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Redirecting Photosynthetic Reducing Power toward Bioactive Natural Product Synthesis

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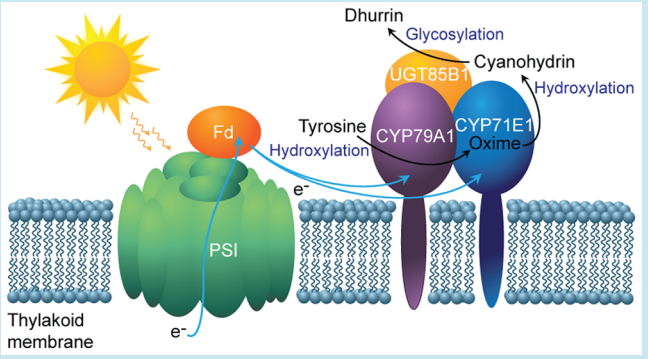
Supporting Information

ABSTRACT: In addition to the products of photosynthesis, the chloroplast provides the energy and carbon building blocks required for synthesis of a wealth of bioactive natural products of which many have potential uses as pharmaceuticals. In the course of plant evolution, energy generation and biosynthetic capacities have been compartmentalized. Chloroplast photosynthesis provides ATP and NADPH as well as carbon sources for primary metabolism. Cytochrome P450 monooxygenases (P450s) in the endoplasmic reticulum (ER) synthesize a wide spectrum of bioactive natural products, powered by single electron transfers from NADPH. P450s are present in low amounts, and the reactions proceed relatively slowly due to limiting concentrations of NADPH. Here we demonstrate that it is possible to break the evolutionary compartmentalization of energy generation and P450-catalyzed biosynthesis, by relocating an entire P450-dependent pathway to the chloroplast and driving the pathway by direct use of the reducing power generated by photosystem I in a light-dependent manner. The study demonstrates the potential of transferring pathways for structurally complex high-value natural products to the chloroplast and directly tapping into the reducing power generated by photosynthesis to drive the P450s using water as the primary electron donor.



KEYWORDS: *light-driven biosynthesis, plant biology, metabolic engineering, natural products production, photosynthesis, chloroplast*

Amino acid[1] and UDP-glucose[2] biosynthesis takes place in the chloroplast and profits from easy access to carbon skeletons derived from photosynthesis and reduced ammonium ions. Amino acids are the precursors of a diverse range of pharmaceutically interesting bioactive molecules such as alkaloids, phenylpropanoids, and cyanogenic glucosides. Pathways resulting in the formation of these complex structures typically include key steps catalyzed by cytochrome P450s localized in the ER. In this study, we have used synthesis of the aromatic defense compound dhurrin (D-glucopyranosyloxy-(*S*)-*p*-hydroxymandelonitrile, a cyanogenic glucoside) found in *Sorghum bicolor* as our model system for P450 action.[3] Our aim was to transfer an entire P450 pathway to the chloroplast and thereby drive product synthesis in a light-dependent manner using photosynthesis.

Dhurrin synthesis from the amino acid tyrosine involves three ER-localized enzymes: two P450 enzymes (CYP79A1[4] and CYP71E1[5]), the NADPH cytochrome P450 oxidoreductase (POR) that provides reducing power from NADPH in single electron transfer steps, plus a soluble cytosolic UDP-glucosyl transferase UGT85B1.[6] CYP79A1 converts L-tyrosine to *p*-hydroxyphenylacetaldoxime, which is further metabolized by CYP71E1 into the cyanohydrin *p*-hydroxymandelonitrile. In the final step, UGT85B1 stabilizes the *p*-hydroxymandelonitrile by glucosylation to yield dhurrin.[6−8] Because all genes encoding the pathway are available, the dhurrin pathway provides an excellent model system to establish proof-of-concept for light-generated biosynthesis of bioactive compounds. Transfer of P450-dependent pathways to the chloroplast is further facilitated by the previous *in vitro* observations that reduced ferredoxin (Fd) generated by photosystem I (PSI) may serve as a direct and efficient electron donor to the two microsomal P450s described, thus bypassing the involvement of POR[9−11] as illustrated in Figure 1a.

As a DNA-containing cellular compartment, the chloroplast encodes only about 120 genes.[12] However, the biosynthetic capacity of the chloroplast is enormous, as is the number of processes that may be modified or introduced by metabolic engineering.[13]

In the chloroplast, photosynthesis is mediated by the photosystems localized in the thylakoids and by soluble electron carrier proteins in the stroma and in the thylakoid lumen.[14] In the thylakoids, photosystem II and I (PSII and PSI)

