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Fusion of Ferredoxin and Cytochrome P450 Enables Direct Light-Driven Biosynthesis

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ABSTRACT: Cytochrome P450s (P450s) are key enzymes in the synthesis of bioactive natural products in plants. Efforts to harness these enzymes for in vitro and whole-cell production of natural products have been hampered by difficulties in expressing them heterologously in their active form, and their requirement for NADPH as a source of reducing power. We recently demonstrated targeting and insertion of plant P450s into the photosynthetic membrane and photosynthesis-driven, NADPH-independent P450 catalytic activity mediated by the electron carrier protein ferredoxin. Here, we report the fusion of ferredoxin with P450 CYP79A1 from the model plant Sorghum bicolor, which catalyzes the initial step in the pathway leading to biosynthesis of the cyanogenic glucoside dhurrin. Fusion with ferredoxin allows CYP79A1 to obtain electrons for catalysis by interacting directly with photosystem I. Furthermore, electrons captured by the fused ferredoxin moiety are directed more effectively toward P450 catalytic activity, making the fusion better able to compete with endogenous electron sinks coupled to metabolic pathways. The P450-ferredoxin fusion enzyme obtains reducing power solely from its fused ferredoxin and outperforms unfused CYP79A1 in vivo. This demonstrates greatly enhanced electron transfer from photosystem I to CYP79A1 as a consequence of the fusion. The fusion strategy reported here therefore forms the basis for enhanced partitioning of photosynthetic reducing power toward P450-dependent biosynthesis of important natural products.

With the continued development of tools for engineering biosynthetic pathways into microorganisms, elucidation of routes leading to plant natural products of high value is attracting considerable interest.1–3 Cytochrome P450s (P450s) are key enzymes in specialized metabolism and are involved in the formation of terpenoids, alkaloids, cyanogenic glycosides, glucosinolates, and phenylpropanoids like flavonoids, coumarins, and stilbenes. They catalyze stereo- and regiospecific hydroxylations, epoxidations, and C−C couplings that are often difficult to accomplish by chemical synthesis. In eukaryotes, P450s reside on the endoplasmic reticulum, and plant genomes often contain several hundred P450-encoding genes.4–6 Biotechnological production of many high-value specialized metabolites thus requires heterologous expression of one or more P450s in highly active form. The yeast Saccharomyces cerevisiae remains the favored host for introduction of plant P450-dependent pathways,1 but cyanobacteria are gaining in popularity and offer unparalleled sustainability because they too are photosynthetic organisms and therefore require minimal nutrient input.7 We recently showed that the thylakoid membranes of both cyanobacteria and plants, which host the photosynthetic apparatus, can also accommodate plant P450s.8–11

Two key issues encountered when engineering P450-dependent pathways are the need for high-level functional expression of the P450 and the provision of sufficient reducing power to drive their catalytic cycle.12 This study addresses the latter. Many alternative P450 reductase systems and P450-reductase fusions have been reported, but only a few have resulted in increased activity.13 Photosynthetic hosts offer several advantages for P450-dependent pathways:4–7 Plant P450s are functionally active when targeted to thylakoid membranes and benefit from the ample supply of electrons and molecular oxygen generated by photosynthetic electron trans-
port (Figure 1a).8,9 Cyanobacteria or plant-cell cultures may thus constitute a useful vehicle for environmentally contained, heterologous production of specialized plant metabolites of high value, such as structurally complex diterpenoids functionalized in P450-dependent reactions.14−18 However, competition from native electron sinks, such as the metabolic reactions involved in CO₂ fixation and redox regulation processes,8 complicates the diversion of electrons from photosynthesis to non-native processes. In an effort to improve partitioning of electrons toward the P450, we have now designed and tested fusions that covalently connect the electron transfer protein ferredoxin (Fd) to the well-characterized P450 CYP79A1 from Sorghum bicolor (Figure 1a).

