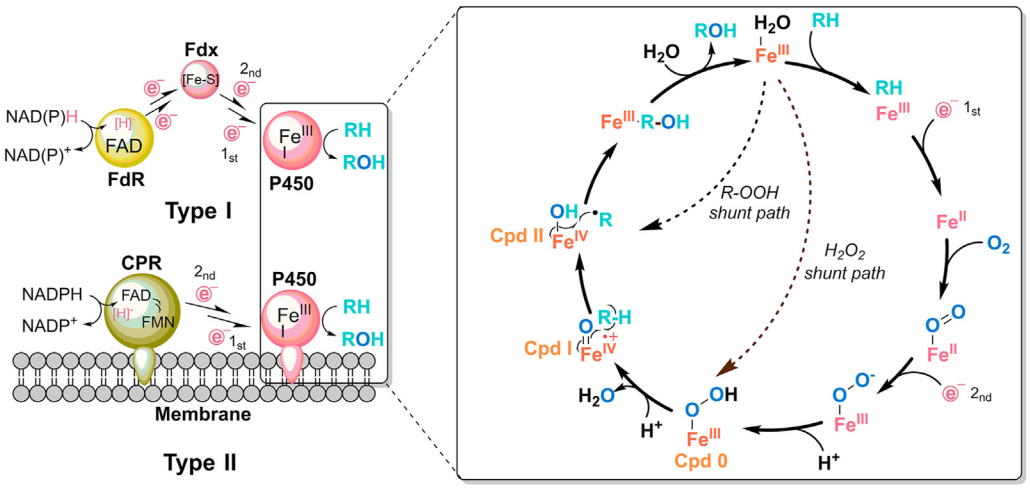

Figure 1 The two major types of algal P450s (left) and their catalytic systems which share a common catalytic cycle (right). The electron transport pathways: as shown in the left panel, NADPH first transfers a pair of electrons in the form of hydrogen ion (H^-) to FAD in either FdR or the FAD domain of CPR; then FAD transfers the two electrons one at a time to the iron-sulfur cluster ([Fe−S]) of Fdx or to the FMN domain of CPR; and finally Fdx or the FMN domain delivers the electron to the heme-iron center of P450 to drive the catalytic cycle as shown in the right panel[33].

produce the substrate radical ($R^{\cdot}$) and CpdII; then, the "OH rebound" reaction occurs to quench the $R^{\cdot}$ and generate the monooxygenation product R−OH (Fig. 1). It is worth noting that for many P450s, the catalytic cycle can be shortcut to the peroxide shunt pathway when using H_2O_2 as the sole oxygen and electron donor, in which H_2O_2 directly activates the resting heme-Fe(III) to Cpd0 (Fig. 1)[34,37]. In rare cases, such as the fatty acid-peroxide (R−OOH) metabolizing P450 family members including CYP5, CYP8A, and CYP74 enzymes, the ferric enzyme can directly cleave the peroxy bond, leading to the formation of CpdII and the fatty acid alkoxyl radical ($RO^{\cdot}$), and finally yields the epoxyalcohol or allene oxide products (Fig. 1)[38].

3. Cyanobacterial P450s in natural product biosynthesis

Cyanobacteria, also referred to as blue-green algae, are a class of prokaryotic algae ubiquitously distributed in fresh and saline waters[39]. Cyanobacteria are thought to be the oldest photosynthetic organisms that played a central role during the evolution of life by contributing to the oxygenation of the early earth. Interestingly, they are also an abundant source of bioactive natural products, including polyketides (PKs), non-ribosomal peptides (NRPs), terpenoids and the hybrids thereof[5,40]. Genome mining revealed that cyanobacteria represent a huge arsenal of P450s, which may function as indispensable roles in both primary and secondary metabolisms[41]. In this section, we will summarize the identified P450-catalyzed reactions involved in cyanobacterial natural product biosynthesis (Table 1).

3.1. *All-trans-retinoic acid*

All-*trans*-retinoic acid (**1**) is a naturally occurring vitamin A-derived pharmaceutical that is highly effective in the treatment of acute promyelocytic leukemia, and it shows the potential to treat some autoimmune diseases, such as psoriasis, systemic lupus erythematosus, inflammatory arthritis, and ulcerative colitis[42,43]. Genome mining of *Synechocystis* sp. PCC 6803 revealed a function unknown P450 named CYP120A1[44]. In an earlier study, CYP120A1 was proposed to participate in the transformation of **1** based on bioinformatics analysis and the co-crystal structure with **1**[45]. Later, an *in vitro* bioassay, using the microsomal fraction of the recombinant *Saccharomyces cerevisiae* WAT11 expressing CYP120A1, showed that CYP120A1 is indeed capable of hydroxylating C16 and C17 of **1** to form **2** and **3** respectively (Fig. 2A)[46]. It is noteworthy that CYP120A1 is the first identified retinoic acid-metabolizing enzyme of non-animal origin[45].

3.2. *Type A malyngamides*

Type A malyngamides are a family of marine cyanobacteria derived natural products that have potent anticancer and anti-inflammatory activities[47]. Recently, malyngamide I (**4**) and malyngamide C (**5**) were isolated from the cyanobacterial strains *Okeania hirsute* PAB and PAP respectively, and their biosynthetic pathways were characterized based on *in vivo* studies. On the early stage, the carbon skeleton is assembled by a polyketide synthase (PKS) and a nonribosomal peptide synthetase (NRPS) to form **6** and **7** with the aid of several tailoring enzymes. Next, P450 MgcT catalyzes the C4=C9 oxidation of **6** to produce the epoxidation product, which is further hydroxylated at the C8 position by another P450 MgcU to form the precursor of **4** in the strain PAB (Fig. 2B). Similarly, in *O. hirsute* PAP, the MgcT counterpart P450 MgiT was considered responsible for the C4=C9 epoxidation of **7** to produce **5** (Fig. 2B)[48].