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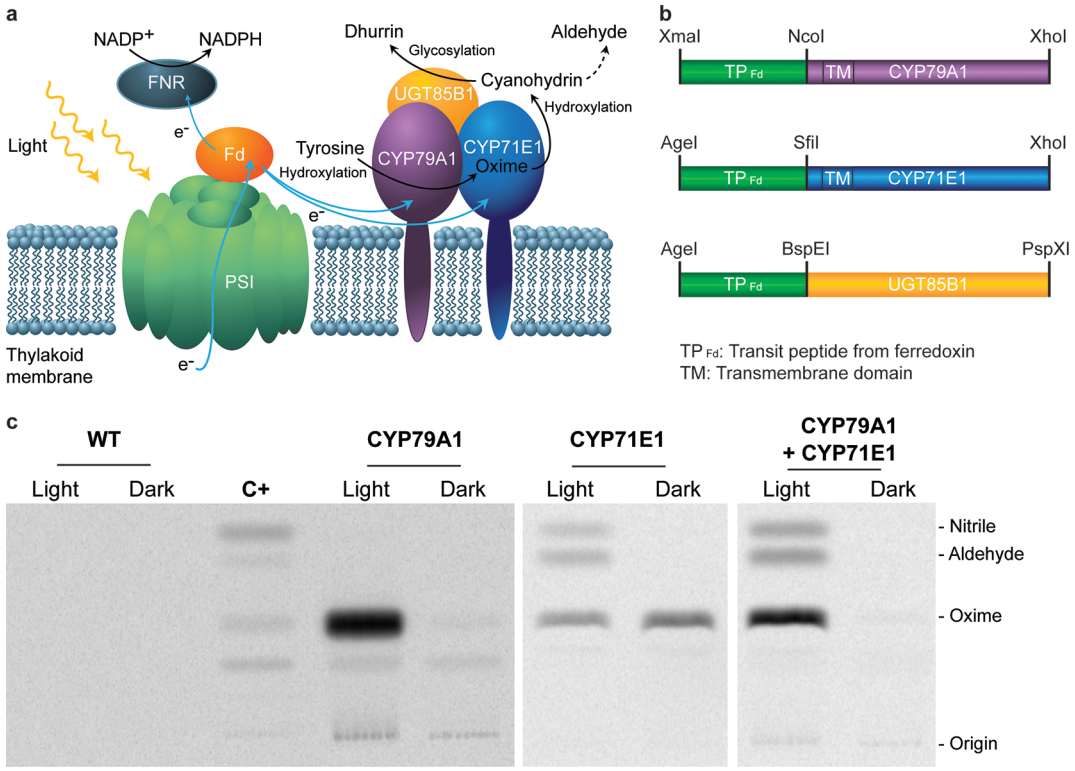

Figure 1. Light-generated enzymatic activities engineered into the chloroplast thylakoids of tobacco. (a) Schematic representation of light-generated synthesis of the cyanogenic glucoside dhurrin based on direct electron transfer from photosystem I. The reducing power (e^-) needed for dhurrin formation in the chloroplast is thus ultimately derived by the water splitting activity of photosystem II. (b) Scheme of the fusion enzymes generated to target the dhurrin pathway into the chloroplast. The transit peptide from Fd (TP_{Fd}) was fused to the cDNA encoding the native enzymes: the two P450s CYP79A1 and CYP71E1 and the UDP-glucosyltransferase, UGT85B1. The restriction enzymes used for the cloning into the pEAQ-HT expression vector are shown. (c) Thylakoids expressing CYP79A1, CYP71E1, or both were isolated and incubated with the appropriate radiolabeled substrate and Fd. The samples containing thylakoids were incubated in either darkness or irradiated (100 photons m^{-2} s^{-1}). The reaction products formed were extracted into ethyl acetate and analyzed by thin layer chromatography (TLC). Thylakoids harboring CYP79A1 catalyzed light-generated conversion of radiolabeled tyrosine into *p*-hydroxyphenylacetaldoxime. Thylakoids harboring CYP71E1 catalyzed light-generated conversion of *p*-hydroxyphenylacetaldoxime to *p*-hydroxymandelonitrile and with *p*-hydroxyphenylacetonitrile as an intermediate. The *p*-hydroxymandelonitrile formed nonenzymatically dissociates into *p*-hydroxybenzaldehyde and hydrogen cyanide. Thylakoids expressing both CYP79A1 and CYP71E1 catalyzed light-generated conversion of tyrosine into the *p*-hydroxymandelonitrile reconstituting the first two enzymatic steps of the dhurrin pathway in the thylakoid membrane. WT tobacco thylakoids were used as negative control, whereas purified yeast spheroplasts expressing CYP71E1 were used as positive control (C+). List of abbreviations used: oxime, *p*-hydroxyphenylacetaldoxime; nitrile, *p*-hydroxyphenylacetonitrile; aldehyde, *p*-hydroxybenzaldehyde.

work in series to abstract electrons from water and use these to reduce $NADP^+$. Photons absorbed by PSII are used to oxidize water through the oxygen-evolving complex connected to PSII and transfer the abstracted electrons to PSI. Upon light excitation, PSI transfers electrons from the lumenal side of the thylakoids to the stromal side to reduce the soluble electron donor, ferredoxin (Fd). Downstream of ferredoxin, electrons are distributed into several pathways. When the Calvin cycle is fully active, the vast majority of electrons are used for NADPH production via the ferredoxin-NADP+ oxidoreductase (FNR). The NADPH provides reducing equivalents to drive the reductive steps of the Calvin cycle.[14,15]Nitrogen and sulfur assimilation constitute additional significant electron sinks. The aim of the current study is to demonstrate that it is possible to express the entire biosynthetic pathway for production of the tyrosine-derived cyanogenic glucoside dhurrin in the chloroplast as a model system for relocation of a P450-catalyzed pathway from the ER to the chloroplast and redirect the electrons from the photosynthetic apparatus to drive the redox reactions of the P450s. The P450s are active when expressed in the thylakoid membrane, are able to function in a light-driven manner, and are not inactivated by the shift in stroma pH from neutral to alkaline following irradiation. This opens the avenue for light-driven synthesis of a vast array of other bioactive natural products in the chloroplast-like structurally complex alkaloids and diterpenoids.