In the current study, we expressed and targeted P450-Fd fusions to the chloroplast in Nicotiana benthamiana. One of the fusion constructs, which could acquire photosynthetic reducing power by direct electron transfer from photosystem I without the need for a dedicated reductase, was better able to compete with endogenous electron sinks and showed higher in vivo activity than the native enzyme. This study thus represents a proof of concept that P450-Fd fusions can interface directly with electron transfer from photosystem I and divert more photosynthetic reducing power to engineered metabolism.

RESULTS

Design of CYP79A1-Ferredoxin Fusion Proteins. The initial step in the biosynthetic pathway leading to the cyanogenic glucoside dhurrin in Sorghum bicolor19−21 is the conversion of L-tyrosine to (E)-p-hydroxyphenylacetaldoxime, which is catalyzed by CYP79A1.21−24 We chose CYP79A1 for our ferredoxin (Fd) fusion studies because it is stable both in its native ER and in photosynthetic membranes8,9 and well characterized.21,22,24 We designed three different fusion constructs—two C-terminal fusions named CΔF and CF and one N-terminal construct FCΔ (Figure 1b). A linker length of 15 amino acids was chosen based on the distances between the C-terminus of CYP79A1 and the N-terminus of Fd derived from docking models, the presence in CYP79A1 of four C-terminal residues with predicted random-coil secondary structure not resolved in crystal structures,25 and the estimated distance from the edge of photosystem I to its Fd binding site (Supporting Figure S1). A Gly/Ser-rich sequence was chosen to avoid secondary structure and maximize the ability of the Fd...
domain to transfer electrons to the heme of CYP79A1 and reduce susceptibility to proteases. Because the native CYP79A1 (named C in Figure 1b) contains an N-terminal transmembrane domain that normally anchors it to the ER membrane, N-terminal fusions to Fd were constructed with CYP79A1 lacking this domain, to avoid placing Fd and CYP79A1 domains on opposite sides of the thylakoid membrane. An additional C-terminal Fd fusion with the same truncated CYP79A1 variant was therefore assembled to control for differences due to a lack of the transmembrane anchor.

Chloroplast Sublocalization of Fusion Proteins. We used Agrobacterium-mediated infiltration of N. benthamiana leaves to generate plants that transiently expressed the CYP79A1 constructs. Subsequently, we fractionated intact chloroplasts prepared from infiltrated leaves and subjected each fraction to immunoblot analysis with an anti-CYP79 antibody to verify that the Fd transit peptide targeted fusion proteins to thylakoid membranes (Figure 2a). As expected, the fusions were found mainly in thylakoid fractions, though both FCΔ and CΔF were also detectable in the stromal fraction.

Immunoblot analyses performed with an antibody raised against ferredoxin showed clear signals for CΔF, CF, and FCΔ constructs, but not the unused construct C (Figure 2b), thus confirming that the translated fusion proteins contained both CYP79A1 and Fd domains. The CΔF, CF, and FCΔ proteins comprised 0.2–2.6% of total thylakoid protein, based on SDS-PAGE densitometry (Supporting Figure S2c). The C protein could not be quantified by this approach because it comigrated with the abundant β-subunit of ATP synthase. A high pigment background (due to chlorophylls and carotenoids) in the isolated thylakoid membranes interfered with standard determination of concentrations of the P450s by CO difference spectra, and we therefore measured their relative levels by immunoblot analysis (Supporting Figure S2a,b and Supporting Table S1).

Fusion with Fd Directs Electrons to CYP79A1 in the Absence of Soluble Fd. We next performed enzyme assays using thylakoid preparations containing C, CΔF, CF, or FCΔ protein to investigate whether the Fd domain fused to CΔF, CF, and FCΔ mediated direct electron transfer from photosystem I to the heme of the CYP79A1 domain. In vitro assays were carried out to assess whether added soluble Fd was required for electron transfer between photosystem I and the P450s (Figure 3). Only the CF fusion protein harboring the full-length CYP79A1 protein was found to exhibit substantial activity in the absence of added soluble Fd. Direct electron transfer between Fd and CYP79A1 domains also operated in assays in which the Fd domain was reduced in a light-independent manner by the enzyme ferredoxin:NADP+ reductase (FDN) in the presence of NADPH (Supporting Figure S3). Based on these findings, we focused subsequent experiments on characterizing the function of the CF enzyme.

