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Review

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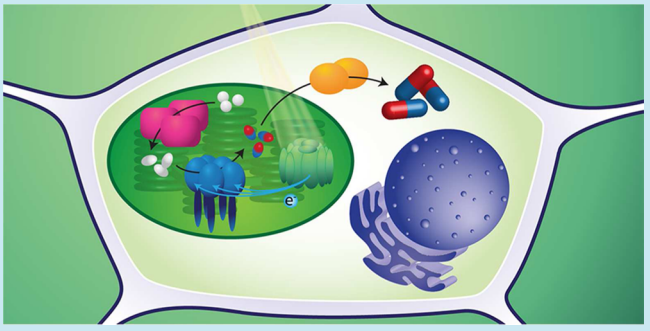
Redirecting Photosynthetic Electron Flow into Light-Driven Synthesis of Alternative Products Including High-Value Bioactive Natural Compounds

Lærke Münter Lassen, Agnieszka Zygadlo Nielsen, Bibi Ziersen, Thiyagarajan Gnanasekaran, Birger Lindberg Møller, and Poul Erik Jensen*

UNIK Center for Synthetic Biology, Interdisciplinary Research Center "bioSYNergy", the VILLUM Research Center "Plant Plasticity", Copenhagen Plant Science Center, Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Copenhagen, Denmark

ABSTRACT: Photosynthesis in plants, green algae, and cyanobacteria converts solar energy into chemical energy in the form of ATP and NADPH, both of which are used in primary metabolism. However, often more reducing power is generated by the photosystems than what is needed for primary metabolism. In this review, we discuss the development in the research field, focusing on how the photosystems can be used as synthetic biology building blocks to channel excess reducing power into light-driven production of alternative products. Plants synthesize a large number of high-value bioactive natural compounds. Some of the key enzymes catalyzing their biosynthesis are the cytochrome P450s situated in the endoplasmic reticulum. However, bioactive compounds are often synthesized in low quantities in the plants and are difficult to produce by chemical synthesis due to their often complex structures. Through a synthetic biology approach, enzymes with a requirement for reducing equivalents as cofactors, such as the cytochrome P450s, can be coupled directly to the photosynthetic energy output to obtain environmentally friendly production of complex chemical compounds. By relocating cytochrome P450s to the chloroplasts, reducing power can be diverted toward the reactions catalyzed by the cytochrome P450s. This provides a sustainable production method for high-value compounds that potentially can solve the problem of NADPH regeneration, which currently limits the biotechnological uses of cytochrome P450s. We describe the approaches that have been taken to couple enzymes to photosynthesis *in vivo* and to photosystem I *in vitro* and the challenges associated with this approach to develop new green production platforms.



KEYWORDS: *photosystems, cytochrome P450, light-driven biosynthesis, natural products, specialized metabolites, metabolic engineering*

Sunlight is an inexhaustible and abundant source of energy. In less than two hours, more solar energy reaches the Earth than what is consumed by humanity in a full year. The average rate at which sunlight strikes the surface of the Earth is around 120 000 TW, a rate that is about 4 orders of magnitude larger than the worldwide energy consumption rate, which is currently around 15 TW.[1−3] With the increasing energy demands, combined with the depletion of the fossil fuel reserves and the major environmental challenges of climate changes, there is a growing need for sustainable energy solutions. Since it is renewable and abundant, solar radiation holds the potential to be a key component in our future energy supply.[1,3,4]

Different approaches for conversion of the harvested solar energy can be used to produce different types of products. While photovoltaic cells can be used in generation of electrical current, natural photosynthesis can be used for production of biomass or chemical products as for instance biofuels.[1,2] Plants, green algae, and cyanobacteria convert solar energy into chemical energy through oxygenic photosynthesis.[5,6] This review focuses on how light energy, through synthetic biology-based applications of photosynthesis and particularly photosystem I (PSI), can be utilized for biotechnological purposes.

Because the amount of light energy captured in photosynthesis often is larger than what can be used in the photosynthetic metabolic reactions, excess excitation energy is lost (*e.g.*, by thermal energy dissipation).[7] This provides the opportunity to take advantage of the excess reducing power and, through bioengineering approaches, direct it toward commercially valuable pathways. By tapping directly into the photosynthetic electron transport chain and redirecting some of the reducing power generated in photosynthesis, efficient production of a desired compound may be obtained using energy that would otherwise have been in excess of cellular

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requirements.[4] Especially, plants produce a diverse variety of chemical compounds, of which many can be utilized as, for example, pharmaceuticals or food additives. Thus, their biosynthetic pathways are of interest in this context.[8−11]

In synthetic biology, natural biological systems are redesigned and new are constructed. The enzymes constituting biosynthetic pathways are regarded as biological building blocks that can be genetically altered or combined in novel ways, developing biological systems not existing in nature. By learning from and exploiting nature's strategies for producing structurally and functionally complex molecules, it is possible to alter pathways and combine enzymes to make modified compounds with altered or enhanced properties, or to synthesize completely novel compounds.[4,12]

By choosing a genetically well-defined and transformable fast-growing photosynthetic host, it may be possible via synthetic biology approaches to construct an *in vivo* system in which a valuable natural compound is synthesized in high amounts fuelled by the excess excitation energy of photosynthesis. *In vivo* light-driven biosynthesis thus constitutes a promising approach for sustainable production of high value natural compounds.[4]