RESULTS AND DISCUSSION

Targeting Novel Biosynthetic Enzymes to the Chloroplast. In order to target dhurrin synthesis to the chloroplast, we engineered the genes encoding the three biosynthetic enzymes to deliver the enzymes to the chloroplast. Briefly, we fused the coding sequence of all three genes to the coding sequence for the N-terminal 52 amino acid transit peptide of the *Arabidopsis* Fd protein FedA (TP_{Fd}) known to direct Fd to the chloroplast[16] (Figure 1b).

The localization of the expressed enzymes targeted to the chloroplast using the Fd transit peptide have been analyzed by immunoblot analysis on chloroplasts containing all three enzymes, showing simultaneous expression and localization of all three enzymes in the chloroplast (Supplementary Figure S1).

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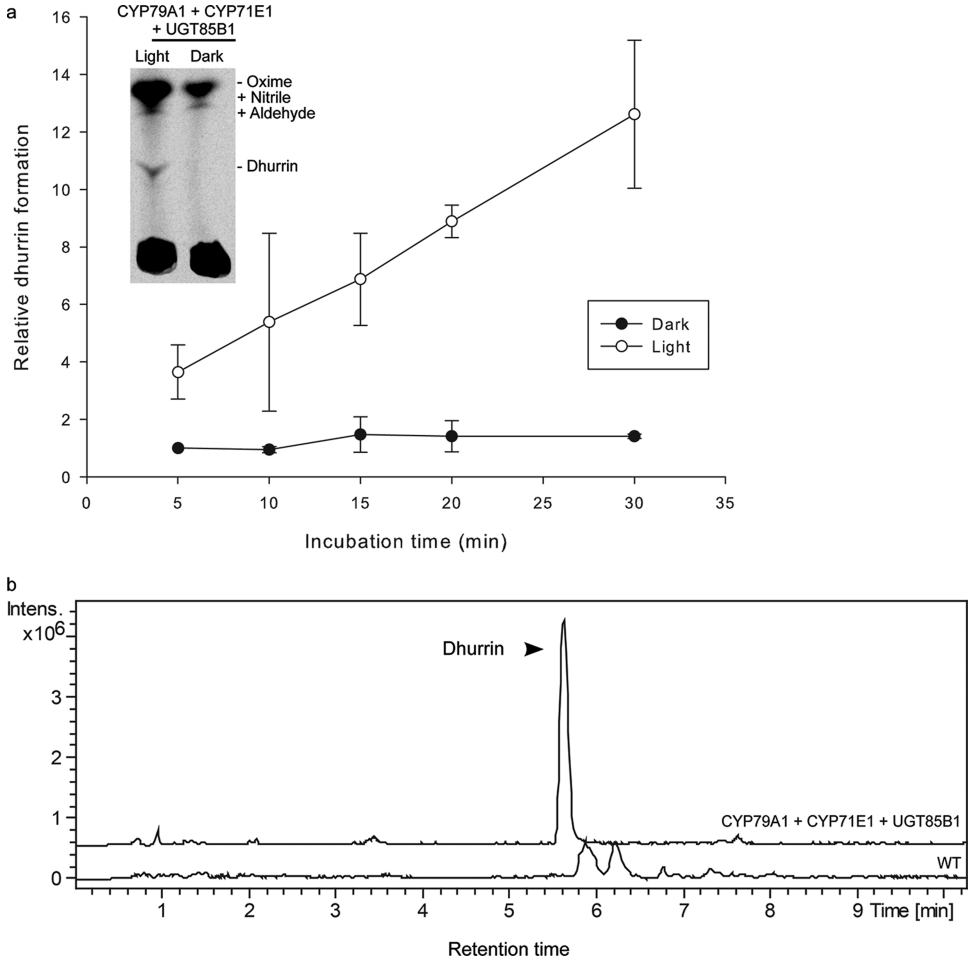

Figure 2. Expression of the entire dhurrin pathway in tobacco leaves and chloroplasts. (a) Light-dependent dhurrin formation in intact chloroplasts containing CYP79A1, CYP71E1, and UGT85B1 following incubation with radiolabeled tyrosine. Chloroplasts were isolated from triple infiltrated tobacco leaves 1 week after infiltration. Aliquots taken at different time points were analyzed by TLC to detect and quantify formation of dhurrin. The result from a typical experiment is shown. The radiolabel incorporated into dhurrin was normalized to the labeling obtained in darkness following 5 min incubtion. The insert shows dhurrin radiolabeling following 30 min incubation in the light and dark as monitored by TLC analysis. (b) Dhurrin formation in intact leaves based solely on endogenous substrates. Dhurrin formation in the tobacco leaves was monitored by LC−MS single ion monitoring (EIC *m*/*z* 334) 1 week following triple infiltration. Wild-type leaves served as negative control. List of abbreviations used: oxime, *p*-hydroxyphenylacetaldoxime; nitrile, *p*-hydroxyphenylacetonitrile; aldehyde, *p*-hydroxybenzaldehyde.

