Anchoring a plant cytochrome P450 via PsaM to the thylakoids in Synechococcus sp. PCC 7002

Lassen, Lærke Marie Münter; Nielsen, Agnieszka Janina Zygaldo; Olsen, Carl Erik; Bialek, Wojciech; Jensen, Kenneth; Møller, Birger Lindberg; Jensen, Poul Erik

Published in:
P L o S One

DOI:
10.1371/journal.pone.0102184

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Anchoring a Plant Cytochrome P450 via PsaM to the Thylakoids in *Synechococcus* sp. PCC 7002: Evidence for Light-Driven Biosynthesis

Lærke Münter Lassen¹, Agnieszka Zygdal Nielsen¹, Carl Erik Olsen¹, Wojciech Bialek², Kenneth Jensen¹, Birger Lindberg Møller¹, Poul Erik Jensen¹*

¹Center for Synthetic Biology "bioSYnergy", the VILLUM Research Center “Plant Plasticity”, Copenhagen Plant Science Center, Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg C, Copenhagen, Denmark, ²Department of Biophysics, Faculty of Biotechnology, University of Wroclaw, Wroclaw, Poland

Abstract

Plants produce an immense variety of specialized metabolites, many of which are of high value as their bioactive properties make them useful as for instance pharmaceuticals. The compounds are often produced at low levels in the plant, and due to their complex structures, chemical synthesis may not be feasible. Here, we take advantage of the reducing equivalents generated in photosynthesis in developing an approach for producing plant bioactive natural compounds in a photosynthetic microorganism by functionally coupling a biosynthetic enzyme to photosystem I. This enables driving of the enzymatic reactions with electrons extracted from the photosynthetic electron transport chain. As a proof of concept, we have genetically fused the soluble catalytic domain of the cytochrome P450 P450 CYP79A1, originating from the endoplasmic reticulum membranes of *Sorghum bicolor*, to a photosystem I subunit in the cyanobacterium *Synechococcus* sp. PCC 7002, thereby targeting it to the thylakoids. The engineered enzyme showed light-driven activity both in vivo and in vitro, demonstrating the possibility to achieve light-driven biosynthesis of high-value plant specialized metabolites in cyanobacteria.

Introduction

Sunlight is an inexhaustible and abundant source of clean energy that, through photosynthesis, can be harvested by plants, algae and cyanobacteria. The amount of light energy captured in photosynthesis is often larger than what can be used by the photosynthetic metabolic reactions. This suggests a potential for utilizing photosynthetic organisms in biotechnology, as the excess electrons generated in photosynthesis could be applied in sustainable production of biofuels and high-value chemical compounds [1]. Using a synthetic biology approach, a photosynthetic organism can be engineered to take advantage of this excess reducing power. By transferring enzymes that catalyse reactions of interest into the vicinity of the thylakoids, it is possible to tap directly into the photosynthetic electron transport chain and redirect some of the electrons towards production of valuable bioactive compounds [1,2].

Plants synthesize a vast array of bioactive molecules, many of which have properties that make them useful as pharmaceuticals, biostics, flavours, fragrances or colours [3,4]. Examples include paclitaxel from *Taxus brevifolia* and vincristine and vinblastine from *Catharanthus roseus*, which are all used in cancer treatment, and the anti-malarial agent artemisinin from *Artemisia annua* [5–7]. Often these compounds are of high market value, but are synthesized at low levels in the plants and are difficult to produce chemically due to their highly complex structures [4,8,9]. A group of enzymes often involved in the biosynthesis pathways of these molecules, catalysing key steps with high regio- and stereospecificity, is the cytochrome P450s (P450s). P450s are typically monooxygenases, catalysing the cleavage of O₂ and incorporation of an oxygen atom in a wide variety of different substrates [10–12]. In plants, P450s are mainly found in the endoplasmic reticulum (ER) and receive the electrons needed for their reactions from NADPH via a NADPH-cytochrome P450 oxidoreductase (POR) [13].


Supporting Information files.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting information files.

Funding: The authors gratefully acknowledge financial support from 1) the VILLUM Center of Excellence "Plant Plasticity", 2) Center of Synthetic Biology "bioSYnergy" funded by the UCPH Excellence Program for Interdisciplinary Research, 3) "Plant Power: Light-driven synthesis of complex terpenoids using cytochrome P450s" (12-131834) funded by the Danish Council for Strategic Research, Programme Commission on Strategic Growth Technologies and 4) ERC Advanced Research Grant No. 323034 “Light driven P450s”. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: peje@plen.ku.dk

Current address: Nixon Group, Sir Ernst Chain Building - Wolfson Laboratories, Imperial College London, London, United Kingdom

Current address: Novozymes A/S, Bagsvaerd, Denmark
In this report it is our aim to construct a microbial in vivo production system for plant specialized metabolites, in which the POR is omitted and instead the electrons for the P450s are supplied from the photosynthetic electron transport chain.

