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Light-Driven Cytochrome P450 Hydroxylations

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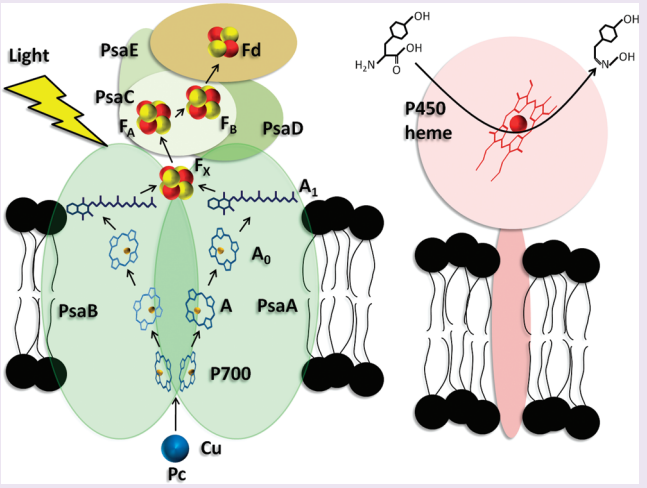
Supporting Information

ABSTRACT: Plants are light-driven "green" factories able to synthesize more than 200,000 different bioactive natural products, many of which are high-value products used as drugs (*e.g.*, artemisinin, taxol, and thapsigargin). In the formation of natural products, cytochrome P450 (P450) monooxygenases play a key role in catalyzing regio- and stereospecific hydroxylations that are often difficult to achieve using the approaches of chemical synthesis. P450-catalyzed monooxygenations are dependent on electron donation typically from NADPH catalyzed by NADPH-cytochrome P450 oxidoreductase (CPR). The consumption of the costly cofactor NADPH constitutes an economical obstacle for biotechnological *in vitro* applications of P450s. This bottleneck has been overcome by the design of an *in vitro* system able to carry out light-driven P450 hydroxylations using photosystem I (PSI) for light harvesting and generation of reducing equivalents necessary to drive the P450 catalytic cycle. The *in vitro* system is based on the use of isolated PSI and P450 membrane complexes using ferredoxin as an electron carrier. The turnover rate of the P450 in the light-driven system was 413 min^{-1} compared to 228 min^{-1} in the native CPR-catalyzed system. The use of light as a substitute for costly NADPH offers a new avenue for P450-mediated synthesis of complex bioactive natural products using *in vitro* synthetic biology approaches.



P450s play a key role in catalyzing regio- and stereospecific hydroxylations of complex chemical structures that are often difficult to achieve using the approaches of chemical synthesis. The catalytic cycle of P450s is dependent on two single electron transfers facilitated by a number of different electron carriers. Some P450s depend on a specific electron carrier, whereas others are more promiscuous.[1] The vast majority of eukaryotic P450s is localized in the endoplasmic reticulum (ER) membrane and is strictly dependent on the ER-localized CPR for activity. The membrane-bound CPR is a diflavin protein catalyzing stepwise one electron transfers from NADPH *via* the FAD- and FMN-cofactors to the heme iron of the P450. CPR has evolved from ancestral gene fusions of flavodoxin (Fld) and ferredoxin-NAD(P)H oxidoreductase (FNR). The N-terminal FMN-binding domain of CPR shows strong structural homology to Fld while the C-terminal FAD- and NADPH-binding domains are structurally and functionally homologous to FNR.[2]

Photosynthesis in plants and cyanobacteria mediates light-driven electron transport from water to $NADP^+$. In plants, the two photosystems involved are localized in the thylakoid membrane within the chloroplast. Photosystem I (PSI) catalyzes light-driven electron transport from plastocyanin in the thylakoid lumen to $NADP^+$ in the stroma. Electrons are transferred stepwise from the P700 reaction center (a chlorophyll *a* dimer) to A_0 (a chlorophyll *a* molecule) and A_1 (a phylloquinone) and from there to the terminal electron acceptors, the [4Fe-4S] clusters F_X, F_A, and F_B. In the P700 reaction center, A_0, A_1 and F_X are bound to the chlorophyll binding heterodimeric reaction center proteins PsaA and PsaB,[3] whereas F_A and F_B are bound by the stromal localized PsaC subunit.[4] The PsaC subunit binds to the PsaA/B heterodimer and mediates binding to PsaD.[5] The soluble [2Fe-2S] iron−sulfur protein ferredoxin (Fd) binds to the stromal localized PsaC, PsaD, and PsaE[6] subunits, and further electron transport proceeds *via* Fd to the FAD-binding protein FNR, which catalyzes the reduction of $NADP^+$ to NADPH.[7] In prokaryotes and some oceanic red and green algae, the small soluble FMN-binding protein flavodoxin is able to substitute for Fd as electron carrier between PSI and FNR during iron starvation.[8,9] PSI generates the most negative redox potential in nature (−1.2 V) and operates with an unprecedented quantum yield close to 1.0, unmatched by any other known biological or chemical system.[10] This makes PSI an excellent energy transducing source to drive enzymatic reactions dependent on a strong reductant. Previously, this has been attempted directly by cross-linking of hydrogenase to PSI or indirectly by combining