Demonstration of Light-Dependent Enzyme P450 Activity. To demonstrate that the chloroplast-targeted P450 enzymes are active in a light-dependent manner, we used *Agrobacterium tumefaciens* to transiently express the TP_{Fd} fusion constructs of CYP79A1 and CYP71E1 independently and in combination using *Nicotiana benthamiana* as the host plant. One week after Agrobacterium infiltration, leaves were harvested, and thylakoids were isolated. Presence of the transiently expressed P450s in the thylakoids was verified by immunoblot analyses using specific antibodies directed against the two P450s (Supplementary Figure S1). Functional expression of the P450s was demonstrated by incubation of isolated thylakoid membranes with radiolabeled substrates in the presence of added Fd and monitoring of product formation by radiolabeled TLC analysis (Figure 1c). Thylakoids harboring CYP79A1 convert tyrosine into the expected *p*-hydroxyphenylacetaldoxime upon light irradiation. Only trace amounts of *p*-hydroxyphenylacetaldoxime were formed after incubation in the dark. Transiently expressed CYP71E1 was also active in the thylakoids in a light-dependent manner as seen from conversion of its substrate *p*-hydroxyphenylacetaldoxime into the *p*-hydroxymandelonitrile with concomitant accumulation of small amounts of the intermediate *p*-hydroxyphenylacetonitrile. *p*-Hydroxymandelonitrile is labile and dissociates into *p*-hydroxybenzaldehyde and hydrogen cyanide (Figure 1c). The positive control (C+) is an enzymatic assay consisting of spheroplasts from yeast expressing CYP71E1 reconstituted with POR and NADPH[17] that is active both in darkness and the light. Thylakoids prepared from wild-type (WT) leaves served as negative control. This demonstrates that *p*-hydroxyphenylacetaldoxime and *p*-hydroxymandelonitrile formation is dependent on light-generated reducing power and exclusively occurs in leaves transformed with CYP79A1 and CYP71E1, respectively. In a subsequent series of experiments, tobacco leaves were co-infiltrated with a combination of the two Agrobacterium strains harboring the CYP79A1 and the CYP71E1 expression vectors. Thylakoids prepared from the double infiltrated leaves converted radiolabeled tyrosine via the *p*-hydroxyphenylacetaldoxime intermediate into the *p*-hydroxymandelonitrile in a strictly light-dependent manner (Figure 1c). Neither *p*-hydroxyphenylacetaldoxime nor *p*-hydroxymandelonitrile was formed by WT thylakoids. The observed accumulation of

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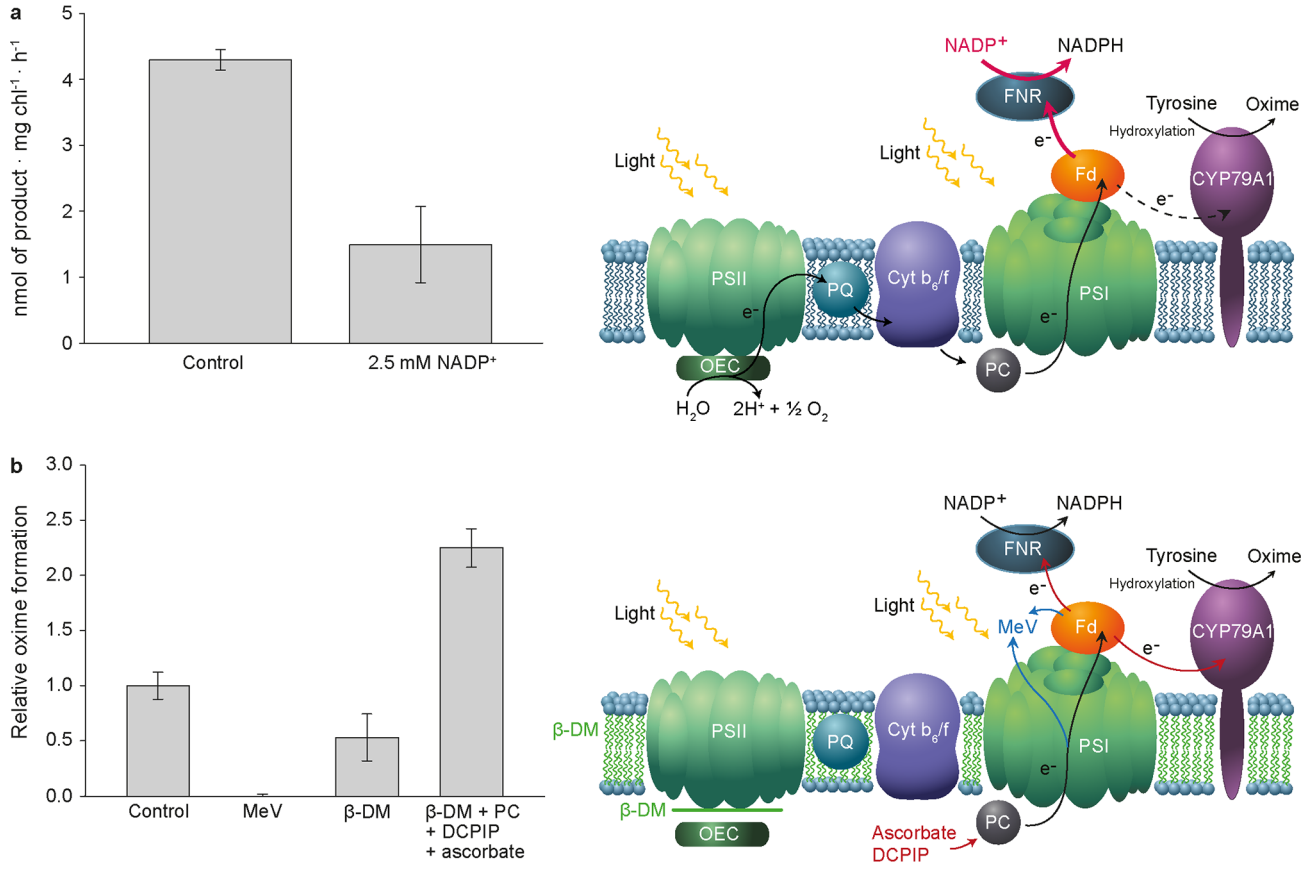