In oxygenic photosynthesis, electrons are extracted from water by photosystem II [PSII] and delivered to photosystem I [PSI] via an electron transport chain [14,15]. PSI utilizes energy from light to excite electrons and transfers them to ferredoxin (Fd), a small soluble redox protein, which in turn reduces proteins of different electron-requiring processes in the stroma of the plastid or cytosol of cyanobacteria, primarily the ferredoxin-NADP+ reductase (FNR), which catalyses the reduction of NADP+ to NADPH. The structure and function of PSI is highly conserved between organisms, the core of the complex even being remarkably conserved between cyanobacteria and higher plants despite a billion years of separate evolution [16,17]. In addition, the high stability and quantum yield of PSI make it useful in bio-engineered production systems [18].

As a model system to investigate the effect of functionally coupling PSI and a plant P450, we have chosen the P450 CYP79A1 from the biosynthesis pathway of the cyanogenic glucoside dhurrin (β-D-glucopyranosyl-(S)-3-hydroxymandelonitrile) in the crop grass Sorghum bicolor. The dhurrin biosynthesis pathway consists of two membrane-anchored P450s, CYP124A1 and CYP71E1, and a soluble UDP-glucosyltransferase, UGT85B1. CYP79A1 is the first enzyme in the pathway and converts the substrate tyrosine into p-hydroxyphenylacetaldoloxime (oxime) [19-20].

We have previously shown that Fd is capable of mediating electron transfer from PSI directly to the CYP79A1 in vitro, thereby enabling oxime production with high efficiency [13]. The versatility of the approach of light-driven P450 reactions was demonstrated by showing that the system was not only functional with the plant CYP79A1, but also with a bacterial P450, the CYP124 from Mycobacterium tuberculosis [21]. More recently, we transiently expressed the entire dhurrin biosynthesis pathway in the tobacco Nicotiana benthamiana, where it was targeted to the chloroplast by transit peptides [2]. The two P450s were inserted in the thylakoid membrane and demonstrated to be driven by the photosynthetic reducing power.

In the present study we aimed at developing an in vivo-functional light-driven microbial production system by stably expressing a fusion protein of the catalytic domain of CYP79A1 and a PSI subunit, functioning as a thylakoid-targeting membrane anchor, in a cyanobacterium. Using a photosynthetic microorganism as host organism provides the opportunity for future scale-up to industrial production levels.

For the cyanobacterium to be viable, only a part of the electrons from PSI should be delivered to the CYP79A1 via freely diffusible Fd. The peripheral, membrane embedded PSI subunit PsAM was chosen as anchor point for the CYP79A1 (Figure 1), as this should allow access of Fd to its docking site at PSI, even if the recombinant PsAM-CYP79A1 subunit remained bound to PSI in a PSI-CYP79A1 supercomplex, and ensure partition of electrons to both the CYP79A1 as well as to other proteins relying on reduction by Fd [22]. Proximity of the CYP79A1 to PSI should be beneficial in the competition for reduced Fd, as close confinement has been shown to be an advantage for the efficient electron transfer between interacting proteins in crowded environments like the thylakoids [23].

The PsAM-CYP79A1 fusion protein was shown to hydroxylate tyrosine to the corresponding oxime both in vivo and in vitro. Despite the fact that only a fraction of the fusion protein was found to be associated with PSI upon purification, the recombinant P450 was active without its native membrane anchor and its activity was indeed sustained by the photosynthetic electrons. Interestingly, the produced oxime was excreted into the growth medium, facilitating simple product isolation.

Materials and Methods

Construction of the psaM-CYP79A1 fusion

A psaM-CYP79A1 fusion construct was designed as a DNA sequence encoding amino acid 1-25 of Synechococcus sp. PCC 7002 PsAM, corresponding to the membrane spanning part of the protein, followed by a sequence encoding amino acid 36-558 of the S. bicolor CYP79A1, corresponding to the part of the CYP79A1 protruding from the membrane in the native protein, codon optimized for expression in cyanobacteria. The construct was commercially synthesized and inserted in the multi cloning site of the pUC57 cloning vector by Genscript (http://www.genscript.com/).

Genomic DNA (gDNA) was prepared from Synechococcus sp. PCC 7002 as described by Bickley and Owen [24]. The 831 bp flanking region upstream and 791 bp flanking region downstream of the psaM gene were amplified from the Synechococcus sp. PCC 7002 gDNA using primer pair b and c, respectively, and the npt cassette, conferring kanamycin resistance, was amplified from the pJHJ08 vector [25], gift from associate professor Yumiko Sakuragi using primer pair a by PCR (primers are listed in Table 1). The fragments were cloned into the pUC57 construct in three consecutive cloning steps at EcoRI, BamHI and BspEI sites as illustrated in Figure S1 to yield the psaM-CYP79A1 fusion construct.

Transformation of cyanobacteria

Transformation of Synechococcus sp. PCC 7002 with the fusion construct to obtain the PsAM-CYP79A1 strain was performed as described by Frigaard et al. [26]. The psaM gene was replaced with the psaM-CYP79A1 gene through homologous recombination. Transformants were selected for kanamycin resistance and segregated for 4 generations before they were screened for the presence of the fusion gene by colony PCR using primer pairs d, e and g (Table 1).