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Table 1. Components Required for Light-Driven CYP79A1 Hydroxylations[a]

	components						
electron mediators	light	Fd	FNR	isiB	$NADP^+$	CPR	pmol oxime produced min^{-1} (pmol CYP79A1)$^{-1}$
Fd + FNR + CPR[c]	X	X	X		X	X	1.1 ± 0.02
Fd + FNR + CPR[c]		X	X		X	X	n.d.[b]
Fd + CPR	X	X				X	2.7 ± 0.1
Fd	X	X					2.9 ± 0.1
Fd		X					n.d.[b]
Fld(isiB) (high concn)	X			X			1.0 ± 0.1
Fld(isiB) (low concn)	X			X			0.04 ± 0.001
Fld(isiB) (high concn)				X			n.d.[b]
Fd (no PSI)	X	X					n.d.[b]
Fd (no CYP79A1)	X	X					n.d.[b]

[a] All experiments were carried out in TNM buffer, 84 pmol PSI and 1.4 pmol CYP79A1 in a total volume of 200 μL. A total amount of 76 pmol Fd, 79 pmol CPR, and 1944 pmol (high concn) or 194 pmol (low concn) Fld(isiB) was used in the experiments. Experiments were done at a light intensity of 151 μmol quanta m^{-2} s^{-1} and L-[U-^{14}C]tyrosine concentration was 0.5 nmol (0.25 μCi). [b] None detected. [c] Including 0.5 mM $NADP^+$.

isolated NADPH-producing chloroplasts with a purified enzymatic system.[11−13]

In eukaryotes, the P450s are mainly localized in the ER, whereas the photosynthetic machinery resides in the chloroplast. Nevertheless, the presence of similar protein domains and electron transport cofactors within the two protein complexes suggested the possibility to link the systems directly if assembled *in vitro*. In this paper, we demonstrate the design of an *in vitro* system based on isolated PSI and P450 membrane protein complexes capable of mediating light-driven P450-catalyzed hydroxylations. The studies were carried out using CYP79A1, which catalyzes the conversion of L-tyrosine to (*E*/*Z*)-*p*-hydroxyphenylacetaldoxime (oxime) in the biosynthesis of the cyanogenic glucoside dhurrin.[14]

Efficient electron donation to P450s is a prerequisite for its optimal activity. The ability of soluble electron carrier proteins to mediate electron transfer between isolated PSI and P450 complexes was examined. Fld(isiB) from the photosynthetic cyanobacterium *Synechococcus* sp. PCC 7002 and Fd from spinach were found to mediate $NADP^+$ photoreduction, but Fld(isiB) at a 10 times lower molar rate compared to Fd (Supplementary Table 1), indicative of more efficient interactions between PSI and Fd than between PSI and Fld(isiB).