Figure 3. Provision of reducing equivalents generated by photosynthetic electron transport is essential for dhurrin formation. Formation of radiolabeled *p*-hydroxyphenylacetaldoxime by thylakoids containing CYP79A1 following incubation with radiolabeled tyrosine was monitored by TLC analysis. (a) Incubation in the presence of 2.5 mM $NADP^+$ as an electron sink as illustrated (red arrows) in the scheme next to the column diagram. (b) Incubation in the presence of 0.3 mM methylviologen (MeV) as a competing electron acceptor as illustrated in blue in the scheme next to the column diagram. Then thylakoids containing CYP79A1 were solubilized in 0.1% *n*-dodecyl *β*-D-maltoside (*β*-DM) to disrupt membrane integrity and detach the oxygen evolving complex (OEC) from PSII as indicated in the scheme (green). Finally, to restore electron transfer through PSI, DCPIP, ascorbate, and plastocyanin were added to provide an independent electron donor system to PSI in the solubilized thylakoids indicated in red in the scheme.

pathway intermediates in these *in vitro* assays on thylakoids shows that not all of these were properly channeled. This most likely reflects unbalanced expression levels of the three biosynthetic enzymes in the course of the transient expression experiments.

Expression of the Entire Dhurrin Pathway in the Chloroplast. The entire dhurrin pathway was reconstituted in tobacco chloroplasts by co-infiltration of Agrobacterium strains containing the expression vectors for CYP79A1, CYP71E1, and UGT85B1 (Figure 1a). The accumulation and proper targeting of all three enzymes was shown by immunoblot analysis of intact and fractionated chloroplasts (Supplementary Figure S1).

Intact chloroplasts isolated from the triple-infiltrated tobacco leaves were tested for their ability to convert radiolabeled tyrosine into dhurrin *in vitro* in darkness or in the light. In the light, the chloroplast incorporated the radiolabeled tyrosine and synthesized dhurrin *de novo*, demonstrating that all three biosynthetic enzymes were functionally active in the isolated chloroplasts (Figure 2a). The catalytic activity of the expressed P450s in the chloroplast is light-dependent. In the dark, a limited amount of dhurrin formation was observed, which could be explained by reducing equivalents retained in the chloroplast. In contrast it seems that dhurrin formation is directly dependent on light. In the TLC solvent system used for dhurrin analysis, *p*-hydroxyphenylacetaldoxime and *p*-hydroxybenzaldehyde co-migrate as a single band in the solvent front. Administration of radiolabeled tyrosine or *p*-hydroxyphenylacetaldoxime to WT chloroplasts did not result in formation of any metabolites related to the dhurrin pathway. Isolated chloroplasts prepared from leaves infiltrated with the single gene constructs or the two P450 constructs in combination showed the ability to catalyze the expected partial reactions of the dhurrin pathway (Supplementary Figure S2).

The impact of light on *de novo* synthesis of dhurrin in the isolated chloroplasts expressing the CYP79A1, CYP71E1, and UGT85B1 constructs was assessed in a time-course experiment following administration of radiolabeled tyrosine (Figure 2a). Formation of radiolabeled dhurrin was detected already after 5 min incubation in both dark and light incubated samples. However, the capacity to synthetize dhurrin in the dark was limited, and the rate was only one-third of the activity in light. Following 30 min incubation, the amount of dhurrin formed in the light was 12-fold higher compared to the dark (Figure 2a). As observed for the triple infiltrated tobacco chloroplasts, analysis of chloroplasts transiently expressing single P450 constructs or the two P450s in combination also showed a residual metabolic activity in the dark upon administration of the proper radiolabeled substrates (Supplementary Figure S2).

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This suggested that the chloroplasts contain a limited amount of stored reducing power (reduced Fd or components able to reduce Fd) able to support a low rate of P450 activity in the dark. The trace activity observed for both P450s in the dark with intact chloroplasts can be explained by residual amounts of reducing power in the form of Fd/NADPH or generation of Fd/NADPH by metabolic activities in the stroma. The time-course study (Figure 2a) showed that these residual amounts of reducing power are quickly exhausted. However, in all cases significantly increased rates were obtained upon irradiation of the incubation mixtures, unambiguously demonstrating that the P450-catalyzed steps in dhurrin synthesis are light-driven when expressed in the chloroplast.

***In Vivo* Production of Dhurrin.** The experiments reported above were all carried out *in vitro* using isolated thylakoids or intact chloroplasts. We next proceeded to investigate whether intact tobacco leaves transiently expressing CYP79A1, CYP71E1, and UGT85B1 were able to produce dhurrin in the absence of any exogenously added substrates. In this assay dhurrin biosynthesis relies strictly upon the endogenous tyrosine and UDP-glucose present in the chloroplast. The leaves were harvested 1 week after co-infiltration with the three constructs, and the extracted metabolites were analyzed by LC−MS (Figure 2b). Dhurrin production was observed in the triple infiltrated leaves and not in metabolite extracts from non-infiltrated WT leaves. This demonstrates that the entire dhurrin pathway is operating in the chloroplast *in vivo* with endogenous tyrosine derived from amino acid biosynthesis in the chloroplast as the sole substrate. Likewise, the experiment demonstrates that the chloroplasts in the intact leaf synthesize sufficient amounts of UDP-glucose to drive the final glucosylation step. Taken together, this demonstrates successful relocation of the dhurrin pathway to the chloroplasts and that the required precursors for dhurrin synthesis are endogenously present in the chloroplast.

