Membrane composition influences expression yield of plant cytochrome P450s in *E. coli* lysate-based cell-free systems

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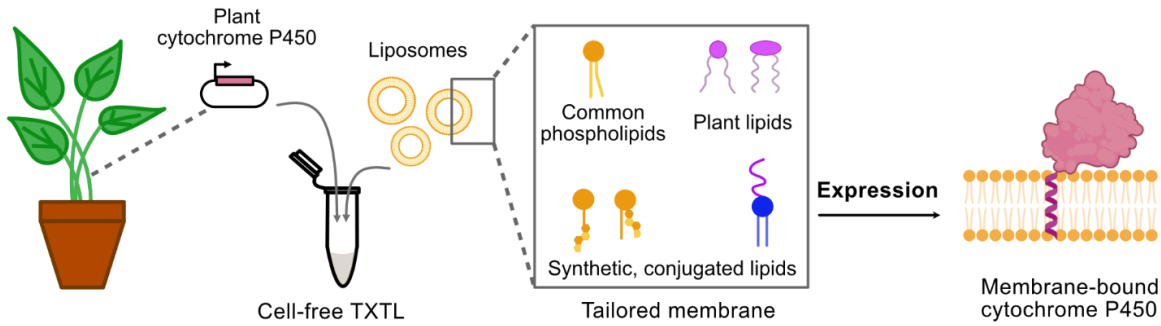
Graphical abstract



Abstract

Plant cytochrome P450 enzymes are central to natural product biosynthesis, but remain difficult to express in microbial hosts due to their transmembrane nature. Lysate-based, cell-free expression systems allow supplementation with artificial membranes to support expression and translocation of transmembrane proteins. We developed a framework to systematically test liposomal membrane compositions to enhance plant P450 expression yield. Adjustments to common phospholipid ratios or addition of plant galactolipids had minimal impact on expression. In contrast, blended liposomes containing Egg PC, sterol-conjugated phospholipids, and PEGylated lipids produced concentration-dependent increases in expression. Expression of an *E. coli* mechanosensitive channel and three plant P450s improved for more than two-fold, with some P450s showing up to 14-fold enhancement. These findings highlight membrane composition as a key determinant of P450 expression yield in cell-free expression systems. While P450s activity was not measured, these findings provide a framework for

future workflows toward achieving functional plant transmembrane enzymes for bioproduction of natural products.

Keywords: liposome, membrane, cell-free systems, TXTL, expression, cytochrome P450s

Introduction

Heterologous production of bioactive, plant natural products has been demonstrated in microbial hosts[1,2], but remains challenged by the difficulties of expressing plant transmembrane cytochrome P450s (CYPs) at high levels[3,4]. CYPs are heme-containing transmembrane enzymes with N-terminal transmembrane helix anchors[5] (**Figure 1A**), which often require specific lipid environments for expression and translocation.[6] *In vitro*, cell-free systems could be used to bypass cellular constraints and enable the expression of CYPs.[7–9] However, establishing a functional transmembrane CYP *in vitro* would require reconstituting proteins into membranes[10] or using an expression platform that allows the addition of artificial membranes for protein translocation[11].

Cell-free gene expression systems (CFEs) from *E. coli* lysate[12] contain bacterial transcriptional and translational machinery and have been used for expressing various membrane proteins[13–17]. The accessible nature of CFEs permits the addition of artificial lipid bilayers in the form of 2D membrane supports[18], lipid rafts[11], synthetic cells[19] and exogenous liposomes[20–22]. Despite these recent advances, approaches for creating membrane-based CFEs for expression of highly-active plant transmembrane CYPs are still underexplored. In microbial hosts, membrane engineering was implemented to

reduce membrane incompatibility that could potentially affect expression, translocation, and function of recombinant membrane proteins.[23–25] In principle, commercially-available lipids with varying structures, charges, chain length and degree of saturations can be used to create artificial membranes. Accordingly, lipid compositions can be tailored to develop CFEs that support expression and translocation of plant CYPs.[26]

Recent efforts to obtain functional expression of eukaryotic, transmembrane CYPs in CFEs have employed a range of expression systems and membrane environments. For example, human CYPs have been expressed in CFEs from a modified Chinese hamster ovary (CHO)[7] and Leishmania cell lysates[8], both in the presence of endogenous microsomes. Likewise, functional reconstitution of plant CYPs was reported in wheat germ extract expression systems supplemented with liposomes from soybean phospholipids, asolectin.[9] While these studies demonstrate that *in vitro* expression of CYPs is feasible when the expression environment matches their native context, such approaches rely on pre-engineered lysates and residual or heterogeneous lipid membranes, limiting the ability to systematically dissect the contribution of individual lipid components on CYP expression. Overcoming this limitation, a generalizable cell-free expression framework that uses defined membrane compositions could enable systematic evaluation on how specific lipid compositions influence the expression yield of CYPs.

Previously, we outlined strategies to enhance cell-free membrane protein expression by augmenting CFEs with membranes, engineering the chemical composition of lipids, and adjusting membrane physical properties.[26] In this study, we tested these strategies by developing a framework for expressing plant transmembrane CYPs in CFEs

through supplementation of tailored liposomes to achieve biologically-relevant properties for CYP localization. To do this, we fabricated the liposomes using commonly-used phospholipids, plant galactolipids, and specialized synthetic lipids (**Figures 1B-C**). We monitored the expression and translocation of CYP into the membrane using a microplate reader by fusing a monomeric green fluorescent protein (deGFP)[19,21] to the C-terminus of plant CYPs (**Figure 1D**). A concentration-dependent fluorescence response was observed upon the addition of tailored liposomes made from natural and specialized synthetic lipids. Interestingly, we found that tailored liposomes supported cell-free expressions of not only an *E. coli* model membrane protein, but also three transmembrane CYPs from multiple families and plant species with up to 14-fold improvement. Taken together, this approach provides a groundwork for achieving high levels of transmembrane CYP expression in membrane-augmented *E. coli* CFEs[26], with potential applications in enzyme structural characterization[13,27], plant biosynthetic pathway prototyping[28], and cell-free bioproduction of natural products[29].