3.3. *Cryptophycins*

Cryptophycins, as exemplified by cryptophycin 2 (**8**), are a class of peptolide natural products isolated from the lichen cyanobacterial symbiont *Nostoc* sp. ATCC 53789 and *Nostoc* sp. GSV224[49]. They are potent anticancer agents by stimulating cellular microtubule instability, inhibiting microtubule assembly, and inducing tubulin self-association[50]. In the biosynthetic study of **8**, a P450 enzyme CrpE was characterized to mediate the formation of the characteristic β-epoxy ring at the C2=C3 double bond towards the substrate cryptophycin 4 (**9**) (Fig. 2C), based on the *in vitro* enzymatic assay using the purified CrpE protein expressed in the recombinant *Escherichia coli*[51]. In addition, CrpE

Table 1 The functions of algal P450s.

P450	Biosynthetic pathway	Function	*In vitro*/*in vivo* characterization	Source	Prokaryotic (P)/ Eukaryotic (E)	Ref.
AB2	Polychlorinated biphenyls/triphenyls	C−C or C−O biaryl ring coupling	Y/Y	*F. ambigua* 108b	P	60
AB3	Polychlorinated biphenyls/triphenyls	C−C or C−O biaryl ring coupling	Y/Y	*F. ambigua* 108b	P	60
CrpE	Cryptophycin 2	Epoxidation	Y/–	*Nostoc* sp. ATCC 53789 and *Nostoc* sp. GSV224	P	51
CYP120A1	All-*trans*-retinoic acid	Hydroxylation	Y/–	*Synechocystis* sp. PCC 6803	P	44–46
CYP110	Long-chain saturated fatty acids	Hydroxylation	–/–	*Anabaena* sp. PCC 7120	P	62,63
*Cke*CYP97A1	Xanthophylls	Hydroxylation	–/Y	*Chlorella kessleri*	E	112
*Db*CYP97A	Xanthophylls	Hydroxylation	–/Y	*Dunaliella bardawil*	E	80
*Db*CYP97B	Xanthophylls	Hydroxylation	–/Y	*D. bardawil*	E	80
*Db*CYP97C	Xanthophylls	Hydroxylation	–/Y	*D. bardawil*	E	80
ESEAS	Epoxyalcohols	Epoxidation	Y/–	*Ectocarpus siliculosus*	E	75
*Eg*CYP97H1	Xanthophylls	Hydroxylation	–/Y	*Euglena gracilis*	E	81
*Hae*CYP97A	Xanthophylls	Hydroxylation	–/Y	*Haematococcus pluvialis*	E	78
*Hae*CYP97B	Xanthophylls	Hydroxylation	–/Y	*H.pluvialis*	E	78
*Hae*CYP97C	Xanthophylls	Hydroxylation	–/Y	*H.pluvialis*	E	78
HctG	Hectochlorin	Hydroxylation	–/–	*Lyngbya majuscula*	P	53
HctH	Hectochlorin	Hydroxylation	–/–	*L. majuscula*	P	53
KfAOS	Jasmonates	Epoxidation	Y/–	*Klebsormidium flaccidum*	E	74
LtxB	Lyngbyatoxin A	C−N bond formation	Y/–	*Lyngyba majuscule*	P	66
MgcT	Malyngamide C	Epoxidation	–/Y	*Okeania hirsute* strains PAB	P	48
MgcU	Malyngamide C	Hydroxylation	–/Y	*O. hirsute* strains PAB	P	48
MgiT	Malyngamide I	Epoxidation	–/Y	*O. hirsute* strains PAP	P	48
P450NS	Oxidized germacrene A	Oxidative cyclization	–/Y	*Nostoc* sp. PCC 7120	P	57,58
*Pu*CHY1	Xanthophylls	Hydroxylation	–/Y	*Porphyra umbilicalis*	E	113
*Ptr*CYP97B1	Xanthophylls	Hydroxylation	–/Y	*Phaeodactylum tricornutum*	E	114
*Ptr*CYP97B2	Xanthophylls	Hydroxylation	–/Y	*P. tricornutum*	E	114

can also convert the **9-**analogues, **10**−**13**, to the corresponding epoxidation products **14**−**17** *in vitro* (Fig. 2C), indicative of a broad substrate spectrum[52].

3.4. Hectochlorin

Hectochlorin (**18**) is another peptolide product identified from marine cyanobacteria *Lyngbya majuscula* and shows remarkable antifungal and cytotoxic properties. Biosynthetically, **18** is produced by a PKS-NRPS hybrid assembly-line, during which the elongating substrates are tethered to a peptidyl carrier protein (PCP)[53]. A few P450s have been reported to be capable of mediating the online oxidations, of which the P450's substrate is linked to an acyl carrier protein (ACP) or PCP, thus involving the protein−protein interactions during the substrate recognition process. The biosynthetic gene cluster of **18** encodes two P450s HctG and HctH[53]. According to bioinformatics analysis and the biocatalytic principles, HctG/HctH were proposed to catalyze such an online hydroxylation at the C3 position of the PCP-tethered substrate **19** to form **20**, prior to the NPRS-mediated cyclopeptide formation (Fig. 2D)[53].

3.5. Fatty acids

The ω-hydroxylated fatty acids are important starting materials in chemical, pharmaceutical, and cosmetic industries[54]. However, the regiospecific ω-hydroxylation of the inert terminal sp^3 C−H bond in a hydrocarbon chain is thermodynamically disfavored for chemical synthesis. Some P450s have long been found to hold the capacity of mediating such ω-hydroxylation reactions towards various fatty acid substrates[55,56]. Genome mining of the freshwater cyanobacterium *Anabaena* sp. PCC 7120 revealed an unknown P450 named CYP110[57,58]. Protein sequence analysis indicated the high similarity of CYP110 with the mammalian CYP4 family proteins and the bacterial P450 BM3 from *Bacillus megatarium*, two classes of identified fatty acid ω-hydroxylases (Fig. 2E). The substrate titration experiments and spectroscopic spin-shift assays demonstrated that the saturated and unsaturated fatty acids of chain lengths C_{10}−C_{20} could bind with CYP110[58]. Thus, CYP110 was proposed to catalyze ω-hydroxylation of long-chain fatty acid (**21**) to form **22** (Fig. 2E)[57,58].