Electron Donors to the P450s in the Chloroplast. To unambiguously demonstrate that the reducing equivalents necessary to drive the catalytic cycle of the two P450s are derived from PSI in the chloroplast, a series of competition experiments were carried out using isolated thylakoids containing CYP79A1. In the isolated thylakoids, the stromal side of the lamellar system harboring PSI, including the site of electron donation from PSI, is freely exposed. The washed thylakoids contain residual amounts of Fd that can shuttle electrons to the P450 enzyme upon irradiation (Supplementary Figure S3). Supplementing the *in vitro* assays with thylakoids with exogenous Fd increased the enzymatic activity of CYP79A1 in a light-dependent manner, demonstrating clearly that Fd reduced by PSI is the electron donor to the P450 enzyme.

The competition between CYP79A1 and $NADP^+$ as electron acceptors for the PSI reduced Fd was tested in the presence of a surplus of exogenously added Ferredoxin-$NADP^+$ oxidoreductase (FNR). Accordingly $NADP^+$ functions as a natural electron sink being converted into NADPH at the expense of reduced Fd produced by light-generated electron transport through PSI. In the absence of POR, the NADPH formed does not function as an electron donor to the P450s. Following addition of high amounts of exogenous $NADP^+$ (2.5 mM), the competition for reducing equivalents resulted in a 64% reduction of the CYP79A1 activity as monitored by *p*-hydroxyphenylacetaldoxime formation (Figure 3a). This demonstrates that CYP79A1 when present in the thylakoids effectively competes for the reducing equivalents generated by PSI. In this study we demonstrate that the electrons driving the P450 activity are derived directly from the reducing power of photosynthesis. The experiment introducing 2.5 mM of $NADP^+$ to act as an electron sink is physiologically relevant since the stromal concentration of $NADP^+$ is around 500 μM and the NADPH/$NADP^+$ ratio is 0.5.[18] In the presence of excess of $NADP^+$, the electron flow carried by the reduced Fd is redirected toward FNR, resulting in a decreased CYP79A1 activity. This shows that CYP79A1 is able to compete for electrons directly from the reduced Fd. Activities of the P450s decreased in the presence of excess of $NADP^+$ and FNR underlining the role of Fd in mediating electron transfer from PSI to the P450s. In a recent study it was also concluded that the presence of membrane or PSI-bound FNR efficiently inhibited electron transfer from reduced Fd to the soluble hydrogenase HydA and thus resulted in low hydrogen production rates observed *in vitro*.[19] Future work with stably transformed tobacco lines will serve to optimize the stoichiometry of the three enzymes, further improving electron transfer to the P450s and thus enhancing dhurrin production.

Methyl viologen (MeV) is an artificial PSI electron acceptor able to capture electrons either from the reduced FeS centers A and B of PSI or from reduced Fd.[20] In the presence of 0.3 mM MeV, no CYP79A1-catalyzed product formation could be detected (Figure 3b). This set of data further confirms that light-generated PSI electron transport is the principal provider of the electrons necessary for the P450 activity. Disruption of thylakoid integrity by solubilization in 0.1% *n*-dodecyl β-D-maltoside (β-DM) inactivates photosystem II (PSII)-mediated electron transport by detaching the oxygen evolving complex from PSII, whereas PSI remains fully functional and active.[21,22] Detergent solubilization also results in partial loss of the soluble PSI electron donor plastocyanin (PC) from the thylakoid lumen. Accordingly, the thylakoids solubilized by β-DM showed a 50% loss of CYP79A1 activity. Furthermore this experiment using detergent to disrupt the thylakoid membrane and PSII demonstrates that light-generated electron transport through PSII is the limiting factor in providing reducing power for CYP79A1 in our *in vitro* assay using thylakoids. The partial electron flow through PSI was restored by supplementing the solubilized thylakoids with an artificial PSI electron donor system consisting of ascorbate, DCPIP, and PC (Figure 3b). The solubilized thylakoids containing CYP79A1 showed a 2.2-fold higher CYP79A1 activity when irradiated compared to the CYP79A1 activity observed following irradiation of control non-solubilized thylakoids.

We here demonstrate that it is possible to redirect and express the entire biosynthetic pathway for production of the tyrosine-derived cyanogenic glucoside dhurrin in the chloroplast. Our study shows that activity of the expressed P450s in the chloroplast is light-dependent directly from the reducing power of photosynthesis and that the required precursors for dhurrin synthesis are endogenously present in the chloroplast.

An important role of P450s is within the biosynthesis of important compounds such as fatty acids, as well as the bioactive steroid hormones, vitamins, antibiotics, and plant defense compounds.[23,24] P450s are also involved in the biosynthesis of many plant terpenoids, the largest class of plant specialized metabolites, with medicinal applications.[25] Medicinal phytochemicals are often only found in small or varying amounts in rare plants and often are difficult to isolate or chemically synthesize.[10] The advantages of chemical

production in a photosynthetic organism are potentially interesting and so far not fully explored. Among the obvious advantages are energy self-sufficiency and self-assembly of enzymes, naturally occurring maintenance and repair systems, and the possibilities for scale-up.