Electron transport from CPR to P450 is dependent on the presence of NADPH.[15] To test the possibility to achieve PSI-mediated photoreduction of $NADP^+$ at a sufficient rate to saturate the demand of the P450 for reducing equivalents, an NADPH regenerating system composed of PSI, Fd or Fld(isiB), FNR, $NADP^+$, and CPR was incubated with CYP79A1. Upon irradiation, the rate of $NADP^+$ photoreduction observed was sufficient to sustain CYP79A1 activity. As summarized in Table 1, the CYP79A1-catalyzed hydroxylation of L-tyrosine to oxime was dependent on irradiation with no CYP79A1 activity being detected in absence of light. Surprisingly, elimination of FNR and $NADP^+$ from the system was observed to significantly increase CYP79A1 activity. This suggested that electron transfer from PSI to CYP79A1 could be facilitated either by a direct interaction between Fd and CYP79A1 or by reduction of CPR through electron donation from Fd to the FNR-like domain of CPR, followed by electron transfer from CPR to CYP79A1. Elimination of either CPR or Fd showed that only Fd was needed to maintain CYP79A1 activity. This demonstrated that reduced Fd is able to serve as a direct electron donor to CYP79A1 (hereafter referred to as the PSI-CYP79A1 system) (Table 1). Light-dependent CYP79A1 activity was also established using Fld(isiB) as an electron carrier between isolated PSI and CYP79A1, although Fld(isiB) was found to be less capable of supporting CYP79A1 activity in comparison to Fd, even at a 25 times higher concentration of Fld(isiB) compared to Fd (Figure 1, panel a). The low CYP79A1 activity using Fld(isiB) as electron carrier was puzzling, as Fld(isiB) was envisioned to serve as a good electron donor to CYP79A1 on the basis of the structural similarity between Fld(isiB) and the P450-interacting FMN-binding domain of CPR.[2,16] When Fld(isiB) was added to the reconstituted native CPR-CYP79A1 system, a clear inhibitory effect on the activity was seen (Figure 1, panel b). The inhibitory effect of Fld(isiB) was evident even at 100 times lower concentration of Fld(isiB) than used in the PSI-CYP79A1 system and could be explained by high affinity binding of Fld(isiB) to CYP79A1, similar to the previously reported high affinity observed for Fld(FldA) and P450c17.[17] In contrast, increased Fd concentrations resulted in a corresponding linear increase in CYP79A1 activity. The increase was linear up to a 1000-fold excess concentration of Fd compared to CYP79A1 and approximately 25-fold to PSI (Figure 1, panel c), demonstrating that electron shuttling by Fd between PSI and CYP79A1 is the rate limiting step. Thus Fd and, less efficiently, Fld(isiB) were found to be functional in shuttling electrons directly from isolated PSI to the CYP79A1 complexes, thereby describing the first functional *in vitro* system using light-driven electron transport to sustain P450 activity in the absence of NAD(P)H.

The experiments identified Fd as the electron carrier of choice in the PSI-CYP79A1 system. The light-driven PSI-CYP79A1 system was further optimized, characterized, and compared to the reconstituted CPR-CYP79A1 system. In these experiments, different substrate concentrations were used. This prevents a direct comparison of the observed rates of oxime formation between experiments. To clarify, the expected rates of oxime formation at the given substrate concentration were calculated on the basis of the hyperbolic fit of the K_M/V_{max} curve (Figure 2, panel c) and listed in the legends of the different figures to demonstrate the coherency. Oxime production by the PSI-

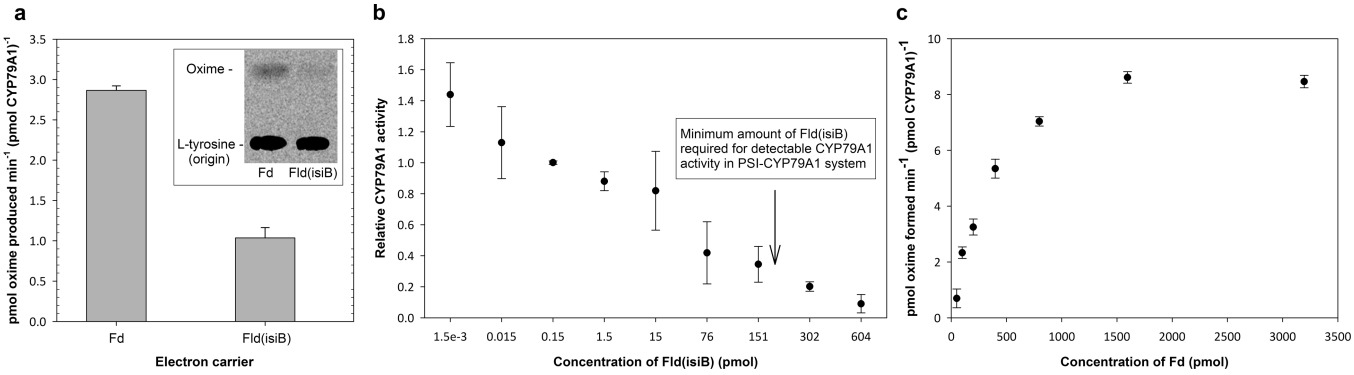

Figure 1. Ferredoxin and flavodoxin can mediate electron transport between photosystem I and CYP79A1. (a) CYP79A1 activity was monitored by the enzyme-catalyzed conversion of L-tyrosine (0.5 nmol L-[U-^{14}C]tyrosine (0.25 μCi)) to (*E*/*Z*)-*p*-hydroxyphenylacetaldoxime (oxime). Light-induced electron transfer from photosystem I to CYP79A1 *via* either ferredoxin (Fd) or flavodoxin (Fld(isiB)) was required for CYP79A1 activity, but Fd was able to support a 3 times faster rate of CYP79A1 activity. The assay was performed at 25 °C at a light intensity of 151 μmol quanta m^{-2} s^{-1}, in the presence of either 76 pmol Fd or 1944 pmol Fld(isiB), respectively. The total amount of radioactively labeled oxime was visualized by TLC (insert) and quantified using autoradiography. (b) By increasing the concentration of Fld(isiB), a clear inhibitory effect on CYP79A1 activity in the reconstituted CPR-CYP79A1 system was observed. CYP79A1 (1.4 pmol) was incubated (30 min, 25 °C) with 519 pmol L-[U-^{14}C]tyrosine (0.25 μCi), 198 pmol NADPH-cytochrome P450 reductase, and 0−604 pmol Fld(isiB). The relative activities were normalized to CPR-CYP79A1 assays performed without Fld(isiB), but at identical concentrations of glycerol. (c) PSI-CYP79A1 activity is dependent on Fd concentration. Assays were carried out in Tricine buffer, pH 7.5 (25 °C, 3 min) with 50−3195 pmol of Fd present and using a total amount of 0.5 nmol (0.25 μCi) L-[U-^{14}C]tyrosine.