Materials and methods

Plasmids construction and preparation. Plasmids were designed with Benchling sequence designer or SnapGene (**Supplementary Table S1**). PCR primers were synthesized by IDT and gene fragments were synthesized by Twist Biosciences or Thermo Fisher GeneArt. DNA fragments were amplified using Phusion DNA Polymerase (Thermo Fisher) for cloning using 5X In-Fusion HD kits (Takara Bio). Plasmids were transformed into chemically competent *E. coli* NEB Turbo cells (New England Biolabs) in

Luria-Bertani (LB) medium or agar plates supplemented with antibiotics (100 µg/mL carbenicillin) and grown at 37 °C overnight. Plasmids were purified using QIAprep Spin Miniprep Kits (Qiagen 27104) according to the manufacturer's protocol. Plasmid concentrations were quantified via spectrophotometry (Nanodrop 2000c, Cat. ND-2000C). Constructs were verified by Sanger (Genewiz-Azenta) or whole-plasmid (Primordium) sequencing and analyzed using Benchling or SnapGene. Prior to use in a cell-free expression reaction, plasmids were purified using a PCR purification kit (Invitrogen PureLink, Cat. K310001) and eluted with nuclease-free water.

Liposome formulation. Liposomes were made using lipids from Cayman Chemicals or Avanti Research (**Supplementary Table S2**). Pre-dissolved lipids in chloroform were mixed (**Supplementary Table S3**) and dried using a slow Argon stream to create thin film layers. The solvent residues were removed with vacuum drying. Thin lipid films were rehydrated using a feeding solution containing the same components as the cell-free reaction except for the DNA and lysate that were replaced by water.[12,30] The hydrated lipid mixture was lightly vortexed and extruded through 100 nm polycarbonate membrane filters (Cytiva-Whatman, Cat. 800309) using a Mini-Extruder (Avanti Research, Cat. 610000) at ambient temperature, followed by sonication for 30 minutes in a water bath ultrasonicator (iSonic P4810). The resulting liposomes were added into the cell-free TXTL reaction.

Cell-free membrane protein expression. Cell-free expression systems used in this study were a sigma 70 master mix derived from *E. coli* lysate (TXTL 2.0)[31], commercially

available as myTXTL (Arbor Biosciences). Membrane proteins were expressed under a sigma 28 cascade, where sigma 28 proteins were expressed through a P70a promoter.[19] A 10 µL reaction was assembled on ice in a clean 1.5 mL Eppendorf tube from the TXTL master mix (75 vol%), and the remaining 25 vol% filled with 0.5 nM p70a-S28, 5 nM p28a-CYP, liposome at a predetermined concentration (mg/mL), and nuclease-free water (**Supplementary Table S4**). In reactions without plasmids and/or liposomes, nuclease-free water was used as a replacement. The reaction was aliquoted into three technical replicates of a 2.5 µL in a V-bottom 96 well plate (Costar, Cat. 3363) sealed with a lid (Costar, Cat. 3080). deGFP fluorescence readouts were collected using a plate reader (Biotek Synergy H1) following a 12-24 hour incubation at 29°C. The following settings were used for the plate reader measurement: excitation: 485 nm, emission: 528 nm, gain: 60, measurement from the bottom. GFP measurements were quantified using a linear calibration standard curve of purified eGFP (Cell Biolabs, Inc.) previously described in Garamella, *et al*.[31]

Statistical analysis and data visualization. All reactions with the expression plasmids and titrated liposomes were background subtracted using controls of 'plain', unreacted TXTL mixed with the corresponding liposome concentration. Statistical significance was calculated using two-tailed unpaired Welch's *t*-tests. Data were plotted using Prism (GraphPad).

Results

Systematic screening of common lipids revealed limited impact on CYP expression

To investigate if cell-free expression of transmembrane CYPs can be improved in the presence of exogenous liposomes, we created liposomes from common phospholipids known to produce well-defined, reproducible membranes.[19] The liposomes were created using a combination of thin lipid film hydration, extrusion and sonication to minimize polydispersity and lamellarity (**Figure 1C**). We formulated liposomes from a representative natural lipid, chicken egg phosphatidylcholine (Egg PC), mixed with a standard phospholipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), in a 90:10 molar ratio. This formulation was previously used to support cell-free expression of an *E. coli* mechanosensitive ion channel (MscL).[19] With this formulation, we monitored the expression of a plant CYP, geraniol 8-hydroxylase from *Catharanthus roseus* (CrG8H), under a sigma 28 transcriptional cascade[19] (**Figure 2A, Supplementary Figure S1A**). Sigma-28 transcriptional cascade was chosen to introduce a modest delay in protein synthesis, which helps stagger the production of CYPs relative to their co-translational membrane insertion and folding.[19,32] All membrane proteins in this study were fused to monomeric deGFP (**Figure 1D**, **Supplementary Table S1**), which acts as a marker for real-time monitoring of expression and translocation using a plate reader.[21] GFP fluorescence has been validated as a reliable marker of membrane protein insertion and folding[33–35], such that increases in fluorescence can be interpreted as indicative of association or insertion of CYPs into the membrane (**Supplementary Figure S2**). In addition, MscL was included as a control since its membrane-assisted expression in

CFEs is well characterized.[19,32,36] Upon the addition of titrated common liposomes, we saw an average of 2-fold improvement in MscL expression compared to baseline at 0 mg/mL liposomes (**Figure 2A, left**). However, the same trend was not observed in CrG8H (**Figure 2A, right**).