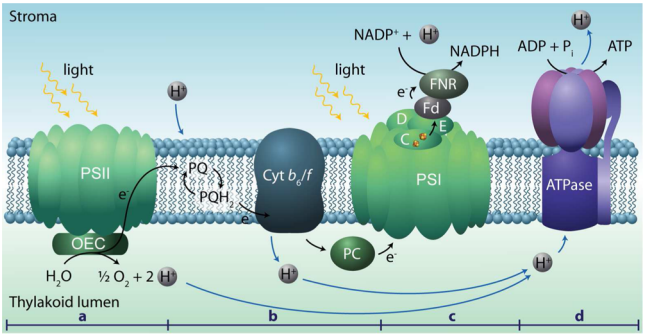

Figure 1. Thylakoid membrane containing the photosynthetic electron transport chain. The stromal subunits of PSI, PsaC, -D, and -E, are indicated. The Fe_4S_4 clusters F_A and F_B, the terminal cofactors involved in light-induced electron transport in PSI, are shown in PsaC. The linear electron flow of photosynthesis is divided in four sections (a−d) described in the main text. ADP, adenosine diphosphate; ATP, adenosine triphosphate; Fd, ferredoxin; FNR, ferredoxin-$NADP^+$ oxidoreductase; NADPH, nicotinamide adenine dinucleotide phosphate; PC, plastocyanin; PSI, photosystem I; PSII, photosystem II; PQ, plastoquinone; OEC, the oxygen-evolving complex of PSII; P_i, inorganic phosphate.

2. PHOTOSYNTHESIS

2.1. Photosynthesis and the Photosystems. Photosynthesis produces and uses the necessary reducing power (NADPH), as well as the chemical energy currency (ATP) for assimilation processes, most notably the fixation of CO_2 to form sugars. Both NADPH and ATP are produced by the photosynthetic electron transport (PET) chain in the light reactions of photosynthesis. In plants and algae, the PET chain is made up of membrane complexes in the thylakoids, the interconnected internal membrane system of the chloroplast, and proteins in the lumen and stroma. The thylakoid lumen is the inner compartment enclosed by the thylakoids, and the stroma is the space between the thylakoids and the envelope membranes, which separate the chloroplast from the cell cytosol, and where CO_2 fixation takes place.[6]

$NADP^+$ is reduced by electrons originating from water, but water is a weak reducing agent; the redox potential E_0' (at pH 7) of the dioxygen/water redox couple is 0.82 V.[13] In contrast, the redox potential E_0' of the $NADP^+$/NADPH couple is −0.32 V,[14] making PET from water to $NADP^+$ an energetically uphill process. To drive this process, the photosystems of the PET chain use the energy from absorbed photons.

Two multicofactor and multiprotein complexes in the thylakoids, photosystem II and I (PSII and PSI), work sequentially to sufficiently energize the electrons from water to reduce $NADP^+$ (Figure 1). Photons absorbed by PSII are used to oxidize water in the oxygen-evolving complex (OEC) connected to PSII (Figure 1, section a). The extracted electrons are passed on to PSI via the plastoquinone (PQ) pool, the cytochrome b_6f complex (Cyt b_6/f) and the lumenal electron transfer protein plastocyanin (PC) (Figure 1, section b). Upon light excitation, PSI transfers the electrons from the lumenal side to the stromal side of the membrane, where it uses them to reduce the soluble electron transfer protein, ferredoxin (Fd). Fd transfers the electrons to ferredoxin-$NADP^+$ oxidoreductase (FNR), which reduces $NADP^+$ to form NADPH (Figure 1, section c). The pH gradient formed over the thylakoid membrane during light-driven electron transport through PSII and PSI is concomitantly utilized by the ATP synthase (ATPase) for ATP formation (Figure 1, section d). When the Calvin−Benson cycle is fully active, the vast majority of ATP and NADPH are used to drive the reductive steps of the CO_2 fixation.

PSI has a quantum efficiency very close to 1, meaning it is very efficient in utilizing absorbed photons for the translocation of electrons from PC to Fd. Additionally; it generates the most negative redox potential in nature.[15] PSI is composed of a core complex responsible for charge separation and the first steps of electron transport and a peripheral antenna system, which is involved in light-harvesting and transfer of excitation energy to the reaction center.[16]

2.2. Coupling of PSI and Hydrogenases. In the research field investigating utilization of sunlight for light-driven production of chemical compounds through engineering of proteins from the photosynthetic machinery, the initial main focus has been on the generation of hydrogen (H_2) as a highly energetic biofuel, using primarily PSI as supplier of electrons. The interest in using PSI can be attributed to the highly negative reducing potential it generates, its high quantum efficiency, high stability, and the ease by which its well-described subunits may be manipulated.[16] A recent review by Winkler et al.[17] focuses on the efforts that have been made to obtain photobiological H_2 production, so here, we provide just a few examples of the progress in this area of research.

A focus area in the development of systems for PSI-driven H_2 production has been the tethering of a hydrogenase to PSI. Ihara and co-workers made the first hybrid complex of PSI and a hydrogenase, fusing a hydrogenase to the PsaE subunit (see Figure 1) of cyanobacterial PSI by genetic engineering and then reconstituting the PsaE-hydrogenase fusion protein into a PsaE-deficient cyanobacterial PSI *in vitro*.[18] Production of H_2 by the fusion protein was observed; however, the H_2 generation was strongly inhibited due to competition for electrons from Fd and FNR. As the long-term aim was the development of an *in vivo* functional system, an alternative construct was made in which cytochrome c_3, which is a natural electron donor to the hydrogenase, was cross-linked to the docking site of Fd in PsaE, thus causing electron flow from PSI via the cytochrome c_3 to the hydrogenase. This gave the hydrogenase a competitive

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advantage and resulted in a 7-fold enhanced H_2 production in the presence of Fd, FNR, and $NADP^+$.[19]