Cyanobacterial strains and culture conditions

Two Synechococcus sp. PCC 7002 strains were used in this study, wild type (WT) and the recombinant PsAM-CYP79A1 strain. Cyanobacterial cells were grown photoautotrophically in medium A [27] containing 1 mg/mL NaNO₃ at 38°C under continuous illumination at 80 μmol·m⁻²·s⁻¹ with aeration by 3% (v/v) CO₂ in air or on A₂ medium. Cell growth was monitored by measuring the absorbance at 730 nm (optical density, OD₇₃₀). The chlorophyll (Chl) content was measured after extraction with 80% acetone as described by Lichtenenthaler [28]. Cultures for experiments and isolation of thylakoids were started at OD₇₃₀ = 0.05. The cells were harvested when they were in the late exponential phase as assessed by measuring the OD₇₃₀.

RNA isolation and RT-PCR

RNA was isolated from 20 mL Synechococcus sp. PCC 7002 cultures grown to the late exponential phase. The cells were harvested by centrifugation at 10,000 × g for 3 min and the pellets were resuspended in 100 μL double-distilled water. 100 μL glass beads and 800 μL TRIzol reagent (Invitrogen) were added and the samples were subjected to 6 rounds of freezing in liquid nitrogen,
thawing and 1 min vigorous vortexing. After 5 min incubation at room temperature (RT), 200 µL chloroform was added and the samples were vortexed for 15 s, and incubated at RT for 3 min. The phases were separated by centrifugation at 12,000 xg for 10 min at 4°C and the upper phases transferred to new tubes.

500 µL isopropanol was added and the samples were incubated for 10 min at RT, and centrifuged at 12,000 xg for 10 min at 4°C. The pellets were washed in 1 mL 70% ethanol by vortexing and centrifugation at 7,500 xg for 5 min at 4°C. The supernatants were discarded and the pellets dried for 15 min before they were dissolved in 100 µL RNase-free water. The RNA was purified using the RNeasy Plant Mini Kit (Qiagen) and the concentrations were measured to be 128 ng/µL for the WT and 202 ng/µL for

Figure 1. Localization of PsaM in PSI and schematic drawing of the thylakoid in the engineered strain. A) and B) The crystal structure of a monomer of photosystem I from the cyanobacterium Thermosynechococcus elongatus seen from the cytoplasmic side of the thylakoid membrane (A) and from the trimer-facing side of the monomer (B). RCSB Protein Data Bank ID: 1JB0. The PsaM subunit is shown in green in both panels and additionally indicated by an arrow in panel A. C) Schematic representation of the PsaM-CYP79A1 fusion protein in the thylakoid membrane. doi:10.1371/journal.pone.0102184.g001

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>aFwd</td>
<td>5’-GGTCCGGAGCCGAAATTCGCTGGG-3’</td>
<td>BspEI</td>
</tr>
<tr>
<td>aRev</td>
<td>5’-GCGGGATCTCAGAAGAATCTCGTAAGAA-3’</td>
<td>BamHI</td>
</tr>
<tr>
<td>bFwd</td>
<td>5’-GCGGATCTCAAACACACTCAGCTTC-3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>bRev</td>
<td>5’-GCGGATCTCAAATTTAAAAAATCATGGCAATATAAAC-3’</td>
<td>EcoRI</td>
</tr>
<tr>
<td>cFwd</td>
<td>5’-GCGGGATCTCAATCTCGACAGAAGCTG-3’</td>
<td>BamHI</td>
</tr>
<tr>
<td>cRev</td>
<td>5’-GCGGATCTCTGTGGATAGGGCCTGA-3’</td>
<td>BamHI</td>
</tr>
<tr>
<td>dFwd</td>
<td>5’-GAAAACGGCAAGGTCTGTA-3’</td>
<td>-</td>
</tr>
<tr>
<td>dRev</td>
<td>5’-ATGTTGGTCAGTTGCGAC-3’</td>
<td>-</td>
</tr>
<tr>
<td>eFwd</td>
<td>5’-AGGCCATCTCTCTCTGAT1GAGAAGAAGAGCCGAGAAGAGAAG-3’</td>
<td>-</td>
</tr>
<tr>
<td>eRev</td>
<td>5’-GGAGGGGAAGAAGCCGAAGAAGAGAAGAGAAG-3’</td>
<td>-</td>
</tr>
<tr>
<td>fFwd</td>
<td>5’-GGCTGCAGCCGAGAAGAAGAGAAGAGAAGAGAAG-3’</td>
<td>-</td>
</tr>
<tr>
<td>fRev</td>
<td>5’-GGCTGCAGCCGAGAAGAAGAGAAGAGAAGAGAAG-3’</td>
<td>-</td>
</tr>
</tbody>
</table>

Restriction sites inserted into the PCR product through the primers are underlined. doi:10.1371/journal.pone.0102184.t001
the PsaM-CYP79A1 using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).

The RNA was prepared for RT-PCR (reverse transcriptase polymerase chain reaction) using the DNase I Amplification Grade Kit (Sigma-Aldrich). cDNA was synthesized from the RNA using the iScript cDNA Synthesis Kit (Bio-Rad) with the primer mixture supplied with the kit (a mixture of oligo(dT) and random hexamer primers). A control sample was made without reverse transcriptase. The cDNA was used as template for PCR using primer pairs e and f (Table 1).