Dependence of Activity on Soluble Ferredoxin. Because Fd interacts electrostatically with redox partners, we examined the effect of the ionic strength (I) on the interaction between Fd and the enzymes C and CF. The activity of C showed a marked bell-shaped dependence on I, reaching a maximum at I = 60 mM, whereas CF was largely unaffected by I (Figure 5a). To test whether fusion with Fd enabled CF to acquire more electrons from photosystem I even in the presence of a competing sink, we examined the effect of I on the light-driven activity in the presence of FNR and the acceptor NADP+. FNR and NADP+ competed strongly with C and CF for reduced ferredoxin, as the catalytic activities of both enzymes were reduced in their presence. However, CF retained more activity (up to 57% (78%) of its peak activity in the presence (absence) of soluble ferredoxin, respectively) than C (18% of maximal or less) under these conditions (Figure 5b).

In Vivo Activity of C and CF. Because in vitro activities of C and CF depended greatly on assay conditions, we examined the in vivo activity of both chloroplast-targeted proteins by quantifying reaction products extracted directly from tobacco. The aldoxime produced by ER-localized CYP79A1 was previously shown to undergo glycosylation in tobacco plants.

To test whether chloroplast-produced p-hydroxyphenylacetaldoxime was glycosylated in our transient transfection experiments, we treated extracts of infiltrated leaves with a commercial 1-β-glucosidase mixture and observed the release of large amounts of p-hydroxyphenylacetaldoxime (Supporting Figure S4). We also synthesized the glucoside p-glycosylphenylacetaldoxime (see Supporting Information, Materials and Methods), which allowed us to quantify both free and

Figure 3. Comparison of in vitro light-driven enzyme activity from N. benthamiana thylakoids harboring CYP79A1 enzyme variants. Assays were carried out in the presence or absence of 8.3 μM soluble Fd (+Fd or −Fd) as an electron carrier. Bars show average activities of four separate thylakoid preparations per construct, normalized to their relative protein levels as determined from immunoblotting experiments. Error bars indicate ± SD of biological replicates.
glycosylated aldoxime using LC-MS/MS. We found amounts of p-glucosyloxyphenylacetaldoxime to be linearly correlated with, and on average 100-fold higher (w/w) than, those of p-hydroxyphenylacetaldoxime (Supporting Figure S5). To ensure that differences in the average metabolite levels detected in C- and CF-expressing plants were not caused by differences in expression of C and CF enzymes, we also quantified the relative amounts of both proteins in total protein extracts from infiltrated leaves by immunoblotting and found average CF expression to be 40% that of C (Supporting Figure S6). The expression-normalized production of free and glycosylated p-hydroxyphenylacetaldoxime showed that the specific activity of CF is 50% higher than that of C (Figure 6).

■ DISCUSSION

This study reports the light-driven activity of three fusions between CYP79A1 and Fd and confirms the establishment of functional electron transfer from photosystem I to the heme domain in the CF enzyme. We expected the CΔF and FCΔfusions to be soluble proteins, but both associated with thylakoid membranes, possibly via hydrophobic interactions with the F-G loop of CYP79A125 and/or electrostatic Arg and Lys interactions with membrane phospho- or sulfolipids.29,30 The CΔF fusion was barely detectable in isolated chloroplasts, but readily so in thylakoid membrane preparations (Figure 2). The FCΔ fusion protein showed the highest expression in both chloroplast and thylakoid preparations, but both it and CΔF gave rise to additional immunospecific bands below the main one (Figure 2b and Supporting Figure S2a). Neither C nor CF, which include the full-length CYP79A1 sequence, appeared susceptible to proteolysis and produced single bands at the expected positions on immunoblots (62 and 73 kDa). We conclude that truncation of CYP79A1 increases its susceptibility to chloroplast proteases, and this might explain the reduced activities of CΔF and FCΔ. Other possible explanations are poor interaction between Fd and P450 domains, aberrant incorporation of heme or 2Fe−2S clusters into the truncated protein, or other post-translational modifications that could affect enzyme activity.