3.6. Polyhalogenated aromatics

Apart from the common monooxygenation reactions, P450s can also mediate various complex structure-reshaping reactions. Enzymatic aromatic/phenolic couplings are fascinating reactions in natural product biosynthesis, because these regio- and/or stereoselective transformations are highly challenging in chemical synthesis. Polyhalogenated aromatics represent a class of anthropogenic chemicals that are widely used in agriculture[59]. Intriguingly, this class of molecules have also been isolated from some marine microorganisms, such as the *Fischerella ambigua* 108b originated polychlorinated triphenyl product **23** and biphenyl products **24** and **25**[60]. The biosynthetic gene cluster of **23**–**25** was recently revealed by genome mining, which is comprised of ten genes including two P450 genes (*AB2* and *AB3*). Based on *in vivo* precursor feeding experiments, AB2 and AB3 were elucidated to

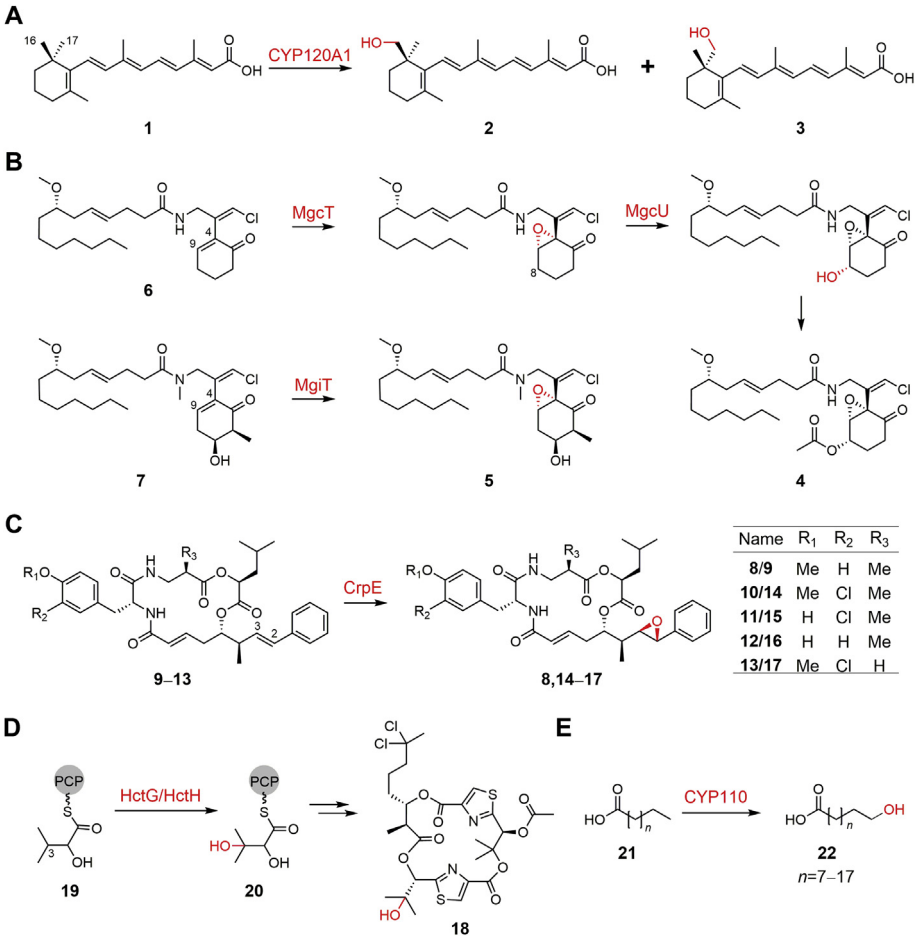

Figure 2 The monooxygenation reactions catalyzed by cyanobacterial P450s. (A) The all-*trans*-retinoic acid (**1**) hydroxylation reactions catalyzed by CYP120A1 from *Synechocystis* sp. PCC 6803. (B) The epoxidation and hydroxylation reactions catalyzed by different P450s during the biosynthesis of malyngamides **4** and **5**. (C) The CrpE-catalyzed epoxidation reactions of cryptophycin 4 (**9**) and **9** analogues (**10**−**13**). (D) The online hydroxylation catalyzed by P450 HctG/HctH during the biosynthesis of **18**. (E) The ω-hydroxylation of fatty acid catalyzed by CYP110.

catalyze the C3−C3′ or C3−O1′ coupling of the monomer **26** to form **24** and **25**, and the O1−C4′/C6′−C3″ coupling of **26** and **27** to yield **23** (Fig. 3A)[60]. The *in vitro* reconstituted enzymatic reactions disclosed more catalytic details, of which AB2 and AB3 can both catalyze the formation of **24** and **25**, but AB2 tends to produce **24** while AB3 prefers the production of **25**[60]. Moreover, the co-incubation of AB2 and AB3 with the monomers **26** and **27** generated the tri-phenol product **23** (Fig. 3A)[60].

Mechanistically, the P450-catalyzed aromatic/phenolic coupling reactions are considered to undergo a biradical coupling pathway. In this case, AB2/AB3 primarily abstracts a single hydrogen from the phenolic hydroxyl group of **26** or **27** to yield the oxygen radicals **i** or **ii**, which can undergo the electron rearrangement to give the corresponding carbon radical **iii** and **iv**. Then, two molecules of **iii** couple to yield **24**, while **i** and **iii** couple to produce **25**. As for the formation of **23**, after the generation of **v** from **i** and **iv**, AB2/AB3 further abstracts the H6' to give a new radical **vi**, which couples with **iii** to yield the tripolymer products (Fig. 3A)[61].

3.7. *Germacrene A*

Germacrene A (**28**), derived from the precursor of farnesyl pyrophosphate (**29**), is a general biosynthetic intermediate of many bioactive sesquiterpenes, such as englerin A and guaianolide, in both plants and microorganisms[62]. In a genome mining study of the cyanobacterium *Nostoc* sp. PCC 7120, a sesquiterpene synthase (NS1) was found to be able to biosynthesize **28**[63]. Stepwise heterologous expression of NS1 and the co-clustered P450NS in *E. coli* revealed that P450NS could mediate an uncommon oxidative cyclization of **28** to yield the more hydrophilic product **30** (Fig. 3B)[64]. It was proposed that P450NS first epoxidizes the C5=C6 double bond to produce the unstable intermediate **i**, which could undergo C−O bond heterolysis to give **ii**. Eventually, **30** is generated after intramolecular electron rearrangement and ring-closure of **ii** (Fig. 3B).