As heterologous expressions of membrane proteins often require specific lipid environments[6], we hypothesized that adjustments to common liposomes to match that of the native *C. roseus* total lipid contents reported in the literature[37] could improve CrG8H expression (**Figure 2B**). One study reported that *C. roseus* has a PC/PE ratio of 1.34 or approximately 57:43, with 16:0 (dipalmitoyl, DP) as the prevalent fatty acid group.[37] Thus, adjustments were made to change the PC/PE ratio to 57:43 (**Figure 2B, top left**), swap the fatty acid tail groups to DP (**Figure 2B, top right**), or a combination of both (**Figure 2B, bottom left**). Despite these adjustments, no improvement on CrG8H expression was observed, while MscL expression appeared to increase by 1.4 to 1.5-fold regardless of the adjustments made (**Figure 2B**). Ultimately, this result could indicate that a rather complex membrane beyond common phospholipids might be required to achieve translocation of CrG8H into liposomes.

Addition of plant galactolipids into liposome did not impact CYP expression

Membrane composition differs across organisms, with galactolipids predominantly found in plants.[26,38] As summarized in our previous review[26], monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) are the two most abundant lipids in plant membranes. Building on our initial evaluation of PC/PE bilayers, we next tested whether the addition of plant galactolipids to PC/PE bilayers would improve CYP expressions

(**Figure 3A**). Similar to PE, MGDG has a conical shape that promotes membrane curvature, while DGDG resembles PC with its cylindrical shape and capability to form bilayers.[39–41] The addition of MGDG and DGDG was intended to make the membrane composition biologically-relevant and more plant-like.

To further explore how structural modifications impact CYP expression, we introduced various PCs, from standard to synthetic[42], while keeping the PC head group constant. The standard PC used was 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), a chemically defined single-component lipid, corresponding to the oleoyl (C18:1) fraction (~32%) of natural Egg PC.[43] The synthetic PCs were 1-palmitoyl-2-cholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (PChemsPC), 1,2-dicholesterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (DChemsPC), and 10-cholesteryloxy decylphosphocholine (Chol-C10-PC). Specifically, PChemsPC and DChemsPC contained modified acyl chains, and Chol-C10-PC incorporates a cholesterol moiety, providing defined structural variations within the PC context.

In this experiment, we maintained a similar fluorescence monitoring workflow and liposome titration as above (**Figure 1D**). Though no significant improvement in CrG8H expression was observed from adding galactolipids, a slight increase in expression was apparent when synthetic PCs were included in the liposome formulations (**Figure 3B**). The fact that all synthetic PCs contain sterol-like moieties suggests that membrane biophysical properties, such as bilayer fluidity, lipid packing, and lateral pressure profiles, may play an important role in CYP translocation.

Specialized synthetic lipids improved expression of various CYPs

Stable lipid membrane is crucial to ensure proper insertion and folding of membrane proteins[44], particularly CYPs, which use a single N-terminal transmembrane helix as a membrane anchor[5]. On top of that, replicating the exact physiochemical characteristics of the membrane and its surrounding outside endogenous hosts is known to be difficult.[26] In this experiment, we attempted to stabilize the membrane by including the specialized, synthetic lipids used in the previous experiment to create blended liposomes (**Figure 4**). To do this, we used Egg PC to form stable bilayers, then added two synthetic, sterol-conjugated PCs (PChemsPC, and Chol-C10-PC)[42] as their cholesterol moieties could fortify the bilayer by preventing curvature deformation.[45] Moreover, sterols in plant lipid membranes have been reported to influence protein functions.[46,47] To decrease non-specific adsorption and prevent vesicle fusions, we also added a PEGylated lipid, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) with a 2000 MW polyethylene glycol (PEG) chain attached to its head group (DPPE-PEG2000).[48] All four lipids were mixed in equimolar ratio to form blended liposomes (**Figure 4A**). Cell-free reactions were set up similar to the experiments above, with fluorescence monitoring and liposome titration workflows maintained (**Figure 1D**).

Following the addition of blended liposomes, MscL showed a concentration-dependent expression profile as liposome concentration was increased gradually, with an average of 9-fold improvement from the no-liposome samples (**Figure 4B, left**). A similar concentration-dependent profile was observed in CrG8H, where we had previously seen no improvement in expression despite supplying various tailored membranes (**Figure 4B,**

right). The addition of blended liposomes resulted in an average of 7-fold increase in CrG8H expression, with an optimum of 12-fold increase at liposome concentration of 8 mg/mL.

With the promising concentration-dependent expression seen in CrG8H (**Figure 4B, right**), we further investigated if the blended liposomes could also improve the expression of plant CYPs of different families and species (**Supplementary Figure S1A**). To test this, we selected a G8H from *Lonicera japonica* (LjG8H), previously expressed in *S. cerevisiae* with a higher 8-hydroxygeraniol titer than that of a CrG8H.[49] Another CYP was a cinnamate 4-hydroxylase from *Lycoris radiata* (LrC4H) that catalyzes the conversion of *trans*-cinnamic acid into p-coumaric acid, a precursor of flavonoids.[50] In the presence of blended liposomes, both LjG8H and LrC4H expressions were improved compared to when no liposomes were added (**Figure 4C**). Similar to CrG8H, LjG8H expression was improved by an average of 9-fold, with a maximum of 14-fold improvement at 8 mg/mL liposome (**Figure 4C, left**). Likewise, LrC4H experienced an increase in expression of 2.6-fold on average compared to samples without liposomes added (**Figure 4C, right**). Together, these results showed that exogenous liposomes can be tailored for improving expression of various membrane proteins in CFEs, including plant transmembrane CYPs. More importantly, we demonstrated that liposomes tailored for stability could be used to assist expression of three plant transmembrane CYPs from multiple families and species.

Discussion

CYPs are an enzyme superfamily found in all domains of life, responsible for catalyzing various oxidation and monooxygenation reactions.[51] Plant CYPs, in particular, hold importance for bioproduction of high-value natural products, and have been engineered into microbial hosts to enable scalable and sustainable biomanufacturing.[52,53] Despite their importance, expressing plant CYPs in microbial hosts remains difficult due to complex biological requirements, such as CYP localization into the membrane.[54] The flexibility of manipulating reaction components in cell-free reactions could enable the creation of biologically-relevant membrane environments for the expression of plant CYPs without sophisticated host engineering. These approaches, previously outlined, include augmenting CFEs with artificial membranes, engineering lipid chemical compositions of the membrane, and adjusting membrane physical properties.[26] In this study, we applied those strategies and demonstrated the expression of three plant transmembrane CYPs in unengineered *E. coli* CFEs. The successful implementation of this approach across CYPs from multiple enzyme families and plant species suggests that tailored liposomes may also support the expression and translocation of other transmembrane CYPs. Beyond that, membrane-augmented CFEs could propel the field of cell-free synthetic biology forward as platforms for both cell-free bioproduction of natural products and cell-free investigation of biological processes involving membrane-bound proteins.