**In vivo PsaM-CYP79A1 activity**

40 mL cultures of WT and PsaM-CYP79A1 were grown as described above. Cells were separated from the growth medium by centrifugation at 5,000 × g for 10 min at 4°C. Metabolites were extracted from the growth medium by vortexing with 1/3 volume of ethyl acetate (EtOAc) three times. The cells were disrupted by sonication (20 cycles of 30 s pulse amplitude 100, 90 s off) in a Q700 sonicator (Qsonica) equipped with a cup horn. Samples were centrifuged at 10,000 × g for 10 min at 4°C and metabolites were extracted from the supernatant into a double volume of EtOAc by thorough vortexing. For both growth medium and cell samples, the EtOAc phase was separated from the water phase by centrifugation at 2,000 × g for 5 min. The EtOAc was evaporated in a Scanpspeed 32 (Scanvac, Labogene). The samples were resuspended in 80% methanol (MeOH), diluted to 20% MeOH in water and subjected to liquid chromatography–mass spectrometry (LC-MS) analysis (final volume 80 µL).

**LC-MS analysis**

Analytical LC-MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Germany) coupled to a Bruker HCT- Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). A Zorbax SB-Aq column (Agilent; 3.5 µM, 2.1 × 150 mm) was used at a flow rate of 0.2 mL min⁻¹. The oven temperature was maintained at 35°C. The mobile phases were: A, 2 mM ammonium acetate; B, methanol. The gradient program was: 0 to 1 min, isocratic 25% B; 1 to 11 min, linear gradient to 60% B; 11 to 12 min, isocratic 98% B; 12 to 20 min, isocratic 25% B. The mass spectrometer was run in positive APCl mode and the recorded mass range was m/z 80-350.

**Isolation of thylakoids and PSI from cyanobacteria**

All steps were carried out in green safelight or dim light with samples kept in the dark at 4°C or on ice. The cells were collected by centrifugation at 7,500 × g for 10 min at 4°C and washed once in 20 mM Tricine-NaOH pH 7.5. The cells were then pelleted again, resuspended in thylakoid buffer (20 mM Tricine-NaOH pH 7.5, 0.4 M sucrose, 10 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 5% (v/v) glycerol, 1× Complete protease inhibitor cocktail (Roche)) and disrupted in a single cycle at 20,000 psi in a TS Series Benchtop cell disrupter (Constant Systems). Unbroken cells were removed by centrifugation at 3,000 × g for 10 min at 4°C and the supernatant was subjected to ultracentrifugation at 180,000 × g for 45 min at 4°C. The pellet, consisting of the thylakoids, was carefully resuspended in thylakoid buffer using a small paintbrush. If not to be used for isolation of PSI, the thylakoids were frozen in liquid nitrogen.

For isolation of PSI, the thylakoids were solubilised in 1% (w/v) n-dodecyl β-D-maltoside (β-DM) at a final concentration corresponding to 1 mg Chl/mL (dilution in thylakoid buffer) by stirring for 30 min on ice. Unsolubilised membranes were removed by ultracentrifugation at 184,000 × g for 30 min at 4°C. Supernatant corresponding to 0.5 mg Chl was applied to 5-step 10–30% sucrose gradients prepared as described by Chitnis and Chitnis (1993) [29]. The sucrose gradients were ultracentrifuged at 270,000 × g for 21 hours at 4°C and green bands were carefully collected using a Pasteur pipette or 0.5 mL fractions were collected using a peristaltic pump.

**In vitro PsaM-CYP79A1 activity measurements**

Thylakoids corresponding to 100 µg Chl were mixed with 2.6 µM [0.25 µCi] L-[-U-14C] Tyrosine (Perkin Elmer) or 0–1000 µM unlabelled tyrosine (Sigma-Aldrich), 0.15 mM spinach Fd, 22.5 µM Synecochococcus sp. PCC 7002 cytochrome c₉ (cyt c₉) expressed in Escherichia coli, 3.6 mM sodium ascorbate, 0.11 mM 2,6-dichlorophenolindophenol (DCPIP), 1 mM dithiothreitol (DTT) and 0.1% (v/v) β-DM in a 20 mM Tricine-NaOH buffer pH 7.5 (total reaction volume 200 µL). Assay samples where incubated at 25°C while illuminated with a Schott KL 1500 light source at 200 µmol m⁻² s⁻¹ for 30 min. The produced oxime was extracted in EtOAc corresponding to twice the reaction volume by vortexing. The EtOAc phase was separated from the assay mixture by centrifugation at 2,000 × g for 5 min, transferred to a new tube and concentrated in a Scanpspeed 32 vacuum concentrator. In experiments using radiolabelled tyrosine as substrate, the sample components were separated by thin layer chromatography (TLC) using a toluene/EtOAc/MeOH (30:8:1 v/v) solvent mixture and a Silica gel 60 F₂₅₄ TLC plate (Merck). A dilution series of L-[-U-14C] Tyrosine was included on the plate to allow quantification. The TLC plate was incubated with a storage phosphor screen for 2 weeks and radiolabelled components were detected and quantified using a Storm 840 PhosphorImager (Molecular Dynamics). In the experiments where unlabelled tyrosine was used as substrate, two samples were prepared for each data point and the EtOAc pooled afterwards. Samples were prepared for and subjected to LC-MS analysis as described above.