CYP79A1 system was found to proceed only during irradiation, and by using periodic light excitation of PSI, it was possible to turn oxime production on and off. No adverse effect on the rate of oxime production in the PSI-CYP79A1 system was observed by using periodic light treatment (Figure 2, panel a). Light intensity was found to be a crucial factor both in generating a continuous and stable production of oxime and in controlling reaction rates (Figure 2, panel b). Light intensities in the mmol quanta m^{-2} s^{-1} range quickly resulted in photoinhibition of PSI (*e.g.*, destruction of the reaction-center chlorophylls by reduced oxygen species), in agreement with previously published results.[18] The highest rate of oxime production was found at a light intensity around 150 μmol quanta m^{-2} s^{-1}. Lower light intensities (<151 μmol quanta m^{-2} s^{-1}) lead to a continuous linear production of oxime, but at lower rates, indicating that electron transfer rate between PSI and CYP79A1 was not saturated. K_M and V_{max} values were determined for the PSI-CYP79A1-catalyzed conversion of L-tyrosine to oxime with Fd as electron carrier (Figure 2, panel c). No apparent change was observed in regards to CYP79A1 substrate binding as the obtained K_M of 0.20 mM was similar to the previously determined K_M of 0.21 mM for the CPR-CYP79A1 system.[14] The turnover of the PSI-CYP79A1 system was 413 min^{-1}, twice as high as the reported value of 228 min^{-1} for the CPR-CYP79A1 system.[14] Inclusion of Fld(isiB) in the PSI-CYP79A1 assay in the presence of Fd as electron carrier had adverse effect on V_{max} for oxime production, which was lowered from 2.90 to 2.28 μM min^{-1}, whereas the apparent K_M was unaffected by Fld(isiB) (Figure 2, panel c). This demonstrated that, at the Fld(isiB) concentrations previously determined to be required for detectable NADP$^+$ photoreduction (Supplementary Table 1), Fld(isiB) inhibited reconstituted CYP79A1 activity. The observed decrease in V_{max} shows that the rate of oxime production is reduced and indicates that Fld(isiB) most likely functions as a noncompetitive inhibitor.

Quantitative comparisons of the PSI-CYP79A1 system with the CPR-CYP79A1 system using identical concentrations of Fd and CPR showed an apparent 10% higher rate of oxime production min^{-1} by the PSI-CYP79A1 system compared to the CPR-CYP79A1 system (Figure 2, panel d). The 10% difference between the systems is significantly lower than the percentage difference in turnover (413 min^{-1} compared to 228 min^{-1}). However, the difference can be explained by the lower substrate concentration used in these experiments as opposed to the saturating substrate concentration used for calculating turnover. Both assays were performed using an excess of reducing equivalents (PSI or NADPH, respectively). When CPR is used as electron carrier protein, P450 activity has been shown to depend on assembly of a binary complex between CPR and P450. CPR-P450 binary complex formation is governed by the concentration of the limiting enzyme component.[19] It is possible to increase CPR-P450 binary complex formation by employing a higher ratio of CPR relative to P450, ultimately resulting in a higher turnover.[19,20] The need for a stoichiometric CPR:P450 ratio higher than 1 most likely reflects that CPR needs to be in an open conformational stage carrying a reduced FMN cofactor to be able to donate electrons to P450s and that only a fraction of the total amount of CPR added is found in this reduced conformational state.[21] To prevent CPR from being the limiting factor for CYP79A1 activity, the comparison of the PSI-CYP79A1 and CPR-CYP79A1 systems was done at saturating concentrations (~300 times excess) of CPR. CPR is most likely the most efficient electron donor for CYP79A1 compared to Fd, as P450 turnover rates peak at a CPR:P450 ratio around 10:1 whereas a higher ratio of Fd to CYP79A1 is needed for similar catalytic rates as seen for the CPR-CYP79A1 system (Figure 1, panel c and Figure 2, panel d).[19,20] Nevertheless, the PSI-CYP79A1 system is able to support a higher turnover of CYP79A1 because CYP79A1 activity increases in a linear manner with Fd concentration (Figure 1, panel c). This linear dependence on Fd concentration provides the opportunity to significantly increase the turnover number of CYP79A1 when it is part of the PSI-CYP79A1 system compared to when CYP79A1 is present in the CPR-CYP79A1 complex. The high ratio of CPR or Fd to CYP79A1 could result in a large population of reduced CPR or Fd, leading to uncoupling. The diffusible reduced oxygen species of superoxide and peroxide formed can be employed as a source of electrons for