In another approach for direct coupling of a hydrogenase to the PSI-mediated supply of photoelectrons, Lubner et al. took advantage of the presence of Fe_4S_4 clusters in both PSI (see Figure 1) and the [FeFe]-hydrogenase.[14,20,21] To enable transfer of electrons from F_B, the terminal Fe_4S_4 cluster in the electron transport chain of PSI, to the distal Fe_4S_4 cluster in the hydrogenase, the two components were covalently coupled to each other via a molecular wire. The molecular wire used was a thiolated organic molecule, which could chemically rescue a PsaC mutant lacking one of the F_B-coordinating cysteines. The same approach was used to anchor the other end of the molecular wire to the hydrogenase, allowing electrons to tunnel through the wire from PSI to the hydrogenase. Self-assembly of the PSI-wire-hydrogenase complex was obtained *in vitro*, and the wiring resulted in high rates of light-driven H_2 production.[20]

Yacoby et al. investigated the *in vitro* hydrogen production by a hydrogenase, reduced by Fd, its natural electron donor, alone or in competition with photoreduction of $NADP^+$ by FNR, using electron delivery from either purified PSI or isolated thylakoids.[22] Presence of FNR and $NADP^+$ caused an inhibition of the hydrogen production of at least 75%. To increase the partitioning of the electrons toward the hydrogenase this was genetically fused to Fd. In competition with FNR and $NADP^+$, but in the absence of free Fd, 90% of the photosynthetic electrons were directed to H_2 production, whereas, in the presence of free Fd, only 60−70% of the electrons were diverted to the Fd-hydrogenase fusion.[22]

PSI-mediated hydrogen production has also been achieved in biohybrid systems, in which photocatalysis of H_2 is taking place on a noble metal (Pt or Au) surface associated with PSI, which supplies an input of electrons. Studies have focused both on connecting metal particles to PSI through molecular wires and on adsorption of nanosized particles onto PSI.[23−25]

Light-driven hydrogen production is thus an extensively researched topic, and a number of interesting systems have been developed. There are, however, several challenges associated with photobiological hydrogen production. The [FeFe]-hydrogenases, which are capable of high rates of H_2 generation, are extremely sensitive to O_2 and irreversibly inhibited and degraded under aerobic conditions, while the less oxygen-sensitive [NiFe]-hydrogenases show a much lower catalytic efficiency and can be strongly product inhibited by H_2.[17,26] The anaerobic requirements of hydrogenases complicate the development of *in vivo* production systems, and most *in vitro* systems would be expensive and difficult to scale up.

3. BIOACTIVE NATURAL COMPOUNDS

3.1. Moving from Bulk Energy Production toward High-Value Fine Chemicals. Despite the challenges in using photosynthesis for H_2 production, the advantages of chemical production *in vivo* in a photosynthetic organism are obvious: energy self-sufficiency (electrons are derived from water), self-maintenance and -repair, system robustness, and possibilities for scale-up. Methods to harness the energy supplied by PET *in vivo* are therefore valuable. Production of high-value bioactive natural compounds in photosynthetic organisms is another area where electrons supplied directly from PET can be utilized advantageously.

3.2. Different Groups of Bioactive Natural Compounds. Plants produce an immense number of bioactive compounds known interchangeably as secondary metabolites, natural products, phytochemicals or specialized metabolites. Whereas primary metabolites are directly involved in growth, development, or reproduction and are found in all, or nearly all, plants, specialized metabolites are not essential to the plant's immediate survival but are important in the interactions between the plant and its environment and allow the plant to adapt to its specific ecological niche.[9,27−30] These compounds often function as chemical defense compounds, for example, to protect against pathogens or herbivores or to suppress the growth of other nearby plants, as attractants of pollinators or seed dispersers, or they can be synthesized in response to environmental stresses such as drought or salt stress or to protect against ultraviolet (UV) radiation.[8,31−34]

More than 200 000 bioactive natural compounds have been identified to date, but the actual number is expected to be much higher.[8,28] Specialized metabolites are often only synthesized in limited taxonomic groups in the plant kingdom and represent adaptations to the specific environments inhabited by a group. The accumulation of the specialized metabolites is also often restricted to specific cell types at specific developmental stages.[8,9,28]

Most plant derived specialized metabolites can be divided into three major groups on the basis of their chemical structures and biosynthesis: (1) the terpenoids, (2) the alkaloids, and (3) the phenylpropanoids and related phenolic compounds.[9,27]

The terpenoids, also known as the isoprenoids, is a very large and diverse group of specialized metabolites all derived from precursors from the mevalonate (MEV) or methylerythritol-phosphate (MEP, also known as DXP) pathways.[9,35] More than 43 000 terpenoids are known, most of which are produced by plants.[36] They consist of joined five-carbon isoprenoid units and are classified according to the number of the units they contain as for instance hemiterpenes (5 carbon atoms), monoterpenes (10 C), sesquiterpenes (15 C), diterpenes (20 C), triterpenes (30 C), and tetraterpenes (40 C).[9,37] Larger terpene molecules have also been reported.[38] The terpenoids have a broad range of biological functions, including defensive and attractive, and have been shown often to act in complex mixtures where their joint function can be synergistic.[27]

Alkaloids are low-molecular-weight compounds containing basic nitrogen atoms. Around 12 000 different alkaloids are known. Most alkaloids are derived from amino acids such as tyrosine, tryptophan, phenylalanine, histidine, and lysine. In plants, they are primarily engaged in defense against pathogens and herbivores; however, alkaloids are also well-known for their pharmaceutical properties.[9,39]

The diverse array of phenylpropanoids and related phenolic compounds are derived from the amino acids phenylalanine and tyrosine synthesized in the shikimate pathway and includes, for instance, the flavonoids, coumarins, lignans, lignins, and stilbenes.[40,41] They have a wide array of different functions and take part in all aspects of plant responses to environmental challenges.