**Immunoblot analysis**

Thylakoid proteins (corresponding to 10 µg Chl) and PSI (proteins corresponding to 30 µg Chl) or sucrose gradient fraction samples (proteins from 80 µL of the 0.5 mL fractions collected from the sucrose gradient, concentrated by acetone precipitation by centrifugation at 20,000 × g for 15 min at 4°C in a final concentration of 80% acetone) were subjected to SDS-PAGE on 12% Criterion XT Bis-Tris gels (Bio-Rad) in XT MES Running Buffer (Bio-Rad) at 200 V for 55 min. Proteins were transferred to a Whatman Protran nitrocellulose membrane using a Criterion blotter (Bio-Rad). The membrane was blocked in 5% (v/v) skimmed milk in PBS buffer containing 0.05% (v/v) Tween-20 (PBS-T) for 1 h at RT and incubated overnight with a rabbit CYP79A1 primary antibody (1:2000 dilution, gift from Tomas Laursen) in 1% (w/v) skimmed milk in PBS-T at 4°C. For the sucrose gradient fraction immunoblot, the membrane was cut in three after the blocking step and the pieces were incubated with the CYP79A1 primary antibody, a rabbit PsaC primary antibody (1:1000 dilution, Agrisera) and a rabbit PsaA primary antibody (1:10,000 dilution, Agrisera), respectively. The blot was washed with PBS-T and incubated with a secondary horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG antibody (Dakopatts, 1:5000 dilution in PBS-T) for 1 h at RT. The membrane was washed again in PBS-T and the secondary antibody detected using SuperSignal West Dura Chemiluminescent Substrate (Pierce) with a BioSpectrum Imaging System (UVP).
BN-PAGE analysis

Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was performed by a procedure adapted from the laboratory of Prof. Jörg Nickelsen, Ludwig Maximilians University of Munich [30]. Briefly, thylakoids corresponding to 500 μg protein were pelleted by centrifugation at 20,000 x g for 20 min at 4°C and resuspended in 50 μL ACA buffer (50 mM Bis-Tris, pH 7.0, 750 mM aminocaproic acid, 0.5 mM EDTA). The thylakoids were solubilized by adding 7.5 μL 10% (v/v) β-DM and incubating on ice for 30 min. Samples were centrifuged at 20,000 x g for 30 min at 4°C and supernatant was mixed with 8 μL loading buffer (750 mM aminocaproic acid, 5% (w/v) Coomassie G-250). 25 μL sample was loaded on a NativePAGE Novex 4–16% Bis-Tris Protein Gel (Life Technologies). Gel electrophoresis was performed to confirm that the fusion gene construct had been integrated in the Synechococcus sp. PCC 7002 as positive selection marker, and colony PCR was performed to confirm that the CYP79A1 gene of PsaM subunit of cyanobacterial PSI. A fusion construct was designed by replacing the native membrane-spanning N-terminal domain of the CYP79A1 with the PsaM subunit of cyanobacterial PSI. A fusion gene of PsaM and a codon-optimized version of the sequence encoding the soluble domain of the CYP79A1 were commercially synthesized, and flanking regions of the PsaM gene and a kanamycin resistance cassette were cloned into the construct (Figure S1).

The marine cyanobacterium Synechococcus sp. PCC 7002 was chosen as photosynthetic host organism. Its available genomic sequence and natural transformability facilitate genetic engineering. Additionally, being tolerant to a broad range of environmental conditions and capable of fast photoautotrophic growth with simple nutritional requirements, this cyanobacterium is a good candidate for use in biotechnological applications. Synechococcus sp. PCC 7002 cells were incubated with the DNA construct to allow uptake and the native PsaM gene was replaced with the PsaM-CYP79A1 gene through homologous recombination.

Verification of the presence of PsaM-CYP79A1 in transformed cyanobacteria

Transformants were segregated for 4 generations using kanamycin as positive selection marker, and colony PCR was performed to confirm that the fusion gene construct had been integrated in the Synechococcus sp. PCC 7002 genome. Using primers binding outside the recombined sequence, it was verified that a DNA construct with a size corresponding to the PsaM-CYP79A1 fusion gene and the kanamycin resistance cassette had replaced the native PsaM gene in the transformed strain (Figure 2A and B). Only one band was seen for each colony of the transformed strain, thus indicating that PsaM had been replaced by the fusion construct in all copies of the genome. Moreover, when the forward primer binding site was located in the CYP79A1 part of the fusion gene or in the kanamycin resistance cassette, colony PCR products were only obtained for the transformed strain (data not shown).