Reconstitution of membrane proteins into native membranes was shown to support structural stability and functional integrity.[10,55] Biocompatible membrane systems including monolayer micelles[56], designer surfactants[57,58], and bilayer liposomes[9] have been widely used to support enzyme activity in aqueous systems. Here, we focus on liposome-based systems to provide bilayer environments analogous to that of cellular membranes and

increase the likelihood of proper CYP expression and folding. In this study, lipid compositions and ratios were adjusted to match that of the total plant membrane composition[37], however, little to no improvements were observed in CYP expression. This could be due to the fact that the reported ratios reflect the total lipid content rather than that of an endoplasmic reticulum (ER) membrane, where plant CYP is localized.[59] Likewise, galactolipids are abundant in the thylakoid membrane of chloroplasts[38], but not in the ER membrane. This is consistent with a previous study showing functional human CYP expression in the presence of ER membrane-derived microsomes[7], providing a native-like environment for CYP localization and interaction with cytochrome P450 reductase (CPR).

Similarly, eukaryotic CYPs expressed in prokaryotic, *E. coli* CFEs without membrane supplementation fail to achieve catalytic activity, whereas expression in a compatible eukaryotic, Leishmania CFEs containing residual endogenous membranes is functional[8]. This highlights the importance of membrane compatibility for proper folding and function. Importantly, our results showed that supplementing *E. coli* CFEs with blended liposomes enhances the expression yields of various plant CYPs, suggesting that systematic membrane designs may help overcome the limitation previously observed in *E coli* CFEs and provide a versatile platform for CYP prototyping. Furthermore, the upregulation of CYP genes in plants[60,61] are closely linked to the production of natural products in response to specific stressors[62]. In relation to this, lipid compositions have also been shown to dynamically change upon exposure to stressors.[37] By conserving PC and PE head groups throughout the experiments while introducing biologically-relevant (galactolipids) and structurally-modified (synthetic PCs) lipids, we were able to attribute

observed effects on CYP expression on specific lipid components, providing a mechanistic baseline for interpreting the role of membrane compositions. Future studies on how dynamic changes in native lipid composition affect expression of CYP could enable precise membrane tailoring to support cell-free expression of plant CYPs.

Recent advances in cell-free engineering have enabled functional expression of CYPs in various CFEs platforms.[7–9] However, the factors governing CYP maturation and activity remain incompletely understood and can differ across platforms. In our *E. coli*-based CFEs, blended liposomes improved expression (**Figure 4**), yet activity remained limited (**Supplementary Figure 1B**), motivating a closer examination of reaction variables and interactions between components. Expression alone is not sufficient as CYPs are hemoproteins that require proper folding, membrane insertion, and incorporation of a heme prosthetic group to catalyze monooxygenation reactions.[63,64] In *E. coli* lysates, heme availability is limited and subject to degradation, while exogenous hemin supplementation paradoxically inhibited CYP expression (**Supplementary Figure 1C**). Alternatively, heme can be added post-translationally to avoid heme interference during CYP translation[8], suggesting that the timing of cofactor supplementation is an important variable in achieving active CYPs. Previous studies also reported that lipid assemblies, though in the micellar context, could influence heme supplementation efficiency by modulating heme solubility and aggregation state, and can act as either reservoirs or sinks for heme, potentially influencing heme availability for protein incorporation.[65,66] Together, these observations suggest that even when CYPs are inserted into membranes, they may misfold or fail to mature into active enzymes if the appropriate cofactors and assembly pathways are lacking. One potential solution to

recover CYP activity in CFEs is through co-expression of aminolevulinic acid synthase (ALAS) to generate 5-aminolevulinic acid, a precursor that endogenous lysate enzymes can convert into heme.[67] Hematin supplementation was also shown to outperform hemin in enhancing CYP expression and function[9], indicating that the choice of heme cofactor can influence CYP bioactivity. Additional characterizations, such as circular dichroism spectroscopy[68] or protease-protection assays[69], could identify misfolded or improperly oriented enzymes, as folding and orientation strongly influence activity. Looking forward, optimizing lysate composition to balance expression, folding, cofactor biosynthesis, and membrane integration will be essential for producing functional transmembrane plant CYPs in cell-free systems.

Activity of Class II plant P450s is dependent on the availability of CPR redox partners to ensure efficient electron transfer from NADPH.[64,70] The importance of CPR in an *in vitro* setup was observable in a study on plant *Sorghum bicolor*, where CPR needs to be supplemented in excess to achieve optimal CYP activity.[71] Similarly, functional expression of eukaryotic CYPs could be achieved via lysate engineering that overexpressed CPR prior to making cell extract[7], or, through co-expression of CPR alongside CYP[8]. Additional parameters, such as CYP-CPR pairing compatibility[8,72,73], their proximity and interactions[74,75], and cofactor balancing[76], should also be taken into account to ensure sufficient electron transfers and substrate conversions. Previous work has shown functional reconstitution of C4H and its cognate CPR in wheat-germ extracts supplemented membranes from soybean asolectin[9], which are rich in ER-like lipids. The ability of CYPs to operate in such complex lipids underscores the importance of not only CPR, but also the surrounding membrane environments for CYP activity. Given that our

membrane-augmented framework uses a composition-defined lipid membrane and enhances expression yields across multiple CYPs, it represents a promising platform for reconstituting transmembrane CYPs in an unengineered, prokaryotic CFEs. In the future, applying the framework to CPRs could help identify a universal formulation that works for both CYPs and CPRs to support functional reconstitution of CYPs. As we are moving towards building complex biosynthetic pathways, dynamic multi-gene programs can be implemented to control expressions of multiple CYPs and CPRs for cell-free bioproduction of plant natural products.[77] Ultimately, the development of membrane-based CFEs for expression of transmembrane CYPs has the potential to advance the field of cell-free synthetic biology by serving both as platforms for bioproduction and investigating biological processes.