Apart from these three major groups, nonprotein amino acids, cyanogenic glucosides, and glucosinolates are other minor but important groups of secondary metabolites.[34,42]

3.3. Applications of Bioactive Natural Compounds. The medicinal potential of plants has been known since ancient times, and plant specialized metabolites are still acknowledged for their pharmaceutical properties. Specialized metabolites often have properties that make them useful as for instance

Table 1. Examples of Well-Known Plant-Derived Specialized Metabolites Produced and Used As Pharmaceuticals, Flavors, and Fragrances

active compound	application	compound nature	plant	reference
linalool	fragrance	monoterpene	many fruits and flowers, *e.g.* strawberry (*Fragaria* × *ananassa*) and *Clarkia breweri*	43−45
menthol	flavor	monoterpene	*Mentha* species	46
gingerols and shogaols	flavor	phenolic compounds	ginger (*Zingiber officinale* Roscoe)	47
vanillin	flavor	phenolic compound	vanilla orchid (*Vanilla planifolia*)	48
paclitaxel	anticancer agent	diterpene	Pacific yew tree (*Taxus brevifolia*)	49
ingenol-3-angelate	anticancer agent	diterpene	petty spurge (*Euphorbia peplus*)	50
forskolin	heart failure treatment	diterpene	Indian coleus (*Coleus forskohlii*)	51, 52
vincristine and vinblastine	anticancer agents	terpenoid indole alkaloids	Madagascar periwinkle (*Catharanthus roseus*)	49, 53
quinine	antimalarial agent	alkaloid	quinine bark (*Cinchona officinalis*)	54
morphine and codeine	analgesics	alkaloids	opium poppy (*Papaver somniferum*)	55
galanthamine	treatment of Alzheimer's disease	alkaloid	Amaryllidaceae family (*e.g.*, snowdrops (*Galanthus* spp.) and daffodils (*Narcissus* spp.))	56
cinnamaldehyde	flavor	phenolic compound	cinnamon (*Cinnamomum verum*)	45
artemisinin	antimalarial agent	sesquiterpene	sweet wormwood (*Artemisia annua*)	57

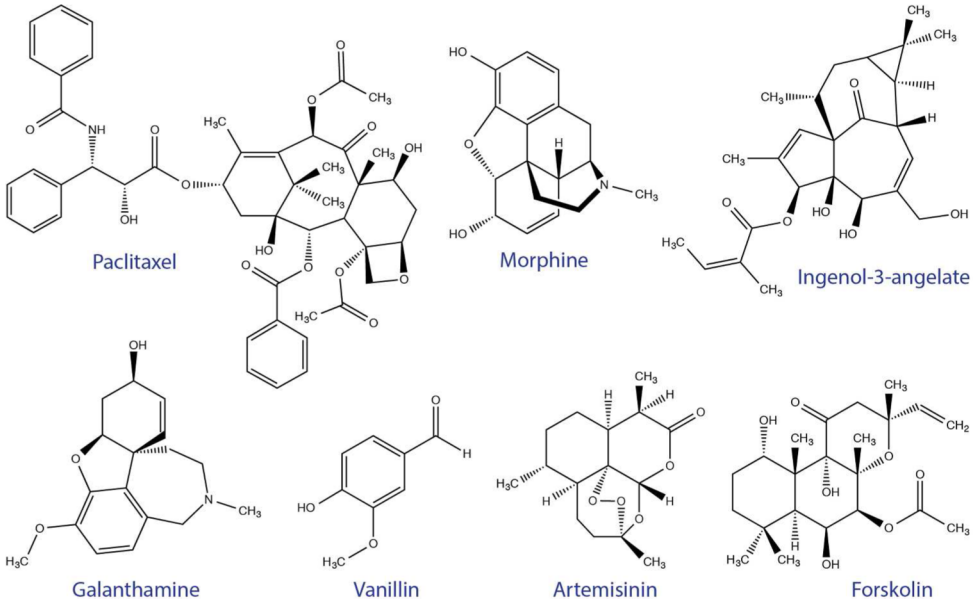

Figure 2. Specialized metabolites often have complex structures difficult to produce by chemical synthesis. Shown here are the terpenoids paclitaxel, ingenol-3-angelate, artemisinin, and forskolin, the alkaloids morphine and galanthamine, and the phenolic compound vanillin.

pharmaceuticals, biopesticides, flavors, fragrances, or food additives. Some notable examples are summarized in Table 1, and examples of structures of a few specialized metabolites are shown in Figure 2.

3.4. High value−low yield. Many bioactive natural products derived from plants have a high market value. Some compounds, such as menthol, are produced in amounts where they relatively easily can be extracted directly from the native plants in quantities that are economically feasible. However, for many specialized metabolites, the biosynthesis in the native plants is tightly regulated, often causing production levels to be low or highly variable and confined to specific growth stages and cell types, thus making extraction, purification, and separation from structurally similar compounds difficult.[4,8,9,39,58]

The structures of specialized metabolites are often highly complex with many chiral carbon atoms and *cis-trans* isomers (Figure 2), which are important for the biological functions of the molecules. This makes them difficult to produce by chemical synthesis, requiring many steps and often resulting in low overall yields.[8,39,59−61]

Plant specialized metabolite biosynthesis pathways are thus appropriate targets for bioengineering and extensive research efforts are focusing on developing new strategies to produce these bioactive compounds, attempting to enzymatically synthesize larger quantities than what is being made in the native plants in other biological production systems.[9,39,62,63]

The potent anticancer drug paclitaxel, also known by the trademark name Taxol, and the effective antimalarial agent artemisinin (Figure 2) are examples of pharmaceutically important compounds for which extensive research efforts are focused on elucidating and expressing the biosynthetic pathways. Both can be prepared by total chemical synthesis but with too high costs and low yields.[39,60,64,65] Biosynthesis of paclitaxel from the common diterpenoid precursor geranylgeranyl diphosphate presumably occurs in 19 steps, resulting in a highly complex structure. Through metabolic engineering it has been possible to produce early intermediates of the pathway in

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E. coli and *Saccharomyces cerevisiae*.[64,66,67] The late artemisinin precursors amorpha-4,11-diene and artemisinic acid, which can be chemically converted to a semisynthetic version of artemisinin, can also be produced in these organisms; however, the complete biosynthesis pathways for both paclitaxel and artemisinin have not been determined yet.[35,68−73]