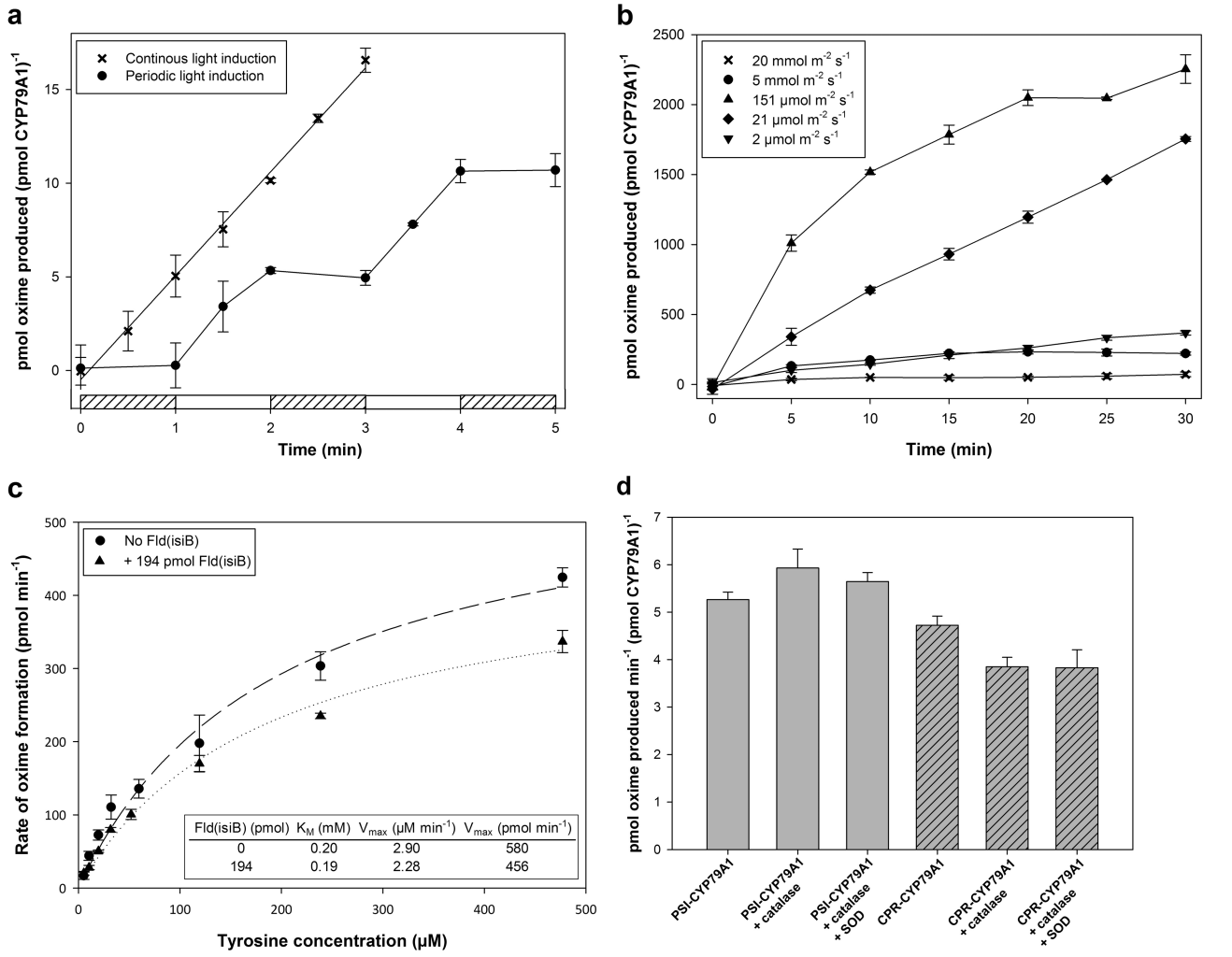

Figure 2. Characterization of the PSI-CYP79A1 system using ferredoxin as electron carrier between PSI and CYP79A1. All experiments were carried out using 1.4 pmol of CYP79A1 and 399 pmol of Fd. (a) Activity of the PSI-CYP79A1 system is strictly light-dependent. Two times 1 min periodic light induction of PSI at a light intensity of 151 μmol quanta m^{-2} s^{-1} show that CYP79A1 hydroxylation of L-tyrosine into oxime is light-dependent in the PSI-CYP79A1 system (●). The rate of oxime formation was similar to what was observed during continuous light induction (×). Experiments were done using 0.5 nmol of L-[U-^{14}C]tyrosine (0.25 μCi in each experiment), corresponding to an expected rate of oxime formation of 5.4 pmol min^{-1} (pmol CYP79A1)$^{-1}$ based on the hyperbolic fit of the K_M/V_{max} curve. Crossed bars at the horizontal axis indicate no light, open bars indicate light. (b) Light is required for PSI to deliver a continuous supply of reducing equivalents to CYP79A1. The light intensity can control the rate of CYP79A1 activity, but excess light results in the onset of photoinhibition of PSI[18] and no further electron transfer from PSI to CYP79A1. PSI is quickly inhibited at high light intensities, whereas a continuous PSI activity at low light intensities can support a high CYP79A1 turnover.[18] Experiments were done using 54 nmol L-tyrosine (0.5 μCi in each experiment), which on the basis of the hyperbolic fit of the K_M/V_{max} curve correlate to 240 pmol oxime produced min^{-1} (pmol CYP79A1)$^{-1}$. (c) K_M and V_{max} of the PSI-CYP79A1 system using Fd as electron carrier. Addition of flavodoxin to the PSI-CYP79A1 system clearly shows an inhibitory effect on V_{max}. The assay mixture contained 1−95 nmol of L-[U-^{14}C]tyrosine (0.5 μCi in each experiment) ± 194 pmol Fld(isiB). (d) Comparison of the light-driven PSI-CYP79A1 system with the CPR-CYP79A1 system. Equivalent concentrations of electron carriers (399 pmol Fd or 415 pmol CPR) were used in the PSI-CYP79A1 or CPR-CYP79A1 assays, respectively. PSI (84 pmol) and NADPH (1.2 μmol) were added in