RT-PCR was used to investigate if the PsaM-CYP79A1 fusion DNA was transcribed in the cyanobacteria (Figure 2A and C). RNA was purified from WT and transformed cultures and used for cDNA synthesis in the presence and absence of reverse transcriptase. PCR using primer pair e with both the forward and reverse primer binding in the PsaM part of the fusion gene resulted in a product in both WT and PsaM-CYP79A1 samples. When primer pair f with the reverse primer binding in the CYP79A1 part of the fusion gene was used, only the sample with the transformed strain resulted in a PCR product. In control PCR samples run in the absence of reverse transcriptase, no bands were seen (data not shown).

The presence of expressed PsaM-CYP79A1 fusion protein in thylakoids and trimeric PSI was confirmed by BN-PAGE purified from the transformed Synechococcus sp. PCC 7002 was analysed by immunoblotting. As positive control, full-length CYP79A1 (~62 kDa) expressed in Nicotiana benthamiana chloroplasts was used. A band of approximately the same size, corresponding to the expected size (~61 kDa) of the fusion of PsaM and the soluble domain of the CYP79A1, was observed in both thylakoids and trimeric PSI of the PsaM-CYP79A1 cyanobacteria, whereas no signal was detected in the WT samples, thus indicating that the PsaM-CYP79A1 fusion was expressed in the transformed cyanobacteria (Figure 2D) and present in at least a fraction of the trimeric PSI complexes.

PsaM-CYP79A1 localization

Having established that the fusion protein is expressed we analysed the localisation of the protein in more detail. In the immunoblot analysis in Figure 2D, the samples contained thylakoids and PSI trimers corresponding to 10 and 50 μg Chl, respectively. The thylakoid sample thus contained much less PSI complexes than the PSI trimer sample. A stronger signal was detected from the thylakoid sample, indicating that a large part of the PsaM-CYP79A1 fusion present in the thylakoids is not associated with the PSI trimers after purification. This was confirmed in an independent experiment by analysing the presence of PSI, PSII and CYP79A1 by immunoblot analysis of representative fractions of a sucrose gradient separating solubilized membrane components of the thylakoids using antibodies against the PSI-subunit PsAl, the PSII-subunit PsbA and the CYP79A1 (Figure 3). While a small fraction of the PsaM-CYP79A1 co-migrated with the PSI trimers, most PsaM-CYP79A1 was detected in fractions of the sucrose gradient shifted towards smaller complexes than the photosynthetic protein complexes (Figure 3A). This indicates that the PsaM-CYP79A1 fusion is not firmly attached to the PSI trimers nor monomers. It is possible that the PsaM-CYP79A1 fusion is associated with PSI in the intact membranes, but most PsaM-CYP79A1 is not bound to PSI upon detergent-mediated solubilisation of the thylakoid membrane.

An increase in the abundance of the PSI monomer band was observed in the sucrose gradient with thylakoids from the PsaM-CYP79A1 strain compared to the gradient prepared from wild type thylakoids (Figure 3B). This increase in PSI monomers in the engineered strain was likewise verified by BN-PAGE (Figure 3C).

In vivo PsaM-CYP79A1 activity

The in vivo enzymatic activity of CYP79A1 in the engineered PsaM-CYP79A1 strain was investigated by analysing extracts of cyanobacteria cells and growth medium by LC-MS. WT and PsaM-CYP79A1 cultures were grown to an OD 730 of 2.5 before they were harvested and metabolites from the cyanobacteria cells and the growth medium were extracted in ethyl acetate and methanol. The samples were analysed by LC-MS in APCl mode. Extracted ion chromatograms (EIC) were created corresponding to [M+H]+ (m/z 152) for p-hydroxyphenylacetaldoxime. The results are shown in Figure 4.
Only the PsaM-CYP79A1 samples contained a product with retention times identical to those of the oxime standard, thus clearly demonstrating that the engineered cyanobacterial strain can convert tyrosine into the corresponding oxime and that the PsaM-CYP79A1 fusion is enzymatically active in vivo in cyanobacteria (Figure 4A). Interestingly, the oxime was detected almost exclusively in the growth medium, implying that the product is excreted from the cyanobacteria (Figure 4B). At OD730 = 2.5, the oxime content was found to be approximately 4 ng/mL culture in the growth medium of the PsaM-CYP79A1 strain. The expression and enzymatic activity of the PsaM-CYP79A1 fusion protein in the cyanobacteria had no apparent impact on growth of the cyanobacteria cells compared with the wild type (data not shown).

PsaM-CYP79A1 activity in isolated thylakoids

Having demonstrated that the PsaM-CYP79A1 fusion enzyme was active in vivo, we now turned to in vitro studies with purified thylakoids. Enzymatic activity assays were performed to verify that the oxime production by the PsaM-CYP79A1 fusion indeed was light-driven. Isolated thylakoids were incubated with tyrosine (radiolabelled or unlabelled) in the presence of electron donors for PSI and Fd for mediation of electron transport from PSI to the CYP79A1 [2,13]. The activity assay was performed under illumination in the presence of detergent to disrupt the thylakoids and allow access of the added electron donors to the oxidizing site of PSI. After completion of the assay, hydrophobic metabolites were extracted from the assay mixture with ethyl acetate. The components of the samples prepared with radiolabelled tyrosine as substrate were separated by TLC and analysed by autoradiography using storage phosphor screens. Unlabelled tyrosine samples were analysed by LC-MS. Oxime produced in the assays was identified through comparison with an oxime standard.