3.8. *Lyngbyatoxin A*

Lyngbyatoxin A (**31**) is an indole alkaloid cyanotoxin that can activate the protein kinase C (PKC) isozyme, and also a potent irritant and vesicant, as well as a carcinogen[65]. Biogenetically, the C−N bond formation is of special interest since such a C−H amination reaction is infrequently occurring in nature. The biosynthetic gene cluster of **31** was mined from a Hawaiian cyanobacterium strain *Lyngyba majuscule*, which contains a P450 encoding gene *ltxB*. Based on the *in vitro* reconstituted enzymatic reaction, LtxB was determined to mediate the conversion of the

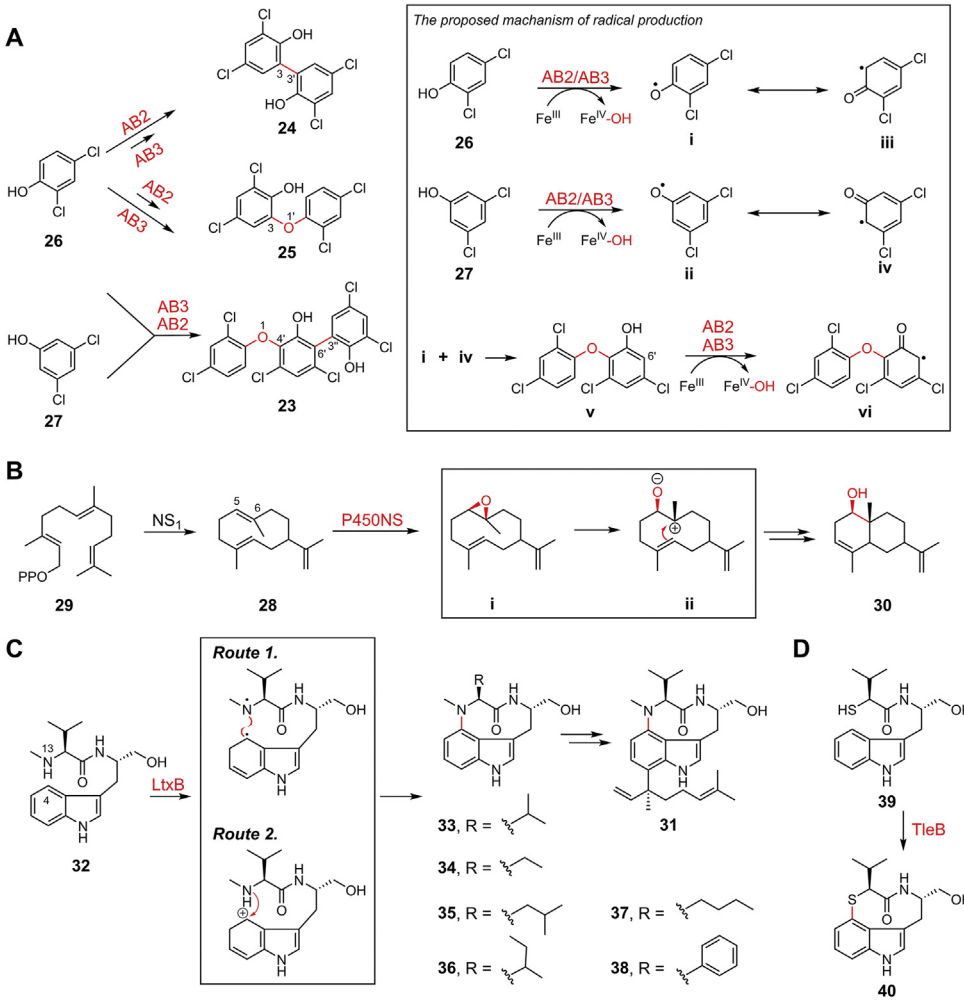

Figure 3 The uncommon reactions catalyzed by cyanobacterial P450s and the related catalytic mechanisms (in box). (A) The C−C and C−O coupling reactions catalyzed by AB2 and AB3 during the biogenesis of polyhalogenated aromatics. (B) The oxidative cyclization reaction mediated by P450NS. (C) The C−N bond formation catalyzed by LtxB in the biosynthesis of lyngbyatoxin A (**31**), and the various C−N bond bearing products (**34**−**38**) formed by LtxB. (D) The C−S bond formation catalyzed by LtxB.

substrate **32** to **33** by catalyzing the rare C4−N13 bond formation reaction. To explore the substrate tolerance of LtxB, several analogues of **32** were synthesized and reacted with LtxB *in vitro*. Intriguingly, all these analogues could be converted into the corresponding C−N coupling products (**34**−**38**, Fig. 3C)[66]. Recently, the mechanistic study of TleB, a homologous protein of LtxB from *Streptomyces*, was conducted by crystal structure analysis, revealing a biradical-coupling process (route 1, Fig. 3C) or an alternative carbocation involved nucleophilic pathway (route 2, Fig. 3C)[65]. Moreover, in a following study, it was revealed that TleB could also catalyze a C−S bond formation towards the substrate *S*-analogue **39** to yield **40** (Fig. 3D)[20]. These results together prove LtxB/TleB to be potential biocatalysts for generation of the C−N/C−S bond containing products.

4. Eukaryotic algal P450s in natural product biosynthesis

Eukaryotic algae encompass diverse photosynthetic species ranging from unicellular microalgae to multicellular giant kelps[67,68]. Continuous natural product prospecting has revealed that eukaryotic algae are also an important source of bioactive compounds. The most attractive merit is that some eukaryotic algae can produce abundant bioactive carotenoid-like pigments and halogenated natural products[69,70]. A recent genome survey disclosed the high diversity of P450s in eukaryotic algae[28]. However, due to the technical limitations in laboratory cultivation and gene-manipulation of eukaryotic algae, the progress on these membrane-bound P450s remains limited, especially for the P450s from giant kelps[25,27]. In this part, a limited number of eukaryotic algal P450s and their catalytic reactions will be reviewed (Table 1).