excess to ensure that the measured rate of oxime formation was solely dependent on the ability of Fd or CPR to donate electrons to CYP79A1. In addition, the significance of uncoupling between CYP79A1 and either CPR or Fd was evaluated by addition of catalase and superoxide dismutase. A total amount of 0.5 nmol of L-[U-^{14}C]tyrosine (0.25 μCi) was used, giving an expected rate of oxime formation of 5.4 pmol min^{-1} (pmol CYP79A1)$^{-1}$.

P450 hydroxylation reactions.[22] Addition of catalase and superoxide dismutase showed a significant reduction in CYP79A1 activity in the CPR-CYP79A1 system, indicating that uncoupling is partly responsible for the observed CYP79A1 activity (Figure 2, panel d). For the PSI-CYP79A1 system, presence of catalase and superoxide dismutase increased CYP79A1 activity, probably by reducing the rate of photoinhibition of PSI.[23]

In this study we have designed an *in vitro* system based on isolated protein complexes able to carry out light-driven P450 hydroxylations using PSI for light harvesting and as electron donor. The system is based on combining isolated proteins that in nature are localized in different compartments of the cell. In general, *in vitro* reconstitution of P450 activity requires a reductase and reducing equivalents, often derived from NADPH. In the PSI-CYP79A1 system, Fd was used as electron carrier between PSI and CYP79A1, circumventing the need for NADPH and the P450 reductase. The PSI-CYP79A1 system was found to have a turnover rate of 413 min^{-1}, twice the rate of the CPR-CYP79A1 system (Figure 2, panel c). Other plant P450s have been found to have 5−10 times or even lower turnover rates than

CYP79A1, for example, CYP703A2 and CYB83B1 have turnover rates of 20 and 53 min^{-1}, respectively.[24,25] That the light-driven system is able to improve the natural high turnover rate of CYP79A1 illustrates the potential gain from coupling P450s directly to an efficient electron donor such as PSI. Another interesting approach toward the same goal has been covalent attachment of a photosensitizer to CYP102A1, thereby enabling direct photooxidation of CYP102A1.[26]