Figure 4. Effects of soluble ferredoxin and detergent on in vitro enzyme activity of C and CF proteins. (a) Light-dependent enzyme activity of thylakoids harboring either C or CF, driven by electron transfer from soluble or fused ferredoxin reduced by photosystem I. (b) Enzyme activity of dark-incubated thylakoids harboring C or CF, driven by electron transfer from soluble or fused ferredoxin reduced by 0.6 μM FNR in the presence of 0.5 mM NADPH. (c) Light-dependent activity of intact (+βDM) or solubilized (+βDM) thylakoids harboring C or CF in the presence of 8.3 μM soluble Fd. Experimental values are means of four technical repeats each, normalized to relative protein levels determined by immunoblot, with error bars showing ± SD. βDM, n-dodecyl β-D-maltoside.

Figure 5. Effect of ionic strength on light-driven enzyme activity of tobacco thylakoids containing C (+soluble Fd) or CF (+soluble Fd). (a) Dependence of C and CF activity on ionic strength, adjusted with NaCl. (b) Dependence on ionic strength of C and CF activity measured in the presence of 0.6 μM FNR and 1.63 mM NADP+ acting as a competing electron sink for reduced Fd. Data are plotted relative to the maximum activity of C obtained in a. Curves show mean activities from (a) four or (b) two technical replicates, normalized to the relative levels of C and CF proteins. Error bars indicate ± SD.

Figure 6. Relative amounts of p-hydroxyphenylacetaldoxime and p-glucosyloxyphenylacetaldoxime extracted from N. benthamiana leaves 5 days post infiltration with Agrobacterium strains bearing plasmids encoding either C or CF, as quantified by LC-MS/MS and normalized to relative protein expression as determined by immunoblot. Equal numbers of leaf samples were analyzed for p-hydroxyphenylacetaldoxime and p-glucosyloxyphenylacetaldoxime in C (n = 37) and CF (n = 40), but p-hydroxyphenylacetaldoxime was below the level of detection in some CF samples. Error bars ± SD. *Statistical significance (p < 0.05) according to two-tailed unpaired t-tests.
proteins, or poor interaction with the PsAC, -D, and -E subunits of photosystem I, which would limit electron transfer from its F₀ [4Fe-4S] cluster (see Supporting Figure S1).31

Cytochrome P450 concentrations are commonly determined by inhibiting the dithionite-reduced enzyme with CO to yield a characteristic reduced-plus-CO vs reduced difference spectrum.32 We were unable to detect characteristic peaks at 450 or 420 nm because the high spectral background from pigments in the photosynthetic membrane swamps the weak signal expected from the P450:CO adduct (data not shown). Though CO spectra can also measure enzyme inactivation, the presence of other hemoproteins compromises their accuracy.32 Since thylakoid membranes contain many hemoproteins, in particular the Cytochrome b₅₆ complex, the method is unlikely to be accurate in this matrix. We instead measured activities in several independent thylakoid preparations and found them to be highly consistent overall, indicating that inactivation of the cytochromes does not cause major variation in this case.