4.1. *Jasmonates*

Jasmonates, taking the 12-oxo-phytodienoic acid (**41**) and jasmonic acid (**42**) as examples, are important hormones distributed in plants and algae. They are also potential therapeutic agents for several types of cancer[71]. During the biosynthesis of **41** and **42**, the fatty acid-derived peroxide oxylipins, such as 13-hydroperoxy-(*E*,*Z*,*Z*)-9,11,15-octadecatrienoic acid (**43**), are the key intermediates[72]. The P450 KfAOS was identified as a CYP74 family member involved in jasmonate biosynthesis from the green microalgae *Klebsormidium flaccidum*. Functional characterization revealed that KfAOS is able to convert **43** into the oxylipin allene

oxide (**44**, Fig. 4A)[38]. Then, the unstable product **44** undergoes a spontaneous rearrangement to form **41**, and such a transformation can be accelerated by an allene oxide cyclase (Fig. 4A)[73,74]. In another study, the CYP74-related P450 ESEAS (CYP5164B1) from the brown algae *Ectocarpus siliculosus* was found to catalyze a similar reaction[75]. As reported, ESEAS is capable of transforming both (9*S*,10*E*,12*Z*)-9-hydro (pero)xy-10,12-octadecadienoic acid (**45**) and (9*Z*,11*E*,13*S*)-13-hydro (pero)xy-9,11-octadecadienoic acid (**46**) into the corresponding epoxyalcohol products **47** and **48**, respectively (Fig. 4B).

Intriguingly, neither redox partners nor electron donor is required for these CYP74-catalyzed reactions. As for the catalytic mechanism, it is proposed that after binding with the fatty acid hydroperoxide (**49**), the heme-Fe(III) of CYP74 (KfAOS or ESEAS) homolytically cleaves the O−O bond to give a [RO˙] radical (**50**) and CpdII (Fe(IV)−OH). Then, the [RO˙] radical reacts with the adjacent double bond to form the epoxy species and relocates the radical to the α-carbon, forming the carbon radical **51**. Thereafter, the [HO˙] of CpdII rebounds to **51** to yield the epoxyalcohol product **52** (for ESEAS). Alternatively, CpdII further abstracts a hydrogen from the α-carbon of **51** to afford the allene oxide product **53** and release a water molecule (for KfAOS) (Fig. 4C)[38,75,76]. Thus, this is a unique class of P450s to catalyze unusual isomerization reactions instead of the normal oxygenation reactions, because no additional electron and oxygen is introduced into the catalytic cycle.

4.2. *Xanthophylls*

Xanthophylls, such as lutein (**54**), are a large class of oxygen-containing carotenoid pigments that play important protecting roles in photosynthesis of eukaryotic algae and higher plants[77,78].