4. CYTOCHROME P450S

While many different kinds of enzymes, such as glycosyl-, acyl-, and *O*-methyl transferases and terpene and polyketide synthases, take part in the formation of bioactive natural products, the cytochrome P450s (P450s) play a key role in catalyzing the regio- and stereospecific reactions functionalizing the core structures of the molecules in the biosynthetic pathways.[31,39,74,75] For instance, in the biosynthesis of the complex diterpene paclitaxel (Table 1 and Figure 2), almost half of the anticipated ∼19 enzymatic steps committed to the paclitaxel pathway from the common precursor geranylgeranyl diphosphate are thought to be catalyzed by P450s.[72,76,77] A simplified schematic representation of a diterpene biosynthetic pathway is shown in Figure 3.

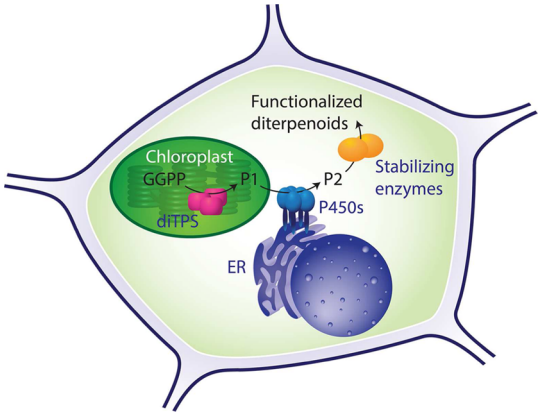

Figure 3. Simplified schematic representation of a diterpene biosynthesis pathway in a plant cell. Geranylgeranyl diphosphate (GGPP), the common diterpene precursor, is synthesized in the chloroplast, where diterpene synthases (diTPS) tailor the molecule by cyclization. The resulting molecule (P1, precursor 1) is functionalized by a number of ER-localized cytochrome P450s, which oxygenate P1 to P2 (precursor 2). Various types of transferases (stabilizing enzymes), for instance methyl-, acetyl-, acyl-, or glycosyl-transferases, decorate the molecule to form the final functionalized diterpenoid.

Cytochrome P450s constitute one of the largest superfamilies of enzymes known with members spread over all biological kingdoms.[78−80] The term P450 refers to a characteristic absorption band obtained at 450 nm when the reduced form of a P450 is incubated with carbon monoxide. P450s contain a heme as a prosthetic covalently bound group, which mediates catalytic activation of oxygen. Although they share a common structural fold, their amino acid sequences are highly diverse with as little as ∼15% identity.[81] P450s are named based on their sequence identity as the letters CYP followed by a number designating the family, a letter specifying the subfamily and finally a number identifying the individual gene.[78,81] In general, prokaryotic P450s are soluble while eukaryotic P450s are membrane-bound, typically to the endoplasmic reticulum (ER) or inner mitochondrial membranes through an N-terminal transmembrane spanning segment.[80,82,83] The catalytic domain of the ER-bound P450s is facing the cytoplasm while the catalytic domain of the mitochondrial membrane-bound P450s is in the mitochondrial matrix.[78,82]

4.1. Function of P450s. P450s are usually monooxygenases, catalyzing the heterolytic cleavage of molecular oxygen and the subsequent exceptionally regio- and stereospecific insertion of one of the oxygen atoms into a substrate, generally a hydrocarbon, while the other oxygen is reduced to water.[78−81]

P450s can modify an immense array of substrates.[80,81] While some P450s are highly selective toward a specific substrate, others are more promiscuous and may react with numerous structurally diverse xenobiotics.[84] The reactions carried out by P450s are highly diverse. Most often, they are C-hydroxylations, but they also include heteroatom oxygenations, dehydrogenations, deaminations, desulfurations, dehalogenations, peroxidations, and epoxide formations.[72,78,79] Though most P450 catalyzed reactions are redox reactions, with the great majority being oxidations, P450s catalyzing nonredox isomerization reactions also exist.[85]

P450s are involved in several functions in different organisms. In addition to the major role they play in the biosynthesis of specialized metabolites, they also participate in for example the biosynthesis of other physiologically important compounds such as fatty acids or steroid hormones important in regulation of growth and development, and in detoxification of xenobiotics such as pesticides, pollutants, carcinogens and drugs.[75,78,80,81,86]

4.2. Classification and Redox Partners. P450s generally receive the two electrons needed for their catalytic reactions from NADPH or NADH. There is, however, a significant variety of electron donor systems involved in the electron delivery.[87]

P450s can be divided into classes based on their redox partners. Class I P450s, which include most bacterial P450s (Figure 4A) and the mitochondrial P450s in eukaryotes, receive

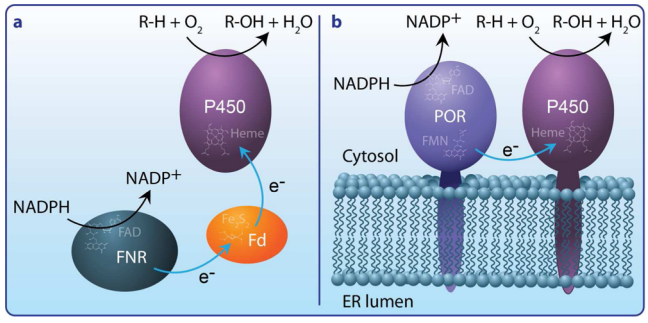

Figure 4. Reductase systems of bacterial class I (a) and eukaryotic class II (b) P450s. (a) The ferredoxin reductase (FNR, contains the cofactor FAD as redox center), transfers electrons from NADH or NADPH to the ferredoxin (Fd, iron−sulfur cluster as redox center), which reduces the P450 (heme as redox center). (b) The NADPH-cytochrome P450 oxidoreductase (POR, contains the cofactors FAD and FMN) transfers electrons from NADPH to the P450.