In comparison to most other industrial enzymes, the *in vitro* use of P450s as bioactalysts is compromised by the requirement for supplementation of stoichiometric amounts of expensive NADPH and the presence of a complex membrane-bound POR-based electron-donating system.[26,27] Here we have overcome these negative attributes by relocating the entire P450-dependent pathway for cyanogenic glucoside production to the plant chloroplast, thus using light-generated water splitting as an essentially limitless source of electrons. The stroma of the chloroplast provides a reducing environment that stabilizes the P450s. Photosynthetic cells thus have a high potential for production of desired bioactive natural products when compared to bacteria and yeasts, where NADPH regeneration is typically insufficient to support high levels of P450 activity.[26−28] It is also important to note that the P450s are active when expressed in the thylakoid membrane, are able to function in a light-driven manner, and are not inactivated by the shift in stroma pH from neutral to alkaline following irradiation. This opens the avenue for light-driven synthesis of a vast array of other bioactive natural products in the chloroplast like alkaloids and diterpenoids. The outlined strategy of light-generated synthesis of bioactive natural products thus represents a novel way to integrate and exploit the photosynthetic toolbox in synthetic biology.

METHODS

Vector Construction. Protein expression vectors for expression of the enzymes of the dhurrin pathway in the chloroplast were based on the pEAQ-HT vector.[29]

Vector Construction. The full-length Fd gene (FedA, NCBI gene ID: 22136515) was PCR-amplified from *A. thaliana* ecotype Columbia cDNA and cloned into *Nde*I-*BamH*I-linearized *E.coli* expression vector pET-15b for the subsequent cloning steps. The fusion constructs were obtained by ligation of the PCR amplified DNA sequences encoding the Fd transit peptide and the full-length enzyme (CYP79A1 or CYP71E1 or UGT85B1) into pEAQ-HT.[29] The nucleotide sequences encoding the full-length native enzyme and the Fd transit peptide corresponding to the first 52 amino acid residues were PCR amplified using the primers listed in Supplementary Table S1. The PCR products of Fd (FedA) transit peptide and the enzyme were digested by restriction enzymes as indicated in Supplementary Table S1. The digested PCR products were directionally coligated into linearized pEAQ-HT generating the fusion DNA construct encoding the native enzyme with an N-terminal Fd transit peptide. All primers and restriction sites used to clone the three cDNAs fused to a ferredoxin transit peptide are presented Supplementary Table S1.

Agrobacterium Infiltration of *N. benthamiana*. Seeds of wild-type *N. benthamiana* were sown in soil (Pindstrup substrate number 2) and grown for 4 weeks in greenhouse with a 16/8 h light/dark cycle and a day/night temperature cycle of 24 and 17 °C.

The vector constructs were introduced into *Agrobacterium tumefaciens* PGV3850 by electroporation as described in Haldrup et al.[30] Transformed *A. tumefaciens* strain PGV3850 was grown in 10 mL of LB media (O/N) at 28 °C, 220 rpm with 25 μg mL^{-1} rifampicin and 50 μg mL^{-1} kanamycin. One milliliter of (O/N) cell culture was used for inoculation of 10 mL of LB media with rifampicin and kanamycin, and the cells were grown (O/N) to reach stationary phase, sedimented by centrifugation at 4,000*g* for 10 min at RT, and resuspended in sterile infiltration buffer (10 mM MES, 10 mM $MgCl_2$, and 100 μM acetosyringone) to reach a final OD_{600} of 0.4−0.6. Cells were shaken in infiltration buffer for 1−3 h before infiltration of *N. benthamiana* leaves with a syringe.[31] The infiltrated plants were placed in the greenhouse at the same growth conditions as described above.

Thylakoid Membrane Isolation. Thylakoid membranes were isolated from *N. benthamiana* leaves 5 d after Agrobacterium infiltration. All steps were carried out on ice and under green light. Leaves were homogenized in buffer (0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM Tricine (pH 7.5), 100 mM sodium ascorbate, and 5 mg mL^{-1} BSA). Following filtration of the homogenate, chloroplasts were sedimented at 5,000*g* for 10 min and lysed by resuspension in 5 mM Tricine (pH 7.9) for 15 min. The thylakoids were sedimented at 11,200*g* for 10 min and resuspended in homogenizing buffer without sodium ascorbate and BSA but supplemented with 20% (v/v) glycerol. Total chlorophyll and chlorophyll *a*/*b* ratios were determined in 80% acetone according to Lichtenthaler.[32]

Chloroplast Isolation and Fractionation. Intact chloroplasts were isolated from *N. benthamiana* leaves 5−7 days after Agrobacterium infiltration essentially as described by Robinson and Mant.[33] Leaves were homogenized in HS buffer (50 mM Hepes, KOH pH 8 and 0.33 M sorbitol). Homogenate was filtered and centrifuged at 3,330*g* for 2 min. The chloroplast pellet was gently resuspended in HS buffer, layered onto precooled Percoll pads, and centrifuged at 1,400*g* for 8 min. Sedimented intact chloroplasts were washed in HS buffer, resedimented at 3,000*g* for 2 min, and resuspended in HS buffer (final chrlorophyll concentration: 1 mg mL^{-1}). To fractionate chloroplast envelope membranes, intact chloroplasts were centrifuged at 2,100g for 1 min and lysed by resuspension in HM buffer (10 mM Hepes, KOH pH 8 and 5 mM $MgCl_2$) + 10 mM EDTA for 10 min. The lysed chloroplasts were centrifuged at 9,830*g* for 2 min, and the supernatant containing the stroma fraction was separated from thylakoid membrane pellet.