Declaration of competing interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

James M. Carothers is an advisor to Wayfinder Biosciences.

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Author contributions

Conceptualization: W.S., P.P-Y., and J.M.C. Experimental design, methodology, investigation, data analysis, visualization: W.S. Liposome preparation methodology, experimentation, and data analysis for Figure 3: D.G. Manuscript writing: W.S. and J.M.C. Manuscript editing: W.S., V.N., P.P-Y., and J.M.C. Project supervision and funding acquisition: V.N., P.P-Y., and J.M.C. All authors approved the submission of the manuscript.

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Supplementary Information

DNA sequences; lipids used in this study; liposome formulations; cell-free reaction conditions; schematics on CYP biocatalysis, testing CYP functionality, data on heme cofactor titration and membrane association.

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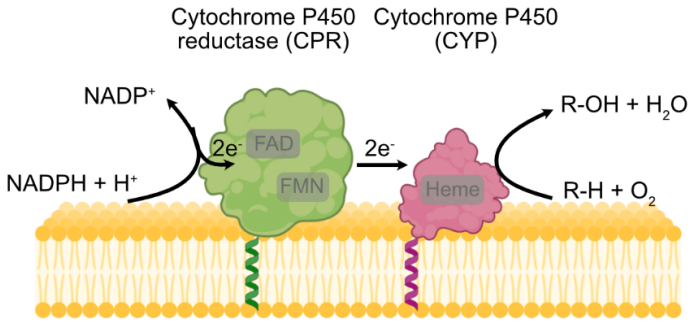
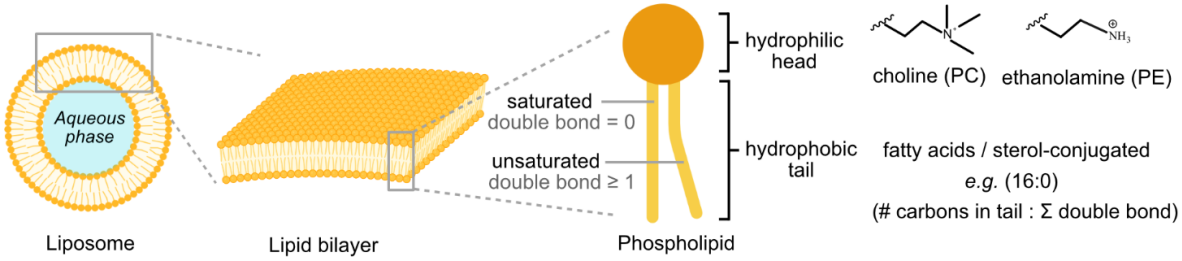
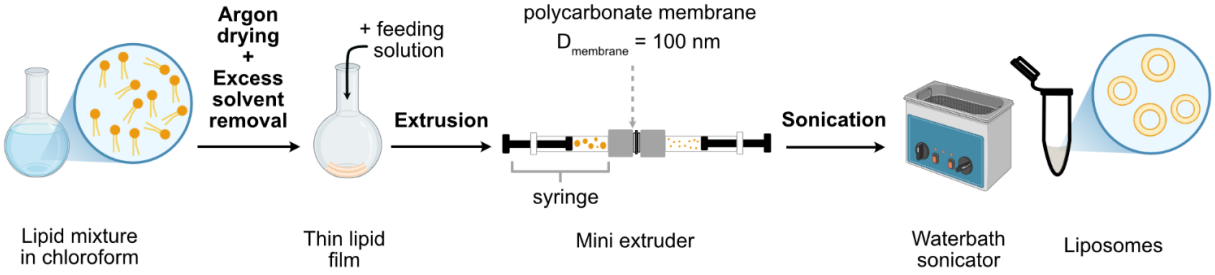
A Schematic of membrane-bound cytochrome P450 and cytochrome P450 reductase



B Schematic of liposome from phospholipids



C Liposome preparation



D Liposome-assisted cell-free expression of membrane proteins

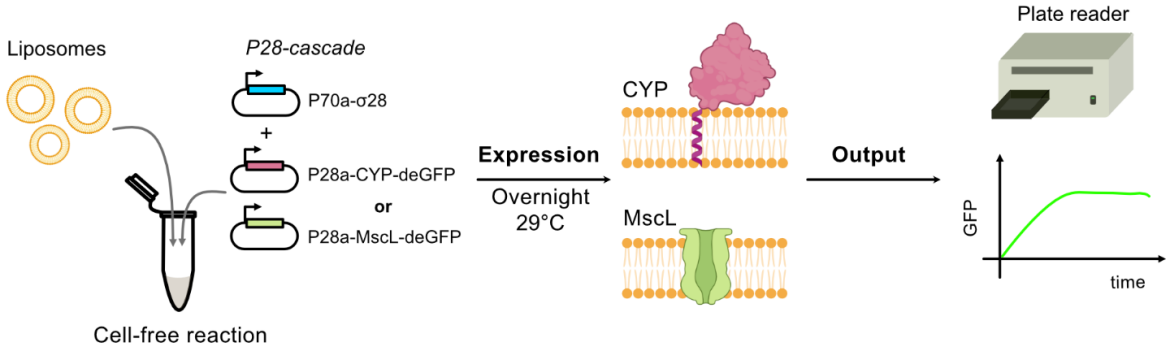
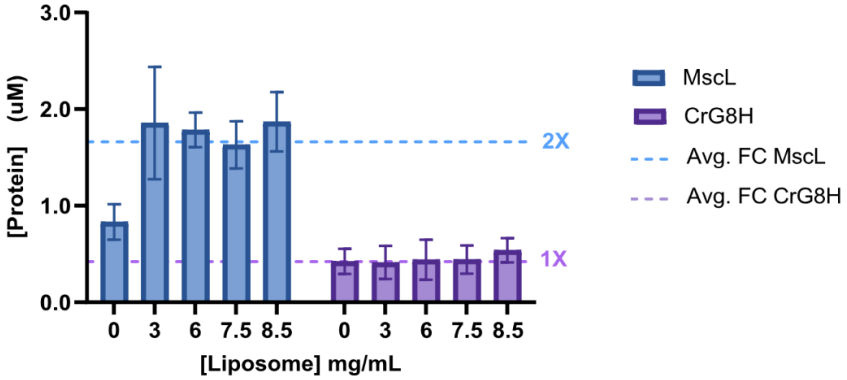