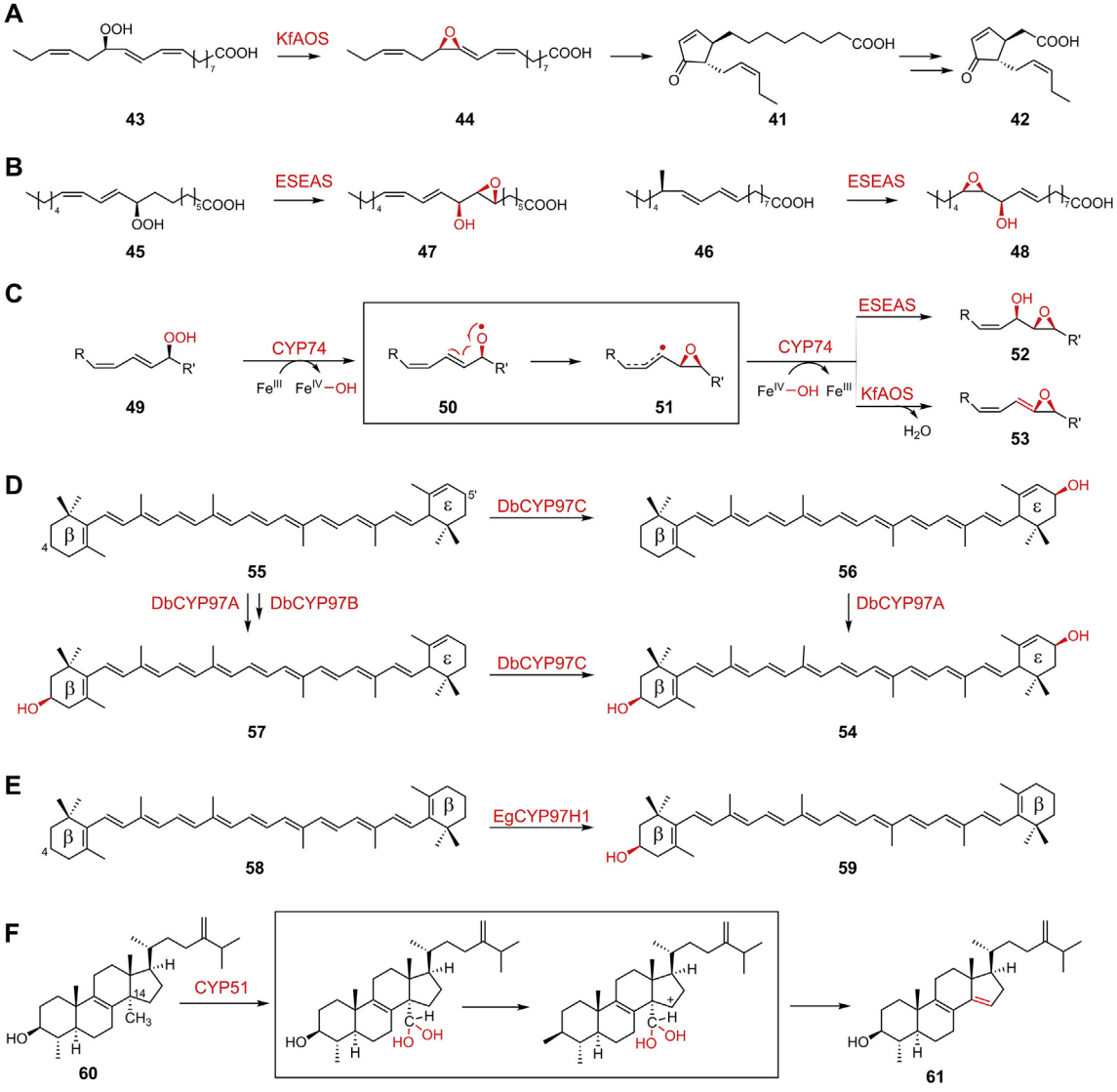

Figure 4 The reactions catalyzed by eukaryotic algal P450s and the related enzymatic mechanism (in box). (A) The oxylipin allene oxide (**44**) epoxidation catalyzed by KfAOS from the green microalgae *Klebsormidium flaccidum* during the formation of **41** and **42**. (B) The epoxidation reactions catalyzed by ESEAS from the brown algae *Ectocarpus siliculosus*. (C) The putative mechanisms of KfAOS and ESEAS. (D) The hydroxylation reactions of α-carotene (**55**), α-cryptoxanthin (**56**) and zeinoxanthin (**57**) catalyzed by DbCYP97C from the green algae *Dunaliella bardawil* during the biosynthesis of lutein (**54**). (E) The hydroxylation reaction of β-cryptoxanthin (**59**) catalyzed by EgCYP97H1 from *Euglena gracilis*. (F) The CYP51 mediated 14α-demethylation reaction during sterol biosynthesis.

Physiologically, **54** and its structural analogues can absorb excess light energy to prevent photo-damages to host organisms. Xanthophylls also have health benefits and therapeutic potentials against neurologic, ophthalmologic, oral, allergic and immune diseases. Biosynthetically, **54** is derived from α-carotene (**55**) with two hydroxylation steps[79]. A recent study on carotene hydroxylases from the green algae *Dunaliella bardawil* revealed a group of P450s (DbCYP97A, DbCYP97B, and DbCYP97C) are responsible for the C4/C5' hydroxylation of **55** to form **54** (Fig. 4D). According to the functional complementation experiments in *E. coli*, the catalytic preferences of the three P450s were revealed: DbCYP97A and DbCYP97C showed a high catalytic activity toward the β-ring and ε-ring of **55** to yield α-cryptoxanthin (**56**) and zeinoxanthin (**57**), respectively; while DbCYP97B displayed minor hydroxylation activity toward the β-ring of **55** (Fig. 4D)[80]. Interestingly, unlike other eukaryotic P450s, the CYP97 proteins lack a transmembrane helix, thus being peripherally bound to the membrane. In addition, another CYP97 member *Eg*CYP97H1 from *Euglena gracilis* was characterized as a β-carotene monohydroxylase, which hydroxylates the C4 position of β-carotene (**58**) to form β-cryptoxanthin (**59**, Fig. 4E). Notably, this is the first example that a P450 functions as a β-carotene hydroxylase[81].

4.3. *Sterols*

Sterols are important structural and regulatory components in eukaryotes[82]. As observed in plants, CYP51 is a necessary P450 for sterol biosynthesis by catalyzing the 14α-demethylation of obtusifoliol (**60**) to yield **61**, *via* the putative geminal diol and carbocation intermediates (Fig. 4F)[83]. However, the algal CYP51 had not been investigated until a recent study carried out in *Nannochloropsis oceanica*. As reported, addition of the CYP51 inhibitor tebuconazole (TEB) into the culture of *N. oceanica* led to the accumulation of the major biosynthetic intermediate of sterols[84]. Accordingly, CYP51 in *N. oceanica* was proposed to play a similar role as their homologous P450s in other eukaryotic organisms.

In summary, as for the algal P450-mediated biosynthesis of essential metabolites, analogous P450-catalyzed reactions can also be found in other non-algal organisms. For example, the all-*trans*-retinoic acid (**1**) metabolizing reaction can be accomplished by both the cyanobacterial P450 CYP120A1 and some human P450s (Fig. 2A)[85]; the C−N bond formation mediated by cyanobacterial P450 LtxB also occurs in *Streptomyces* (Fig. 3C)[86]; and the uncommon rearrangement reactions catalyzed by the eukaryotic algal CYP74 family member have also been elucidated in other higher plants (Fig. 4A–C)[87]. These cross-species functional similarities may suggest some evolutionary relationship between algal and non-algal organisms. By contrast, there also exist some algae-specific reactions that have not been observed in other organisms, such as the tripolymer production co-mediated by the cyanobacterial P450s AB2 and AB3 (Fig. 3A). Besides, algae have evolved many unique P450s giving rise to the specialized natural products of algal characteristics, as exemplified by **4**, **5**, **8**, and **18**. With no doubt, these algal P450s significantly expand the catalytic diversity of the whole P450 superfamily.

5. Algae as photo-bioreactors of heterologous P450s

Algae are photoautotrophic organisms that can use the solar energy and recycle CO_2 to synthesize primary and secondary metabolites (Fig. 5)[31], but the solar-to-biomass energy conversion rate is quite low with the energy converting efficiencies only ranging from 1% to 2%[88]. In fact, in the photosynthetic pathway, the photosystem II can efficiently absorb the solar energy for photolysis of water molecule to generate electrons for downstream metabolic pathways. However, the total cellular biosynthetic electron-demand is far less than the produced electrons[88,89]. Therefore, theoretically, these excessively absorbed solar energy can be further utilized by introducing heterogenous catalytic elements provided that they can interact with the photosystem proteins to acquire electrons.

With regard to P450s, considering the facts that 1) the P450-catalyzed reactions need to consume a lot of NAD(P)H to gain the reducing power (electrons) to drive the catalytic cycle (Fig. 1); 2) excess reducing power is generated during algal photosynthesis[90]; and 3) P450s can use the photosynthesis-associated Fdxs to shuttle the required electrons (Fig. 5), a methodology which can channel the excess reducing power from the algal photosystem to the P450 catalytic system would be highly valuable for heterologous P450s to produce high-value products by directly using the solar energy[91]. Technically, a heterologous P450 can be readily fused with the photosynthetic system by anchoring it into the thylakoid membrane that harbors the whole photosynthetic pathway (Fig. 5). For instance, a eukaryotic membrane-bound P450 can be directly anchored into the prokaryotic thylakoid membrane by its native membrane-associated N-terminal sequence; while the prokaryotic Type I P450 could be re-located into the thylakoid membrane by fusing it with the subunit of photosystem protein (Fig. 5). Based on this strategy, several light-driven P450 reaction systems have been successfully developed in different algal species[31,91–96]. In this section, we will discuss the state-of-the-art algal photo-bioreactors for light-driven P450 reactions.