Immunoblotting. Protein extract samples were electrophoresed on 12% Bis-Tris SDS-PAGE gels (Criterion, Bio-Rad) in MOPS buffer for 1 h at 200 V. Protein transfer from SDS-PAGE to nitrocellulose membranes (40 min at 100 V) was performed according to the manufacturer's instructions (Criterion blotter, Bio-Rad). The membranes were blocked (1 h, RT) in 5% skimmed milk dissolved in PBS + 0.05% Tween-20 (PBS-T), washed, and incubated (O/N) with primary antibodies to CYP79A1 and CYP71E1 diluted 1:5000, UGT85B1 1:1000, Rubisco Rbc-L subunit (Agrisera) 1:10000, PSI-D 1:20,000, Fd 1:1000, FNR 1:1000 in 5% skimmed milk PBS-T. The blots were washed and incubated with polyclonal goat anti-rabbit immunoglobulins/HRP (DAKOCytomation) for 1 h at room temperature at a dilution of 1:2000.

Secondary antibodies were detected using a chemiluminescent detection system (Super-Signal, Pierce) according to the instructions of the manufacturer. The chemiluminescent signal produced was recorded digitally using a cooled CCD camera with the AC1 AutoChemi System (Ultra-Violet Products Ltd., Cambridge, U.K.). The exposure time was set to 5 min, with accumulative snapshots at 30 s intervals. Signal intensity was

analyzed using the LabWorks Analysis Software (Ultra-Violet Products Ltd., Cambridge, U.K.).

Enzyme Activity Assays in the Thylakoid Membrane. CYP79A1 Activity Assay. CYP79A1 catalyzes the conversion of L-tyrosine to (*Z*)-*p*-hydroxyphenylacetaldoxime. CYP79A1 activity in thylakoid membranes was measured in 20 mM Tricine buffer (pH 7.5) supplemented with 100 μM spinach Fd and 2.6 μM [U-^{14}C]L-tyrosine (specific activity 482 mCi/mmol from Perkin-Elmer) in a total volume of 200 μL. The samples were kept at 25 °C, and the reaction was started by illumination with a Schott KL 1500 light source fitted with a single gray filter (Schott NG4), resulting in a light intensity of 100 μmol photons m^{-2} s^{-1}. The reaction was stopped after 30 min, and the reaction product ((*Z*)-*p*-hydroxyphenylacetaldoxime) was extracted in 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).

CYP71E1 Activity Assay. CYP71E1 catalyzes the conversion of (*Z*)-*p*-hydroxyphenylacetaldoxime to *p*-hydroxymandelonitrile. CYP71E1 activity in thylakoid membranes was measured in same way as for CYP79A1 except that the substrate used was [U-14C]*p*-hydroxyphenylacetaldoxime and the product detected was *p*-hydroxybenzaldehyde, the dissociation product of *p*-hydroxymandelonitrile.

[U-^{14}C](*Z*)-*p*-Hydroxyphenylacetaldoxime was prepared from [U-^{14}C]L-tyrosine following incubation with recombinant CYP79A1 expressed in *E. coli*.[34]

Enzyme Activity Assays in the Chloroplast. Assays with intact chloroplasts (20 μg of chlorophyll) were performed in the presence of 8 mM MgATP and the appropriate radiolabeled substrate as described in the thylakoid assays. Illumination, temperature, and extraction of the products were as described above.

UGT85B1 Activity Assay. UGT85B1 glucosylates the *p*-hydroxymandelonitrile to form dhurrin. The activity assay was adapted from Kannangara et al.[35] Assay mixtures containing isolated intact chloroplasts (20 μg Chl) in the presence of 100 mM Tris-HCl (pH 7.5), 3.3 μM UDP[^{14}C]glucose and 5mM *p*-hydroxymandelonitrile were irradiated as described above. Incubations were stopped by adding 2 μL 10% (v/v) acetic acid. Products formed were analyzed by TLC (silica gel 60 F254 plates; Merck). Formation of radiolabeled dhurrin was monitored following development of the TLC in ethyl acetate/acetone/chloroform/methanol/water (20:15:6:5:4 v/v). Radiolabeled products were visualized and quantified using a STORM 840 PhosphorImager (Molecular Dynamics, http://www.moleculardynamics.com).

LC−MS Analysis. Infiltrated leaves were harvested from plants, snap-frozen in liquid nitrogen and grinded. Dhurrin was extracted in 85% MeOH, 0.5% formic acid and heated for 3 min at 95 °C. The sample was centrifuged at 8.000g for 3 min and the supernatant was filtered, concentrated in a Scanspeed 32 (Scanvac, Labogene) to a final volume of 30 μL and subjected to LC−MS analysis as described in Saito et al.[36]

■ ASSOCIATED CONTENT

Supporting Information

Primers and restriction sites used to clone the three cDNAs fused to a ferredoxin transit peptide, localization of transient expressed proteins constituting the dhurrin pathway in tobacco leaves, activities of the two P450 enzymes clearly stimulated by light, P450 activity dependence on Fd as the main electron donor. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.Z.N., P.E.J., B.L.M., K.J., and L.M.L. conceived the project and designed the experiments. A.Z.N., B.Z., and K.J. performed the experiments. C.E.O. performed the LC−MS analysis. L.M.L. conceived and realized the figure graphics. A.Z.N., P.E.J., and B.L.M. wrote the paper.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

DCPIP, 2,6-dichlorophenolindophenol; β-DM, *n*-dodecyl β-maltoside; ER, endoplasmic reticulum; Fd, ferredoxin; FNR, ferredoxin-NADP$^+$ reductase; MeV, methylviologen; NADP$^+$/NADPH, nicotinamide adenine dinucleotide phosphate; P450, cytochrome P450; PC, plastocyanin; POR, P450 oxidoreductase; PSI, photosystem I; PSII, photosystem II; TLC, thin layer chromatography; TP, transit peptide; WT, wild-type

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