Figure 1. Membrane-assisted expression of plant cytochrome P450 in cell-free systems. **A**) Membrane-bound plant CYP on lipid membrane with a CPR, facilitating electron transfers from NADPH for monooxygenation of natural products. **B**) Tailored liposomes from phospholipids. Phospholipid consists of a hydrophilic head group and a hydrophobic tail group. Different chemical moieties on the heads and tails influence membrane properties. **C**) Liposome preparation workflow. Various lipids are mixed in chloroform at certain molar ratios. Solvent is dried with a flow of Argon, and further removed with vacuum drying. Feeding solution is used to hydrate the thin lipid film to form liposomes. The resulting liposomes are extruded through a polycarbonate membrane (D = 100 nm) with a mini extruder, and sonicated using a water bath sonicator. **D**) Expression of membrane proteins in CFEs. Plant CYP and mechanosensitive ion channel proteins (MscL) from *E. coli* are fused to GFP and expressed in CFEs in the presence of exogenous liposomes. Some elements of this figure were created in BioRender. Sugianto, W. (2026) https://BioRender.com/x76szq5

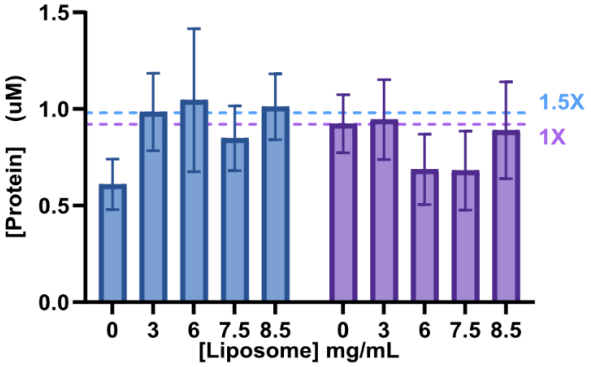
A Liposomes from common lipids EggPC/DOPE in 90:10 molar ratio supported MscL expression



B Ratio and tail group adjustments to common membranes did not impact CrG8H expression

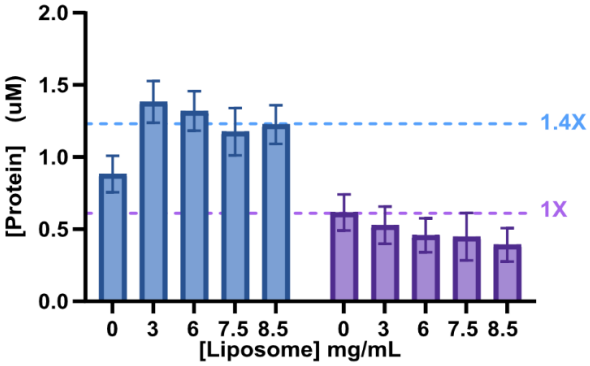
Adjust ratio

EggPC/DOPE in 57:43 molar ratio



Adjust fatty acid tails

DPPC/DPPE in 90:10 molar ratio



Adjust ratio and fatty acid tails

DPPC/DPPE in 57:43 molar ratio

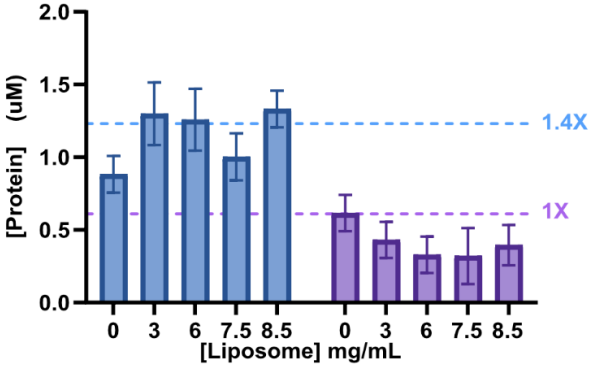


Figure 2. Expression of membrane proteins on liposomes from common lipids. **A**) MscL and a plant CYP, geraniol 8-hydroxylase from *Catharanthus roseus* (CrG8H), are expressed in CFEs supplied with liposomes. Liposomes are made from common phospholipids, such as Egg PC and DOPE at 90:10 molar ratio. Dashes represent average expression fold change compared to baseline (0 mg/mL liposome), blue: MscL, purple: CrG8H. **B**) Adjustments to common liposomes to mimic native *C. roseus* membranes. Adjustment is made to match the PC/PE ratio of *C. roseus* membrane[37] (top-left). Fatty acid tails are adjusted from one unsaturated bond on each tail (18:1, DO) to two saturated tails (16:0, DP), while maintaining the original ratio in Panel A (top-right). Both ratio and lipid tails adjustments are implemented (bottom-left). Values represent the mean ± standard deviation from n = 3 technical replicates.