Figure 5A shows the in vitro formation of radiolabelled oxime by WT and PsaM-CYP79A1 thylakoids in the light or in the dark. Only the PsaM-CYP79A1 thylakoids were able to produce oxime.
Oxime formation was only seen in light-incubated samples with a 27 fold increase in signal compared to dark-incubated samples, thereby demonstrating that the oxime formation was light-driven and indicating that the PsaM-CYP79A1 was indeed fuelled with electrons delivered from PSI.

Figure 5B shows the oxime production in PsaM-CYP79A1 thylakoids as a function of tyrosine concentration. An increase in the production level was seen up to a concentration of 0.5 mM tyrosine. At higher concentrations, the PsaM-CYP79A1 appears to be substrate inhibited. At a substrate concentration of 0.5 mM...
tyrosine, approximately 12 ng oxime was produced by thylakoids corresponding to 100 μg Chl during a 30 min assay.

**Discussion**

**Light-driven oxime production by an engineered cyanobacterium**

In this work, we have genetically linked the catalytic domain of the CYP79A1 from the plant *S. bicolor* to the PSI subunit PsAM in *Synechococcus* sp. PCC 7002 with the aim to target the CYP79A1 to the vicinity of PSI to obtain light-driven P450 biosynthesis. The PsAM-CYP79A1 fusion protein was found to be located in the thylakoids of the cyanobacterial host with a smaller fraction directly attached to the PSI complex. The fusion protein was functional in vivo with the CYP79A1 enzymatic activity being sustained by endogenously produced tyrosine and the product, p-hydroxyphenylacetoxime, being excreted into the growth medium (Figure 4). Through in vitro assays, the enzymatic reaction was confirmed to be light-driven, indicating that the electrons powering the PsAM-CYP79A1 catalytic cycle were indeed photosynthetic electrons delivered from PSI (Figure 5).

Cytochrome P450s are key enzymes in the biosynthesis of the majority of the numerous bioactive specialized metabolites with medicinal properties produced by plants. For many of these compounds, the biosynthesis in the plants is tightly regulated with the production levels often being low or highly variable, dependent on induction by abiotic or biotic factors and confined to specific growth stages and cell types, thus making extraction, purification and separation from structurally similar compounds a challenge [3,4,8,9]. This study demonstrates that it is possible to express the P450s in the thylakoids of a cyanobacterium to obtain a light-driven production system as an environmentally friendly alternative to production through chemical synthesis.

As the P450 fold is highly conserved, and since delivery of electrons from PSI to both the CYP79A1 from *S. bicolor* and the CYP124 from *M. tuberculosis* via Fd have been shown to function in vitro, it is probable that the light-driven biosynthesis approach is generally applicable to a variety of P450s [13,21,31].

In addition to uncoupling from the tight metabolic regulation that the enzyme is often subjected to in the native host, an advantage of heterologous expression in a microorganism is the potential for future scale-up of production to an industrial level. The commercial use of cyanobacterial production systems is still in its developing phase, but recently cyanobacteria have attracted interest due to their green biindustrial potential. The simple nutritional growth requirements, constituting only water, CO₂ and a few mineral nutrients, with sunlight as the only necessary energy source, suggest a potential for inexpensive and renewable production of for instance biofuels and valuable natural products. Cyanobacterium-based production systems thus offer an alternative to the carbohydrate-fed approaches necessary for the microorganisms primarily employed in biotechnology [32–35].

The oxime produced by the PsAM-CYP79A1 in this study was excreted into the growth medium, a phenomenon that has also earlier been observed for heterologous production of caffeic acid via Fd [36,37]. However, more knowledge about the secretion mechanism is needed before we can take full advantage of this phenomenon.

**Electron partitioning and fusion construct design**

For the cyanobacterium to be viable while supplying electrons for the PsAM-CYP79A1, electron delivery should be possible not only to the P450, but also to other processes in the thylakoid environment dependent on reduction by Fd. It was thus decided to attempt to obtain direct electron transfer from the terminal Fe₄S₄ clusters of PSI to the CYP79A1, as has been the aim for in vitro coupling of hydrogenases or metal catalysts to PSI, but rather to rely on electron transfer via Fd [38–42]. It was chosen to anchor the CYP79A1 to the peripheral PsAM subunit of PSI where the P450 would be expected not to prevent access of Fd to its docking site at PSI [22]. Though most CYP79A1 appeared not to be directly associated with PSI, at least after purification, the engineered CYP79A1 was found to be located in the thylakoids and active without its native membrane anchor, demonstrating the
possibility of engineering P450s without inactivating the enzymatic capability. This suggests that it may be possible to develop other P450 fusion proteins for this light-driven setup, possibly providing increased production levels through improved competitiveness for reduced Fd.

Association of PsaM-CYP79A1 and PSI?