The PSI-CYP79A1 system is based on isolated membrane proteins solubilized by nonionic detergents (*n*-dodecyl-*β*-D-maltopyranoside for PSI[27] and Triton X-100 for CYP79A1[28]) and using the soluble protein Fd as an electron carrier. Next generation further improved systems may be envisioned obtained by direct anchoring of the soluble domain of the P450 to one of the subunits of the PSI complex which might serve to further increase P450 activity. The latter design would however involve the design of a separate fusion protein for each P450 to be used. The PSI-CYP79A1 system used here would be expected to work with a number of different P450s because of the evolutionary conserved surface charge distribution of the P450s.[29] The PSI-CYP79A1 system is thus expected to be a modular system where the CYP79A1 used in this study is envisioned as replaceable by any other eukaryotic P450. Most likely, substitution of Fd with other photosynthetic electron carriers would also expand the versatility of the PSI-P450 system, given that changes in overall charge distributions of electron carriers affects their ability to donate electrons to P450s.[30] Thus this system is predesigned for future endeavors to produce valuable bioactive natural products of high chemical complexity using the principles of synthetic biology and the "share your parts" idea. The isolated PSI and P450 protein complexes are envisioned to constitute new important biobricks in the open source collections of standard biological parts, *e.g.*, as outlined in the International Open Facility Advancing Biotechnology (www.biofab.org).

The current demonstration of a highly active light-driven P450 system with Fd as the direct electron donor to the P450 would also suggest that plants engineered to express P450s in the chloroplast with the catalytic domain exposed to the stoma would be able to use Fd as electron donor. This approach would bring the PSI and P450 into the same plant organelle. Currently, five P450s have been shown to be targeted to the chloroplast envelopes of *Arabidopsis thaliana*, but none have their catalytic domain exposed to the stroma, a specific requirement for Fd interaction.[31] The maximum turnover rate for the PSI-CYP79A1 system was achieved at an 1135:1 ratio of Fd to CYP79A1, indicating that a large excess of Fd is required for adequate eukaryotic P450 activity using nonphysiological electron donors.[32] Quantification of the stromal content of Fd in maize leaves was estimated to be 37 μM, a sufficiently high stromal concentration to sustain P450 activity at a high level.[33] A future goal would therefore be to express an eukaryotic P450 in the chloroplast with the catalytic domain exposed to the stroma and targeted to either in the stroma lamella or the inner envelope of the chloroplast.

METHODS

Isolation of Thylakoid Membranes and Preparation of the PSI Complex. Barley plants were grown for 10 days at 20 °C in a 16/8 h light/dark regime at 130−150 μmol quanta m^{-2} s^{-1}. The topmost part of 12 h dark adapted leaves were used for isolation of thylakoids as described previously.[27] Total chlorophyll and chlorophyll *a*/*b* ratio were determined in 80% acetone according to Lichtenthaler.[34] PSI was isolated using sucrose gradients following solubilization of the thylakoids using *n*-dodecyl-*β*-D-maltopyranoside.[35] Total chlorophyll of the isolated PSI was determined and the PSI concentration calculated on the basis of 1 mg of total chlorophyll mL^{-1} equals 3.33 mg PSI protein mL^{-1} corresponding to approximately 5−6 μM PSI. PSI was stored at −80 °C.

Purification of Flavodoxins. An N-terminal His-tag was incorporated in the full-length flavodoxin Fld(isiB) (Genbank M88253.1) from *Synechococcus* sp. PCC 7002 by cloning into the expression vector pET-15b (Novagen). Cloning was carried out by introduction of *NdeI* sites at the beginning of the coding regions and *Bam*HI sites downstream of the stop codons. The construct was transformed into *E. coli* Xja(DE3) cells (ZYMO research) which were grown in the presence of 0.67 mg L^{-1} flavin mononucleotide but otherwise treated according to manufacturers instruction. The his-tagged protein was purified using Ni-NTA Agarose (Invitrogen) according to manufacturers instruction and the purified proteins were transferred to 50 mM Potassium Phosphate Buffer (pH 7.9) with a final concentration of 10% (v/v) glycerol using protein desalting spin columns (Pierce). The purity of the protein was monitored by SDS/PAGE and by the ratio of absorbance maxima at 369 and 464 nm ($A_{369/464}$ = 0.85 or less). Flavodoxin concentration was determined by measuring the absorbance at 464 nm (molar extinction coefficient = 8420 M^{-1} cm^{-1}). Isolated flavodoxin was stored at −80 °C.

$NADP^+$ Photoreduction Measurements. $NADP^+$ photoreduction activity was determined from the absorbance change at 340 nm in a reaction mixture (total volume 500 μL) containing TNM buffer (20 mM Tricine (pH 7.5), 40 mM NaCl, 8 mM $MgCl_2$, 0.1% (v/v) *n*-dodecyl-*β*-D-maltopyranoside, 2 mM sodium ascorbate, 0.06 mM 2,6-dichlorophenolindophenol (DCPIP)), 0.5 mM $NADP^+$, 1.1 nmol spinach plastocyanin, 0.38 μM spinach FNR (Sigma-Aldrich), and 84 pmol PSI. Spinach Fd (Sigma-Aldrich) (76 pmol) or Fld(isiB) (76−1568 pmol) was added to the reaction. The light-induced production of NADPH was measured at 340 nm in a Shimadzu UV-2550 spectrophotometer. The sample was kept at 25 °C and irradiated from the top with a Schott KL 1500 light source fitted with two red filters (Schott RG660 and Corning 2-58). NADPH production was quantified using the molar extinction coefficient of 6620 M^{-1} cm^{-1}.