The CF fusion was preferentially reduced by electrons transferred to its fused Fd domain by photosystem I, and only a minor fraction of its reducing power was derived from soluble Fd (Figure 7a). The most likely explanations for this preference are steric hindrance by the Fd domain limiting access of soluble Fd to the heme-proximal surface and much faster kinetics of electron transfer from photosystem I to the CF enzyme due to reduced dependence on diffusion. While our evidence does not exclude other possibilities, a number of observations support this interpretation. First, the addition of high concentrations of soluble Fd resulted in only limited enhancement of CF activity (Figure 4a). Second, the same trend was observed when FNR and NADPH were used to reduce Fd, with activities of C and CF being equal at ~4 μM soluble Fd in both experiments (Figure 4a,b). Since FNR is a soluble enzyme, it is not affected by steric hindrance in the spatially highly organized environment of the thylakoid, which consists of ~70% (mol/mol) protein33 and allows only limited diffusion.33 Third, CF activity depends on colocalization with photosystem I in the thylakoid membrane, even when soluble Fd is present (Figure 4c). Solubilization of the membrane effectively causes its constituent proteins to be diluted, which is consistent with a decrease in activity of CF if it can only be reduced via direct interaction of its Fd domain with photosystem I. In contrast, the concentration of soluble Fd (8.3 μM) is unchanged by solubilization of the membrane, and therefore so also is the activity of the C protein. Finally, the higher specific activity of CF relative to unfused C under in vivo conditions (Figure 6) indicates that the differences observed between C and CF in vitro relate to the specific assay conditions used. Since the concentrations of Fd available to CF and C differ in principle, membrane colocalization of CF with photosystem I must compensate for its inability to interact with soluble Fd.

Unfused C protein showed typical saturation behavior as Fd concentrations were increased in light- but not in FNR-driven assays (Figure 4a,b). This difference probably results from slower reduction of Fd by FNR, because of thermodynamically uphill electron transfer from NADPH (Em = −320 mV) to Fd (Em = −433 mV).35 From its saturation curve in light-driven assays we estimated the K_M of C for Fd to be 5.0 ± 0.6 μM. For comparison, FNR—which consumes the majority of reduced Fd in vivo—has a K_M for Fd of 2.8 μM.35 Such a high affinity toward an unnatural interaction partner can be rationalized by similarities between cytochrome P450 reductases (POR) and chloroplast Fd’s. POR contains a small FMN-binding domain responsible for electron transfer to P450s, which is structurally homologous to the small electron carrier protein flavodoxin (Fld).36 Since Fld and Fd act interchangeably in some photosynthetic organisms,37 both carry highly negatively charged surfaces, and both interact electrostatically with photosystem I and FNR,38 their ability to support catalysis by many P450s is consistent with the hypothesis that these small electron carrier proteins evolved to interact with proteins through charge and surface shape complementarity in a relatively unspecific manner.42

The light-driven activity of C showed a bell-shaped dependence on ionic strength (I) (Figure 5a). Similar I dependencies have been reported for Fd-dependent FNR activity and for reduction of plastocyanin by cytochrome c, and the increase in activity observed likely reflects increased dissociation rates due to suppression of nonspecific electrostatic interactions.43–45 In contrast, the activity of the CF protein showed less dependence on ionic strength, probably because the covalent association of the interacting partners obviates the need for long-range electrostatic steering. The CF fusion is better able to compete with FNR for electrons (Figure 5b), but reduced CF activity in the presence of soluble Fd shows that the latter competes for interaction with photosystem I (Figure 7b).

This work has important implications in relation to the choice of assay for assessment of P450-reductase fusions. Such
enzymes are often evaluated in comparison with a stoichiometric reductase:P450 mixture, which can be misleading, since in vivo reductase:P450 ratios are often far from 1.46 and the activities of fusion enzymes tend to increase linearly with concentration, while differing reductase:P450 ratios show nonlinear effects on rates.47 The choice of ionic strength is likewise important, because high ionic strengths would favor intramolecular electron transfer by less electrostatic-reliant fusion enzymes (Figure 5). In this study, we showed that our CF fusion can acquire more reducing power in the presence of a strong competing electron sink than unfused C, which partly explains its increased in vivo activity. We should note, however, that in vivo concentrations of FNR and Fd (10−100 μM and 300−800 μM, respectively, reported for chloroplasts and cyanobacteria)48–50 would be difficult or impossible to replicate in vitro. Consequently, we stress the importance of backing up in vitro comparisons with in vivo experiments whenever possible.