electrons from an iron−sulfur cluster-containing ferredoxin (Fd), which is reduced by a flavin adenine dinucleotide (FAD)-containing ferredoxin reductase (FNR or FdR) that extracts electrons from NADPH or NADH. The P450, ferredoxin and ferredoxin reductase are all soluble in bacteria, while in eukaryotes the P450 is membrane-bound and the reductase is membrane-associated.[78,81]

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The most common class of P450s in eukaryotes, which includes the majority of P450s involved in specialized metabolism in plants, is class II, in which the enzymes are located in the ER membranes. Here, electrons are delivered to the P450 by a NADPH-cytochrome P450 oxidoreductase (POR), a diflavin enzyme (Figure 4b).[78,81,88]

POR is thought to have evolved from a fusion of a flavin mononucleotide (FMN)-containing flavodoxin and a FAD-containing ferredoxin-NADP+ reductase (FNR).[88,89] Upon transfer of the electrons from NADPH through the FAD and FMN cofactors of POR to the P450, POR changes conformation from a closed to an open structure to expose the reduced FMN cofactor and facilitate interactions with the P450.[82] A single POR may serve as redox partner to several different P450s.[87,88] A possible mechanism for control of the POR-P450 interactions could be an increase in the affinity of a P450 for the POR by substrate binding-induced conformational changes in the P450.[88]

Apart from these two most common classes, several additional P450 classes can be designated based on various other electron transfer systems.[78,87] For example, some P450s are reduced by an FMN-containing flavodoxin (Fld) instead of ferredoxin in a system similar to the class I system, and some can extract electrons directly from NADPH. Other systems consist of reductase-dependent fusions of the P450 and a ferredoxin or flavodoxin, or self-sufficient fusions of POR and the P450, while yet other systems are even more unusual.[78,81,87] The electron transfer to the P450 is often the rate-limiting step in P450 catalysis.[90]

Figure 4 shows a schematic representation of the bacterial class I and eukaryotic class II reductase systems. P450 redox partners have been comprehensively reviewed by Hannemann et al., and an in depth overview of the reductase systems can be found in this article.[78]

4.3. Plant P450s and Biosynthesis of Natural Bioactive Compounds. While the genes encoding the P450s (*CYP* genes) exist in nearly all organisms, the number of these genes is significantly higher in plants than in other organisms. The model plant *Arabidopsis thaliana* has 245 full-length *CYP* genes, while only 57 *CYP* genes are found in human, 102 in mouse (*Mus musculus*), 109 in the filamentous fungus *Aspergillus nidulans*, and 20 in *Mycobacterium tuberculosis*.[74,91−94] Some of the P450s in plants are related to P450s in other organisms and have a conserved function in, for example, metabolism of sterols or fatty acids, while about two-thirds are plant-specific and involved in natural product biosynthesis.[80]

4.3.1. Heterologous Expression of Plant Cytochrome P450s in Microbial Hosts. Of the many enzymes participating in specialized metabolite biosynthesis, the P450s and their redox partners can be particularly challenging to express functionally, especially in prokaryotic expression hosts.[72,95] Expressed unaccompanied, P450s often show little or no activity if interactions with redox partners endogenous to the host are not effective, and coexpression or purification and subsequent reconstitution with their redox partner is thus often necessary.[96,97] Expression of the redox partners can, however, also be a challenge in microbial systems.[95]

The limitations of expressing plant and other eukaryotic P450s in prokaryotes include improper translation and membrane insertion, and lack of post-translational modifications.[39,72] Incorrect folding and problems with incorporation of the heme can also hinder successful prokaryotic expression of eukaryotic P450s, but this can be improved with the coexpression of a chaperonin system.[98−100]

Eukaryotic P450s are, as previously mentioned, bound through one transmembrane domain to membranes of the mitochondria and ER, and the eukaryotic signal peptides directing the proteins to these organelles may not be efficiently recognized as membrane localization signals in prokaryotes. This can lead to low expression levels of the P450s, but modifications of the N-terminal sequence to either direct the full-length P450 to a membrane localization in the plasma membrane or modification and truncation of the N-terminal sequence including the membrane anchor for soluble expression in the cytosol has in some cases circumvented this problem.[100−103]

E. coli itself does not contain any P450s naturally, thus providing a clean background. A few of the many existing plant P450s have been successfully expressed in *E. coli*.[72,80] Successful expression of P450s resulting in *in vivo* activity in *E. coli* have focused on changing the membrane anchor or fusing the P450 to the redox partner or the enzyme producing the substrate of the P450.[39,104] Strain and promoter variation, codon optimization, and similar strategies have also been used to improve expression and activity.[39,104] Silent mutations in the gene sequence to prevent formation of secondary mRNA structures causing difficulties in ribosome binding and processing has also proven helpful to enhance P450 expression in *E. coli*. Mutation of the second codon to encode an alanine has been shown to be particularly efficient in this context.[99,100,105]

Eukaryotes such as the yeast *S. cerevisiae* can better support the expression of the membrane-localized P450s, and of the microorganisms often used in biotechnology, yeasts have been shown to be well-suited expression systems for P450s.[39,96,106] Many of the functionally expressed *Arabidopsis* P450s have thus been expressed in yeast.[96] As plants are a natural background for P450 expression, engineered model plants could be suitable host organisms for heterologous expression of P450s.[107,108]