From the immunoblot analysis in Figure 2 and 3, the main part of the PsaM-CYP79A1 fusion protein was found not to co-fractionate with the PSI complex. It could be that most PsaM-CYP79A1 has never been associated with PSI, but it is also possible that the PsaM-CYP79A1 is loosely connected to PSI in the intact membranes, but that the complexes disintegrate upon membrane solubilisation. The added size of the CYP79A1 domain on the PsaM protein could result in steric hindrances and thereby weakened interactions between the PsaM and the remaining PSI complex.

The PsaM subunit has previously been found to stabilize, but not be strictly needed, for the assembly of PSI trimers in a psaM– mutant of Synechocystis sp. PCC 6803 [43]. The increased abundance of PSI monomers observed following expression of PsaM-CYP79A1 (Figure 3) could thus be explained by a reduction in the number of stable trimers due to the absence of the PsaM-CYP79A1 fusion protein in a majority of the PSI complexes. Alternatively, trimers containing the PsaM-CYP79A1 could be present in the membrane, but the inter-complex interactions weakened due to the CYP79A1, causing the unstable trimers to fall apart during purification.

No impact on growth was detected for the PsaM-CYP79A1 strain compared to the WT despite the observed destabilization or reduced abundance of PSI trimers in the thylakoids of the PsaM-CYP79A1 strain. It is to be noted that normal growth was also observed in the Synechocystis sp. PCC 6803 psaM– mutant [43].

Increasing the yield of light-driven biosynthesis

The compound produced by light-driven biosynthesis in this project, α-hydroxyphenylacetaldoxime, is chosen for proof-of-concept. However, for a compound produced for a commercial application, the product titer would have to be increased from the current production level of ng/mL culture to be of industrial relevance. Key parameters in this context will be optimization of the expression level of the enzymes in the pathway, the supply of substrate and delivery of electrons to the P450s. Upregulation of the expression of Fd may lead to an increased supply of electrons to the P450s, and engineering of the P450 and/or Fd to increase the affinity of the two proteins for each other may also increase the ability of the P450 to compete for reduced Fd with FNR. Another approach that earlier has proved to yield an increase in production for a P450 was co-expression of a cytochrome b5, which can stimulate the P450 reaction rate through several possible mechanisms [44,45].

The flavodiiron proteins Flv1 and Flv3 have been shown to function as a sink for excess electrons produced in photosynthesis in cyanobacteria, reducing O2 to water without production of reactive oxygen species [46]. As the light-driven approach can also be seen as an additional electron sink, it could be speculated that knocking down those flavodiiron proteins would increase the need for an alternative sink and that this perhaps could lead to an increased supply of electrons to the P450s.

For a pathway containing multiple enzymes, the flux through the pathway will be important to optimize. This can be approached by adjusting the relative expression level of the enzymes, e.g. by changing promoters and ribosome-binding sites, and increasing the product channelling, which may be improved e.g. by scaffolding of the enzymes [47–49]. Balancing the protein expression can however be a challenge, as engineering of cyanobacteria is still not as well established as engineering of classic model microorganisms such as E. coli, which has a less complex metabolism, and even characterized genetic elements can result in unpredictable expression levels [50].

Conclusion and Perspectives

In this study, we have engineered a fusion enzyme of the catalytic domain of a plant P450 and a PSI subunit and expressed it in Synechococcus sp. PCC 7002. The PsaM-CYP79A1 fusion enzyme is present in the thylakoids and proved to be functional with light-driven enzymatic activity detected both in vivo and in vitro. The biosynthesized oxime was found to be excreted into the growth medium, enabling easy product isolation. These works demonstrate the possibility of functionally coupling plant enzymes requiring electrons in their reactions to PSI and utilize the reducing equivalents generated in the photosynthetic electron transport chain to obtain light-driven biosynthesis in photosynthetic microorganisms.

It is envisioned that the approach presented here can be developed as a light-driven and environmentally friendly production system for bioactive compounds relevant to the pharmaceutical or chemical industries. In contrast to most enzymes used as biocatalysts in vitro for industrial purposes, P450s require the costly cofactor NADPH and a reductase for electron donation, an issue that is overcome in the light-driven in vivo system presented here [51]. Cyanobacteria can be efficiently cultivated at industrial scale and the use of a cyanobacterial host thus provides the potential for future scale-up of the system.

Supporting Information

Figure S1 Cloning of the PsaM-CYP79A1 fusion construct. Using PCR, a kanamycin resistance cassette was amplified from a vector containing the cassette [pJHJ08, [25]) and flanking regions of the psaM gene were amplified from Synechococcus sp. PCC 7002 gDNA. Primer sequences are shown in Table 1. The PCR products were inserted in a commercially synthesized fusion construct of the psaM and CYP79A1 genes contained in the pUC57 cloning vector. The final construct was transformed into Synechococcus sp. PCC 7002 for replacement of the native psaM gene by homologous recombination. (TIF)

Author Contributions

Conceived and designed the experiments: LML AZN KJ BLM PEJ. Performed the experiments: LML CEO. Analyzed the data: LML AZN PEJ. Contributed reagents/materials/analysis tools: WB. Contributed to the writing of the manuscript: LML AZN PEJ.

References