Purification of the Cytochrome P450 System. *Sorghum bicolor* CYP79A1 and CPR were expressed and isolated as previously reported following solubilization of the yeast microsomes using Triton X-100 and affinity purification of the N-terminally strepII-tagged proteins.[28] $Fe^{2+}\cdot CO$ vs Fe^{2+} difference spectroscopy was performed (total volume 500 μL) with CYP79A1 diluted either 10 or 25 times in 50 mM potassium phosphate buffer (pH 7.9). CYP79A1 was quantified using the extinction coefficient of 91 mM^{-1} cm^{-1} at 450 nm.

Experimental Setup of the PSI-CYP79A1 System. PSI-CYP79A1 assays (total volume of 200 μL) were performed in either TNM buffer with 1 mg mL^{-1} 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) or in 20 mM Tricine buffer (pH 7.5) supplemented with 0.11 mM DCPIP, 3.6 mM sodium ascorbate, 0.1% (v/v) *n*-dodecyl-*β*-D-maltopyranoside, and 1 mg mL^{-1} DLPC. Protein amounts used were as follows unless otherwise stated in the figure legends: 84 pmol PSI, 1.1 nmol spinach plastocyanin, 1.4 pmol CYP79A1, and 399 pmol Fd or 7−1944 pmol Fld(isiB). One −95 nmol L-[U-^{14}C]Tyrosine (1−95 nmol, 0.25−0.5 μCi) was added to each experiment (see figure legends for the specific substrate concentrations used in the separate experiments). The sample was kept at 25 °C and irradiated from the top with a Schott KL 1500 light source fitted with two red filters (Schott RG660 and Corning 2-58) and one gray filter (Schott NG4), resulting in a light intensity of 151 μmol quanta m^{-2} s^{-1}, unless otherwise specified in the figure legend. At the end of the incubation period (up to 30 min), irradiation was stopped and reaction products were extracted into twice

the reaction volume of EtOAc. The upper phase was recovered by centrifugation (2,000*g*, 5 min) and applied onto TLC plates (silica gel 60 F_{254}, Merck) which were developed in toluene/EtOAc/methanol (30:8:1 v/v). The radioactively labeled products formed were visualized and quantified using a STORM 840 phosphoimager (Molecular Dynamics). K_M and V_{max} determinations for individual components of the PSI-CYP79A1 assay were preformed both in the presence and absence of 194 pmol Fld(isiB). The effects of radical oxygen and H_2O_2 formation in the PSI-CYP79A1 system were tested by addition of catalase (Sigma-Aldrich C30) and superoxide dismutase (Sigma-Aldrich S8160) to a final concentration of 25 μg mL^{-1}.

Experimental Setup of the CPR-CYP79A1 System. CYP79A1 inhibition studies were monitored in assay mixtures (total volume 200 μL) containing TNM buffer, 1.2 μmol NADPH, 1 mg mL^{-1} DLPC, 0.5 nmol L-[U-^{14}C]tyrosine (0.25 μCi), 198 pmol CPR, 1.4 pmol CYP79A1, and 0−604 pmol Fld(isiB). Following incubation (25 °C, 30 min), reaction products were extracted into twice the reaction volume of EtOAc and quantified as described above. Comparison of the CPR-CYP79A1 system to the PSI-CYP79A1 system was done using 1.2 μmol NADPH, 1 mg mL^{-1} DLPC, 0.5 nmol L-[U-^{14}C]tyrosine (0.25 μCi), 415 pmol CPR, and 1.4 pmol CYP79A1 in 20 mM Tricine (pH 7.5) at 25 °C. A similar setup was used for examining the effect of Catalase and Superoxide dismutase on the CPR-CYP79A1 system activity. Both enzymes were used in a final concentration of 25 μg mL^{-1}.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

P450: cytochrome P450; CPR: NADPH-cytochrome P450 oxidoreductase; PSI: Photosystem I; ER: endoplasmic reticulum; Fld: flavodoxin; Fd: ferredoxin; FNR: ferredoxin-NADPH oxidoreductase; oxime: (*E*/*Z*)-*p*-hydroxyphenylacetaldoxime

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