5.1. *Caffeic acid*

Caffeic acid (**62**), a phenolic natural product synthesized by all plant species, shows antioxidant, anti-inflammatory and anticancer activities and is widely used in cosmetics industry[97–99]. At present, the commercial **62** is chemically synthesized, which is suffering from the low yield and serious environmental concerns[91,100]. The P450 CYP98A3 (C3H) from *Arabidopsis thaliana* was identified as a caffeic acid synthase by hydroxylating the C3 position of *p*-coumaric acid (**63**). Previously, functional expression of C3H failed in a bacterial host due to its instability, low abundance, and membrane-bound nature[91]. In a pilot study, the possibility of using cyanobacterium as a "photo-bioreactor" to produce **62** was explored. When the codon-optimized C3H-encoding gene was introduced into *Synechocystis* sp. PCC 6803, the plant P450 was successfully expressed and located onto the thylakoid membrane[91]. Together with other biosynthetic enzymes, the expected product **62** was produced in the cyanobacterium under the photosynthetic growth conditions (Fig. 5). This study demonstrated the algal thylakoid to be a suitable platform for studying the membrane-bound P450s[101].

5.2. *Dhurrin*

Dhurrin (**64**) is a cyanogenic glycoside produced by many plants, which could comprise up to 30% of the dry mass of etiolated sorghum seedlings[102]. Compound **64** exhibits strong antimicrobial properties while its cytotoxicity also discourages herbivory of some insects and animals. CYP79A1 is involved in the **64** biosynthetic pathway from *Sorghum bicolor* that converts L-

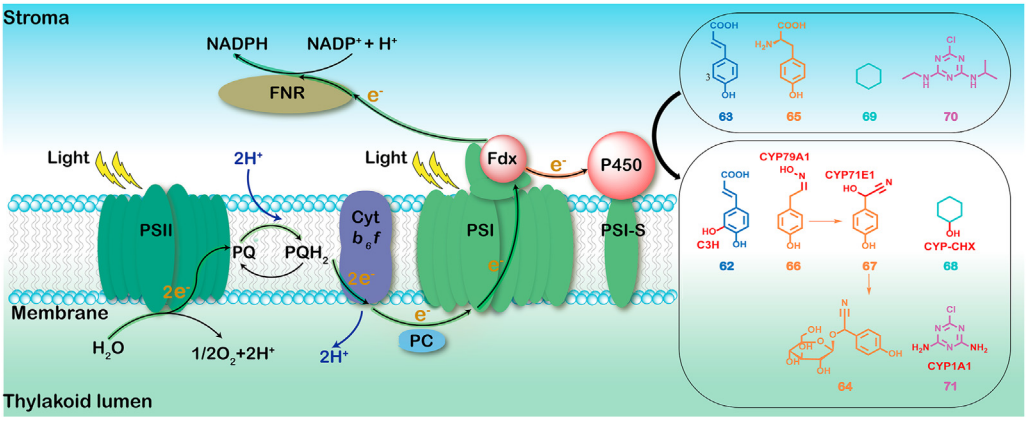

Figure 5 The algal photosynthetic pathway and its application for driving the P450-catalyzed reactions (adopted from Ref. 31 Copyright © 2021 American Chemical Society). During the photosynthesis, photosystem II (PSII) conducts the water photolysis and produces the two electrons which are then transferred to plastoquinone (PQ). PQ gains the electrons and two protons to form PQH_2, which is then oxidized by cytochrome *b6f* complex (Cyt *b6f*) to capture the electrons and restore PQ. Thereafter, the electrons in Cyt *b6f* is stepwise transferred to Fdx *via* the electron-transfer protein plasticyanin (PC) and photosystem I (PSI). Then, Fdx transfers the electron to ferredoxin−$NADP^+$ reductase (FNR) to produce NADPH. When the heterologous P450 is present, Fdx can direct partial electrons to P450s to drive various oxidative reactions.

tyrosine (**65**) into the N−OH bearing product **66**. When the soluble catalytic domain of CYP79A1 was fused with the cyanobacterial photosystem I derived subunit to locate it onto thylakoid, the eukaryotic P450 was successfully expressed in *Synechococcus* sp. PCC 7002; and it showed the light-dependent activity both *in vitro* and *in vivo* (Fig. 5)[93]. During the biosynthesis of **64**, another P450 named CYP71E1 can further oxidize **66** into the nitrile-carrying compound **67**. Thus, to achieve total biosynthesis of **64**, CYP79A1 and CYP71E1 were co-expressed in *Synechocystis* sp. PCC 6803, and the results showed that the reconstituted activities of the two P450s were strictly light dependent. When the glycosyltransferase UGT85B1 was further introduced, the final product **64** was successfully produced in the cyanobacterial host (Fig. 5)[95]. In another study, CYP79A1 was also successfully expressed in the eukaryotic green algae *Chlamydomonas reinhardti*. As expected, after the CYP79A1 encoding gene was incorporated into the chloroplast genome of *C. reinhardti*, the P450 enzyme was stably expressed and located in the chloroplast membrane[94].

5.3. *Artificial chemicals*

In addition to bioactive natural products, the cyanobacterial hosts have also been developed to produce high-value chemicals by running the light-driven P450 reactions. Cyclohexanol (**68**) is a key precursor in chemical/pharmaceutical industry for the production of *ε*-caprolactone, adipic acid and several polymers[103]. However, **68** is intricate to acquire through chemical synthesis mainly due to the difficulty in the inert sp^3 C−H bond activation; thus, enzymatic synthesis provides an alternative solution to address this issue[104]. A P450 enzyme CYP-CHX from *Acidovorax* sp. CHX100 was previously identified to be such a hydroxylase that can hydroxylate cyclohexane (**69**) to **68**[105]. To achieve the production of **68**, the P450 was expressed in the cyanobacterium *Synechocystis* sp. PCC 6803, and gram-scale of **68** was successfully produced by supplying **69** to the recombinant cyanobacterial culture (Fig. 5). Essentially, it was confirmed that the production of **68** was light dependent[106].