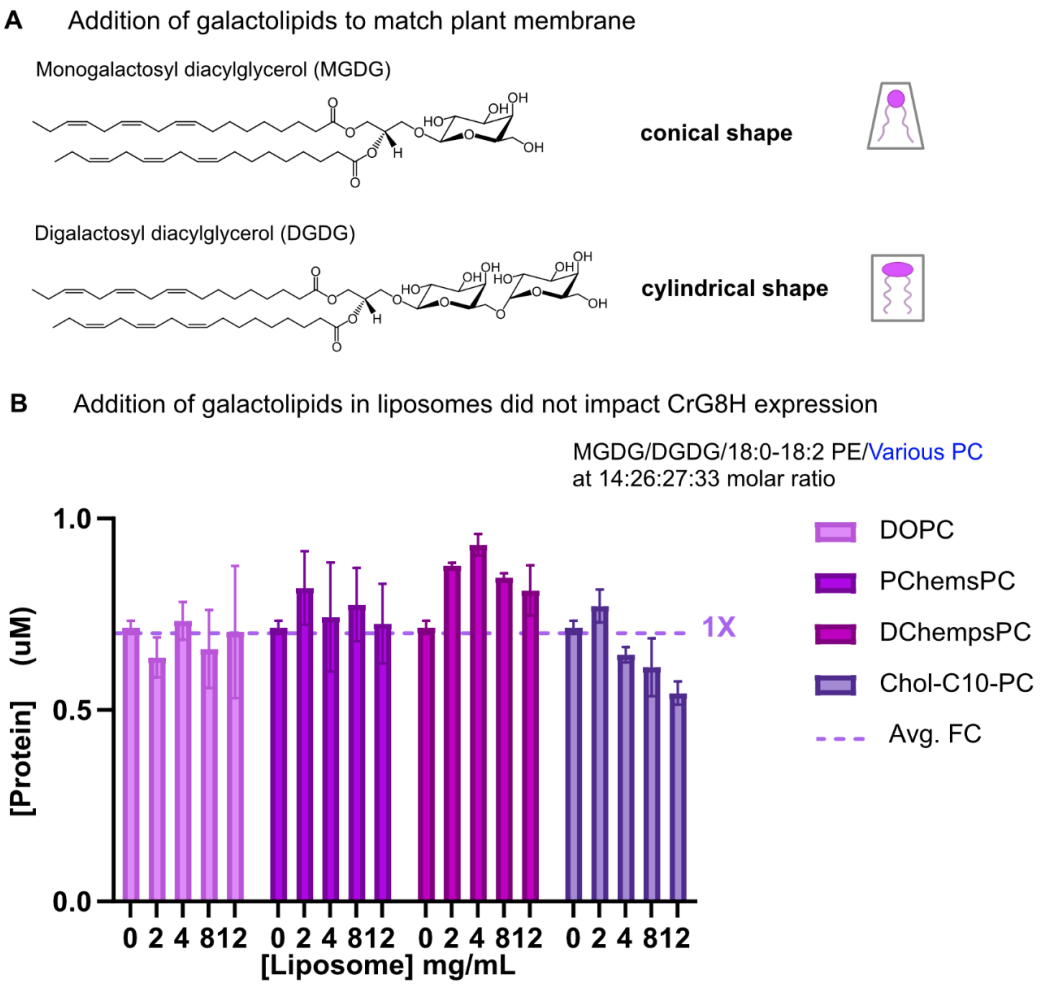
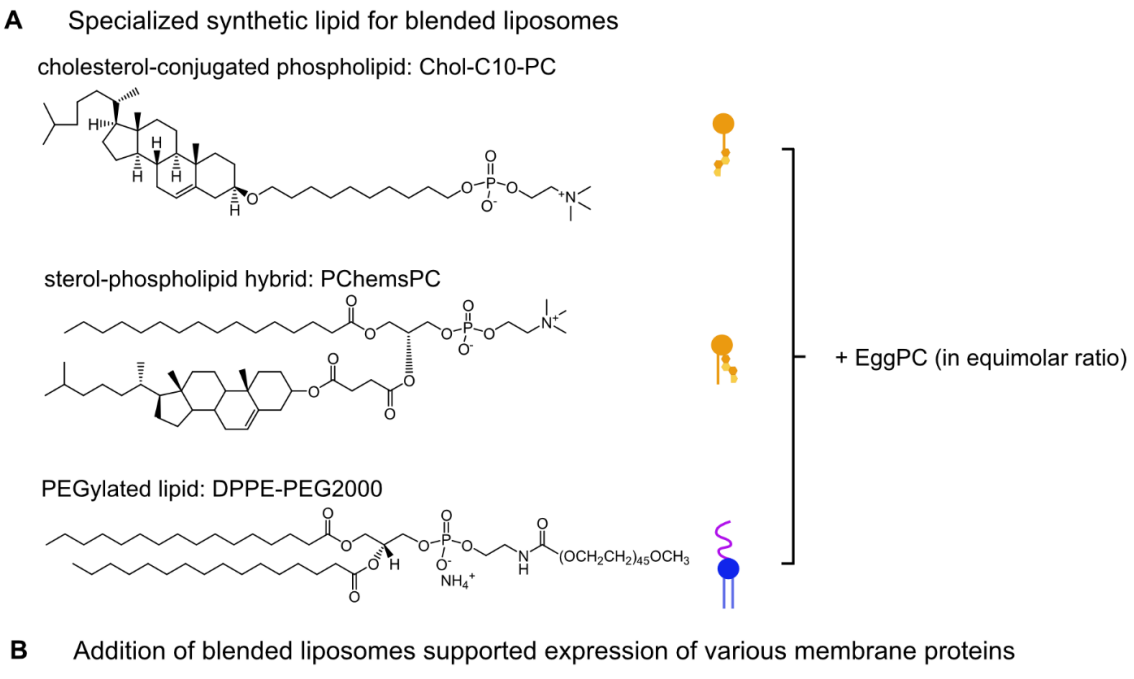
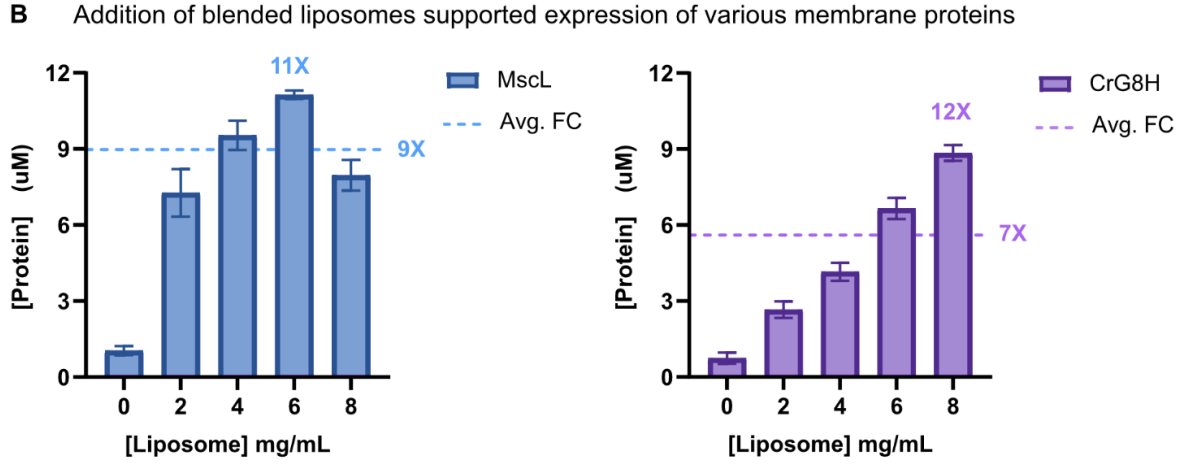