4.4. Supply of Reducing Equivalents. Although the unique catalytic abilities of P450s provide a great potential for applications as industrial biocatalysts, P450s are only used scarcely in biotechnology. Reasons for this are reviewed by O'Reilly and co-workers and include instability under the conditions they are exposed to during isolation, storage and use, intolerance of organic solvents, narrow substrate specificity, requirement of costly cofactors, and limitations in electron supply.[90] The NADPH and NADH cofactors are expensive, and additionally, in the absence of substrate, NADPH or NADH can potentially inactivate the P450, which makes close monitoring of the cofactor/substrate ratio necessary in biotechnological *in vitro* production systems.[109,110] However, due to the stability issues under *in vitro* conditions, a production system based on intact microbial cells, where the P450s are kept under physiological conditions, is often the approach of choice.[111] In microbial production systems such as bacteria and yeast, the native NADPH regenerating metabolism is typically not sufficient to support high levels of P450 activity. One approach of cofactor supply, which can be used both *in vivo* and *in vitro*, is enzyme-coupled cofactor regeneration, but nonenzymatic NAD(P)H regeneration systems have also been developed.[112,113]

To avoid the use of NADPH and NADH, several approaches relying on application of alternative electron supplies have been investigated. These include zinc dust as electron source with

cobaltsepulchrate as mediator or electrochemical methods (*e.g.*, with the P450 immobilized on platinum electrodes).[90,109,113,114] The peroxide shunt pathway, in which P450s are able to use peroxides such as hydrogen peroxide as a source of both oxygen and electrons, has also been employed. This system eliminates the use of NADPH and a reductase, and thus also the rate-limiting electron transfer steps, but is limited by peroxide-mediated heme degradation, which causes rapid inactivation of the P450.[115]

Another approach, which has so far not been used widely, is coupling of P450s to the photosynthetic electron generation. Retargeting of the P450s to the thylakoids either in chloroplasts or in cyanobacteria will position the P450 in an environment where reducing power is generated continuously and should thus ensure ample supply of reducing equivalents.

5. REDIRECTING PHOTOSYNTHETIC REDUCING EQUIVALENTS

The photosynthetic electron transport chain would be an excellent producer of reducing power to directly support P450s. Also, since availability of dioxygen for oxygenase activity in addition to host respiration has been proposed as a challenge in P450 heterologous expression in yeast or bacteria,[116] P450 expression in an oxygenic photosynthetic organism would serve to alleviate such an issue.

Though most plant P450s are located in the ER membranes, P450s are also known to exist naturally in chloroplasts, presumably employing the soluble electron transfer partners ferredoxin and ferredoxin reductase.[94]

5.1. Combining Cytochromes P450 with the Photosynthetic Machinery. The potential of directly combining P450-mediated monooxygenations with photosynthesis was first investigated in 1996 by Kim et al.[117] By combining spinach chloroplasts with microsomes from yeast expressing a fusion enzyme of rat CYP1A1 and a reductase, they developed an *in vitro* system where, under illumination, $NADP^+$ was photosynthetically reduced to NADPH. Via the reductase, this NADPH then served as electron supply for the P450, thus enabling light-driven conversion of the P450 substrate 7-ethoxycoumarin to 7-hydroxycoumarin.

Recently, the approach of employing photosynthetic electrons in P450 reactions *in vitro* was further developed and simplified by Jensen et al.[118] It was found that electrons supplied by PSI from barley (*Hordeum vulgare*) could be transferred directly to *Sorghum bicolor* CYP79A1 by ferredoxin with high efficiency, thus eliminating the need of an NADPH recycling system and POR. The turnover rate of this system, with CYP79A1 catalyzing the hydroxylation of L-tyrosine to *p*-(*E*)-hydroxyphenylacetaldoxime, was reported to be twice as high as when the native reductase was used for electron supply to the P450.[118]

The versatility of this system was tested by replacing the plant CYP79A1 with another P450, the soluble bacterial CYP124 from *Mycobacterium tuberculosis* catalyzing ω-hydroxylations of methyl-branched lipids.[119] This demonstrated the flexibility in the PSI-driven P450 approach and suggested the potential of a modular system, in which production of diverse compounds could be possible dependent on the employed substrate and P450.

As described above, some classes of P450s, including the CYP124 from *Mycobacterium tuberculosis*, natively use ferredoxin or flavodoxin as electron donor with electrons supplied from NAD(P)H via a ferredoxin reductase.[78,119] In such systems, other Fds than the endogenous have been reported to support electron delivery to the P450 *in vitro*.[120] In accordance with this, it has been demonstrated that the P450 CYP106A2 from *Bacillus megaterium* could be reduced by Fd and Fld from the photosynthetic electron transport chain of the cyanobacterium *Anabaena* sp. PCC 7119, which received electrons from NADPH via FNR, showing that hybrid systems combining electron carrier proteins from different electron transfer chains can be made.[89]

In a similar setup, genetic fusion constructs of the rat CYP1A1 with Fd and FNR from the photosynthetic electron transfer chains of maize and pea, respectively, were produced by Lacour and Ohkawa.[121] P450-Fd, P450-FNR, P450-Fd-FNR, and P450-FNR-Fd fusions were expressed in *S. cerevisiae*. *In vitro* assays showed that the highest catalysis rate could be obtained with the P450-Fd-FNR construct with NADPH as electron donor. The P450-Fd and P450-FNR constructs showed low activities similar to the P450 in the absence of any electron transfer proteins, demonstrating that the simultaneous presence of Fd and FNR was necessary to obtain an efficient electron transport chain.[121]

O'Keefe et al. heterologously expressed the CYP105A from the soil bacterium *Streptomyces griseolus* in tobacco, finding that only localization in the chloroplast supported enzymatic activity, N-dealkylation of a sulfonylurea (2-methylethyl-2,3-dihydro-*N*-[(4,6-dimethoxypyrimidin-2-yl)aminocarbonyl]-1,2-benzoisothiazole-7-sulfonamide-1,1-dioxide).[120] This suggests that the endogenous P450 reductases localized on the cytoplasmic surface of the ER could not function as electron donor for this soluble bacterial P450 in the cytoplasm and that the activity of the chloroplast-targeted version was supported only by electron delivery by reduced Fd. Thus, bacterial P450s can be expressed in plant chloroplasts and be driven by electrons from the PET chain with Fd as mediator.