5.4. *Pollutant degradation*

The numerous man-made chemicals, such as the widely used pesticides and antibiotics, have greatly promoted social development in the last century. However, the environmental pollution caused by artificial chemicals also threats the living world. The ubiquitously distributed algae have been designed as pollutant degrading-hosts by expressing biocatalysts which can mediate biodegradation reactions. Atrazine (**70**) is one of the most widely used pesticides in the world, and has been recognized as a serious environmental pollutant[107]. Previous studies have shown that the human or mammalian P450 CYP1A1 has the capacity of converting **70** into the deisopropylated and deethylated product of atrazine (**71**), both *in vitro* and *in vivo*[108,109]. When CYP1A1 from *Rattus norvegicus* (brown rat) was expressed in the cyanobacterium *Synechococcus* sp. PCC 7002, it was found that the protein could be located onto thylakoid membrane through its native N-terminal membrane-bound domain. Importantly, *Synechococcus* sp. PCC 7002 expressing CYP1A1 successfully degraded **70** into **71** in a light-dependent manner (Fig. 5)[107].

Taken together, these results demonstrate the great potential of both cyanobacteria and eukaryotic algae as light-driven P450 bioreactors and biosynthetic platforms for the production of high-value added pharmaceuticals/chemicals (or the degradation of pollutants) in a cost-effective and eco-friendly manner. Intriguingly, a recent study showed that the expression of the above-mentioned CYP1A1 in *Synechococcus elongatus* PCC 7942 led to not only an improvement of P450 activity, but also a significant increase in the quantum efficiency of photosystem[89]. It was hypothesized that the introduced heterologous metabolism might provide an outlet for the excessively captured energy, thus reducing the flow to the photoprotective pathways of energy consumption. Such a balance on the absorbed light energy and metabolic capacity is physiologically important for the photosynthetic microorganism.

Despite these significant progresses, there remain a number of substantial obstacles to develop the algal “bioreactors” into “cell-factories” for industrial applications. The major problem is the low

catalytic efficiency when expressing the heterologous P450s in algae. Therefore, increasing the compatibility of the heterologous P450s with the photosystem, thus improving the electron utilizing efficiency for P450s from photosystem, would become one of the main directions of P450 photo-bioreactor improvement in the future[90]. At present, some efforts have optimized the electron transfer from photosystem I to P450 by fusion-expression of P450 and Fdx[90,110], or through lowering the affinity of Fdx and FNR by point mutation of FNR to redirect the electrons to P450s[111]. We envision that more pathbreaking strategies/approaches are required and will emerge to solve this central problem associated with the algal photo-driven P450 bioreactors or cell-factories.

6. Questions and challenges

In the post-genome era, big data analysis of algal genomes and transcriptomes has helped revealing more roles of P450s in algal living activities including both primary and secondary metabolisms. However, when comparing with other organisms, such as human, mammals, plants, fungi and bacteria, our understanding on the functions and mechanisms of algal P450s is very limited. Despite the recent significant progresses on algal P450s as summarized in this review, the breakthrough in algal P450 studies has yet to come due to some unsolved bottlenecks, especially for the eukaryotic algal P450s. The main problems associated with algal P450 functional studies lies in: 1) For cyanobacteria, genome-editing remains a time-consuming task because of its low growth rate. For some species of diploid or multiploid, the genetic manipulation is even more complicated. Besides, the expression levels of heterologous P450 genes in cyanobacteria are usually low, thus leading to inefficient synthesis of the products of interest; 2) For eukaryotic algae, genetic manipulation and heterologous P450 expression are even more difficult due to the cellular and genomic complexity, low growth rate, membrane-bound protein limitation, and unavailability of the whole genomic blueprints (especially for giant kelps); 3) For both cyanobacteria and eukaryotic algae, genome mining and biosynthetic gene cluster analysis revealed that many P450 genes are not clustered with other biosynthetic genes, making it difficult to predict and identify their catalytic functions[28,41]; and 4) even though the current studies have shown great potential for algae as the light-driven "bioreactors" in the production of useful pharmaceuticals/chemicals, the productivity and compatibility of algal hosts for heterologous P450 expression are still too low to be applied in practical applications.

7. Conclusions and prospects

Algae are a huge group of life on the earth and play essential roles in global substance- and energy-cycles. P450s are one of the key catalytic elements for algae by mediating various common and uncommon reactions[13]. Functional and mechanistic characterization of algal P450s will provide us the knowledge on what they do and how they work in the native metabolic networks of algae. In turn, the knowledge would enable us to employ these algal P450 biocatalysts or the algal photosynthetic platform to produce natural and unnatural but useful products, such as pharmaceuticals and fine chemicals, with the accumulating methodologies and tools of synthetic biology. In recent years, more algal P450s have been functionally and mechanistically elucidated, which expands our understandings on catalytic diversity of the whole P450 superfamily. In addition, the photosynthetic algae have shown their great potential as "photo-bioreactors" for heterogenous P450s of different origins to produce high-value products.

Finally, we conclude that P450 studies in algae are still on the early stage. Comparing with other organisms, there currently lacks a general model strain for either prokaryotic or eukaryotic algal P450 studies. However, once developed, an algal platform strain might be advantageous over *E. coli* and *Sacchromyces cerevisiae* because it would optimally have all the necessary elements, including the endomembrane system, excess O_2 supply, and different sources of reducing power, for P450 expression and functionalization. It is still questionable whether the native algal P450s can directly interact with the photosystem to channel the solar energy derived electrons to drive their catalytic cycles; and whether the Fdx-associated photosystem can be universally used as a general electron donor of P450s to replace the functionality of redox partner proteins and NAD(P)H. Taken together, we anticipate that the studies on algal P450s and "photo-bioreactors" will become more popular and feasible in the near future, along with the expansion of the synthetic biology toolbox, and the development of higher-throughput omics technologies and higher-efficiency genome-editing methods.

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Author contributions

Xingwang Zhang, Shengying Li, and Chunxiao Meng conceived the review. Shanmin Zheng, Jiawei Guo and Fangyuan Cheng summed up the literatures and prepared the manuscript. Xingwang Zhang, Shengying Li, Zhengquan Gao and Lei Du revised the manuscript. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no competing financial interest.

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