A
Addition of galactolipids to match plant membrane
Monogalactosyl diacylglycerol (MGDG)
conical shape
Digalactosyl diacylglycerol (DGDG)
cylindrical shape
B
Addition of galactolipids in liposomes did not impact CrG8H expression
MGDG/DGDG/18:0-18:2 PE/Various PC
at 14:26:27:33 molar ratio
DOPC
PChemsPC
DChempsPC
Chol-C10-PC
Avg. FC
1X
[Protein] (uM)
1.0
0.5
0.0
0 2 4 8 12 0 2 4 8 12 0 2 4 8 12 0 2 4 8 12
[Liposome] mg/mL

Figure 3. Addition of plant galactolipids to improve CYP expression. **A**) Structures of plant galactolipids. Monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) are prevalent, non-ionic plant lipids.[39] MGDG with one galactose head group is conical, whereas DGDG with two galactose moieties is cylindrical in shape. **B**) Expression of CrG8H with plant galactolipids in the membrane. PC and PE are maintained in the mixture to ensure formation of membrane bilayers and curvatures. Bilayer-forming PC lipids, from standard to synthetic PCs, are also varied in the composition. Dashes represent average expression fold change compared to baseline (0 mg/mL liposome). Values represent the mean ± standard deviation from n = 3 technical replicates.


A
Specialized synthetic lipid for blended liposomes
cholesterol-conjugated phospholipid: Chol-C10-PC
sterol-phospholipid hybrid: PChemsPC
PEGylated lipid: DPPE-PEG2000
+ EggPC (in equimolar ratio)


B
Addition of blended liposomes supported expression of various membrane proteins
[Protein] (uM)
[Liposome] mg/mL
MscL
Avg. FC
11X
9X
CrG8H
12X
7X

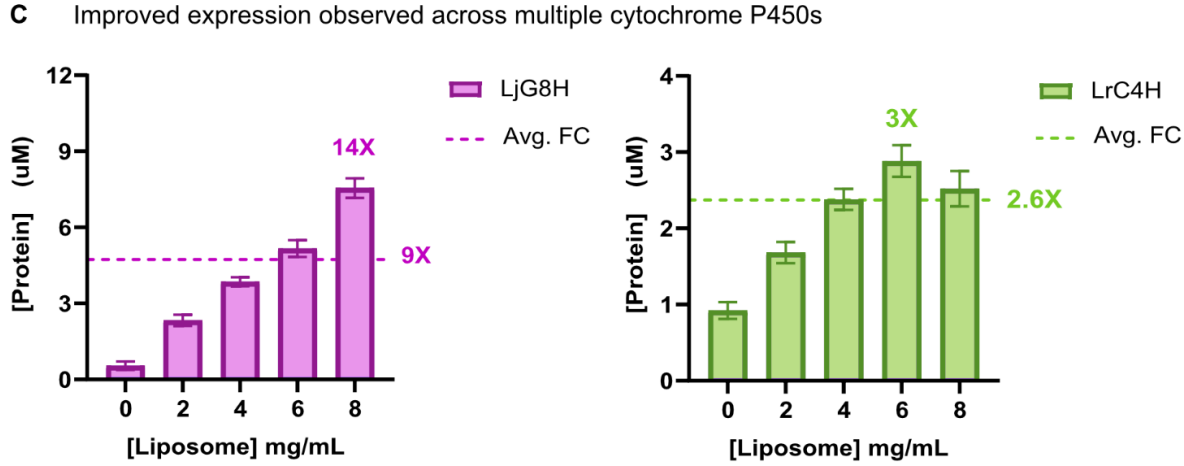

C
Improved expression observed across multiple cytochrome P450s
[Protein] (uM)
[Liposome] mg/mL
LjG8H
Avg. FC
14X
9X
LrC4H
3X
2.6X

Figure 4. Specialized synthetic lipids improved the expression of plant CYPs. **A**) Structures of specialized synthetic lipids. Synthetic lipids are combined at equimolar concentration with EggPC to create stabilized, blended liposomes. **B**) Expression of both MscL and CrG8H significantly improved with the addition of blended liposomes. **C**) Blended liposomes can be used to improve expression of other CYPs, including G8H from another species (*L. japonica*)[49] and another hydroxylase, C4H, from *L. radiata*[50] for production of p-coumaric acid. Dashes represent average expression fold change compared to baseline (0 mg/mL liposome). Values represent the mean ± standard deviation from n = 3 technical replicates. Some elements of this figure were created in BioRender. Sugianto, W. (2026) https://BioRender.com/x76szq5