Since light-driven cytochrome P450 pathways have key applications in live cell-culture production systems, we compared the in vivo activities of unfused C and the CF fusion. A preliminary time course analysis of p-hydroxyphenylacetaldoxime content in leaf extracts at 1−5 days post infiltration showed levels peaking on day 3, with no significant change on the fourth and fifth days (data not shown). The levels of C and CF proteins detected 5 days post infiltration indicated that the plants converted p-hydroxyphenylacetaldoxime to other compounds, and this was confirmed by enzymatic deglycosylation of extracted metabolites (Supporting Figure S4). We found free aldoxime to accumulate to 100-fold lower levels than, and be linearly correlated with, its glucoside (Supporting Figure S6), which implies highly active glycosylation machinery for detoxification of foreign compounds, such as oximes.51,52 By quantifying both, we demonstrated that the catalytic activity of the fusion enzyme is superior to that of unfused CYP79A1 (Figure 6), thanks to its covalent association with Fd, thus confirming that the thylakoid membrane possesses the plasticity required to allow interaction of heterologous enzymes with photosystem I.

This study represents the first report of enhanced light-driven activity of a P450-reductase fusion in vivo. Improved light-driven hydrogenase activity through Fd fusion in vitro was previously demonstrated.53 We did not explore the effect of linker length or composition in the present study, but both were recently found to influence electron transfer rates in fusions between the E. coli flavodoxin reductase and Fd.54 Though five residues were sufficient to support activity of a CYP11A1 adenodoxin fusion,55 both studies found higher activity with longer linkers.54,55 Assembly of a complex between the model P450,un and its reductase system (putidaredoxin and putidaredoxin reductase) by an alternative approach whereby they were fused with subunits of the PCNA trimer gave a 100-fold rate increase, and this could be further increased by optimizing the linker, further demonstrating the rate enhancements achievable by proper linking of P450 and reductase.56,57 Consequently, we consider it likely that our reported CF fusion can be improved further by exploring alternate linker designs.

**Conclusion.** This study reports the in vitro and in vivo effects of introducing a P450-ferredoxin fusion protein into the thylakoid membranes of chloroplasts. The fusion CF could obtain electrons directly from photosystem I, and its activity was affected little by soluble Fd. The fusion enzyme also retained higher activity in the presence of competing electron sinks, probably because it is colocalized with photosystem I in the thylakoid membrane. Our P450-ferredoxin fusion approach thus enables direct coupling of photosynthetic electron transfer to P450s involved in desired biosynthetic pathways introduced into higher plant chloroplasts, algae, or cyanobacteria. To our knowledge, this is the first report of a fusion between a ferredoxin and a eukaryotic P450 involved in specialized metabolism. The CF fusion protein had a higher specific activity than unfused CYP79A1 in tobacco leaves, but was less abundant following transient expression. Minimal interaction between the fusion enzyme and soluble Fd makes the catalytic activity achieved by the fusion enzyme in vivo especially remarkable, since its fused Fd domain is present in substoichiometric amounts relative to free Fd. Evolution has tuned the distribution of photosynthetic reducing power to balance maximal biomass accumulation with the necessary redox regulation of metabolic processes. Thus, successful exploitation of photosynthetic organisms for light-driven production of high-value specialized metabolites or biofuels such as H2 will require strategies that modulate the distribution of reducing power. Our work indicates that Fd fusions constitute a transferable approach to channeling of photosynthetic reducing power into non-native pathways.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00190.

Materials and methods, Supporting Tables S1 and S2, Supporting Figures S1−S6 (PDF)

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Notes

The authors declare no competing financial interest.

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