Using transient expression in *Nicotiana benthamiana* as experimental system, it was recently shown that it is possible to transfer the P450-catalyzed dhurrin pathway[122−124] from the cytosol of *S. bicolor* to the tobacco chloroplast.[108] This was achieved using gene constructs encoding fusion proteins between the transit peptide of the chloroplast stroma localized Fd and the coding regions of two P450 enzymes and a UDP glucosyl transferase, which constitute the dhurrin biosynthetic pathway.[108] The three enzymes were successfully expressed in the chloroplast of transiently transformed tobacco leaves. The chloroplast was able to provide the heme cofactor for proper assembly of the P450s and also the tyrosine and UDP-glucose substrates. The energy-demanding P450-catalyzed synthesis of dhurrin was driven by directly tapping into light-driven reduction of Fd by PSI. Thus, P450s normally residing in the ER membranes can be targeted to the chloroplast, driven by photosynthesis, and be able to biosynthesize dhurrin. This constitutes a proof-of-concept for establishing new biosynthetic pathways in plant chloroplasts and thereby bringing the biosynthetic machinery close to the source of reducing power and place of synthesis of precursors for many specialized metabolites. The next obvious step will be to establish stably transformed plants, algae or cyanobacteria.

6. CONCLUSIONS, PERSPECTIVES, AND CHALLENGES

Plants produce a wide variety of bioactive natural compounds, many of which are used as pharmaceuticals and have a high value. The naturally occurring abundance of these compounds in the plants are, however, often low or highly variable, and

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development of alternative production systems are accordingly beneficial. In this context, the cytochrome P450s, which constitute a large enzyme family in plants and catalyze key hydroxylation steps in the biosynthesis of the bioactive natural compounds, require electrons often delivered from NADPH or NADH by a reductase, a process that at least in a biotechnological context often is limiting. The use of photosynthetically produced reducing power to directly support the activity of P450s is a promising approach for production of complicated-to-produce bioactive products or biosynthesis intermediates interesting for the chemical or pharmaceutical industries.[118,119] Thus, production systems based on photosynthetic hosts offer alternatives to the classical biotechnological hosts such as bacteria and yeast. In addition, production in a photosynthetic organism offers a true sustainable alternative, since the precursor compounds, the energy and reducing power are derived from photosynthesis via water splitting, CO_2 fixation and light-driven electron transport.

The chloroplast is a well-suited compartment for expression of foreign genes. Chloroplast genome transformation has been used successfully to introduce new genes and multigene constructs into the plastid genome, which is attractive for several reasons: the plastids have a potential for high-level foreign protein expression, it is possible to express multiple genes from operons, and there are no reported position effects or epigenetic gene silencing mechanisms in the chloroplast.[125] Integration of foreign DNA into the plastid genome occurs exclusively by homologous recombination, facilitating precise engineering approaches.[126]

There are, however, also several challenges associated with development of light-driven specialized metabolite production systems. Although P450s targeted to the chloroplast will be close to the source of reducing power of the cell, an important challenge will be to direct a sufficient fraction of the electrons generated in the PET chain toward the P450s. *In vivo* the introduced P450s will compete with other proteins reduced by Fd for the available reducing equivalents, most notably with FNR catalyzing the reduction of $NADP^+$, but also with enzymes involved in several other essential processes (*e.g.*, cyclic electron transport, biosynthesis of chlorophyll, lipids, and glutamate, and reduction of nitrite, sulfite, and thioredoxins).[127−131] With inspiration from photobiological hydrogen production, a strategy to increase the electron flow toward a P450 could be to fuse it to Fd, which was shown to increase the *in vitro* competitiveness of a hydrogenase by Yacoby and co-workers.[22] Alternatively, Fds have to be redesigned to preferentially interact with the P450s.

The substrate of the first enzyme specific to the pathway also needs to be produced in the chloroplast in sufficient amounts to sustain the pathway, and it may be necessary to upregulate the substrate biosynthesis to obtain a satisfactory production level of the target compound.[71]

Many specialized metabolite biosynthetic pathways have not been fully discovered yet, and finding the optimal combination of enzymes may require considerable efforts before a heterologous light-driven production system for a high-value natural compound can be developed.[9] Another challenge for specialized metabolite production in all heterologous host systems is the potential metabolic crosstalk between endogenous and engineered pathways. Additionally, toxicity of foreign compounds can be encountered; however, if the compound is an intermediate, balancing the activity of the enzymes producing and consuming it can be a way to minimize the problem.[132]

The plug-and-play approach of synthetic biology opens a variety of possibilities for production of specialized metabolites. In addition to formation of novel or altered compounds, many other approaches, such as manipulation of production levels through compartmentalization, synthetic protein scaffolds or dynamic regulation of enzyme expression, can be applied to optimize the metabolic potential of the cell for production of a specific product and simultaneously present the opportunity to learn more about how these systems function in nature.[133,134]

AUTHOR INFORMATION

Corresponding Author

*E-mail: peje@life.ku.dk.

Notes

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ABBREVIATIONS

ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPX, 1-deoxy-D-xylulose-5-phosphate; ER, endoplasmic reticulum; FAD, flavin adenine dinucleotide; Fd, ferredoxin; Fld, flavodoxin; FMN, flavin mononucleotide; FNR, ferredoxin-$NADP^+$ oxidoreductase; MEP, methylerythritol-phosphate; MEV, mevalonate; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; P450, cytochrome P450; PC, plastocyanin; PET, photosynthetic electron transport; POR, NADPH-cytochrome P450 oxidoreductase; PSI, photosystem I; PSII, photosystem II; PQ, plastoquinone; UV, ultraviolet

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