SI APPENDIX: Includes detailed Materials and Methods and four figures.

Bilin-dependent regulation of chlorophyll biosynthesis by GUN4

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SI APPENDIX MATERIALS AND METHODS

***Chlamydomonas* Strains and Growth Conditions.** *Chlamydomonas reinhardtii* wild-type strain 4A+, *hmox1* and *hmox1* complemented strains *ho1*C2 were described previously (1). The *gun4* mutant (CAL005_01_22, 4A+ background) was from Chlamydomonas Resource Center. All strains were maintained at 22~24℃ under cool-white fluorescent light (~10 μmol photons m^{-2} s^{-1}) on TAP (Tris-acetate-phosphate) agar plates with a revised mineral element recipe (2). For phototrophic growth comparisons, cells were resuspended in TP (without acetate) medium, spotted onto TAP (or TP) agar plates and grown in darkness or under different light fluence rates. For protein extraction and immunoblot analyses, cells were grown in TAP medium under continuous white light (~30 μmol photons m^{-2} s^{-1}) until mid-logarithmic phase, and then cells were diluted in TAP medium to a density of ~1.0×10^6 cells/mL. Half of the culture was acclimated in the dark for 24 h, while the other half was grown in the dark for 12 h and then exposed to light (~30 μmol photons m^{-2} s^{-1}) for various amount of time (typically less than 12 h). Cells were then harvested and immediately processed for protein extraction.

Generation of hmox1 gun4 double mutant and complemented strains. Single mutants *hmox1* and *gun4* were used to generate *hmox1 gun4* double mutant. Briefly, *hmox1* (mt-, progenies derived by crossing 4A- to *hmox1*+, paromomycin resistance) and *gun4* (mt+, 4A+ background, zeocin resistance; from Chlamydomonas Resource Center) were grown in TAP medium under low fluence rate ~30 μmol photons m^{-2} s^{-1} until reaching mid logarithmic phase. Cells were harvested by centrifuging at 1000 *g* for 5 min and washed with TAP-N medium (TAP medium without nitrogen source). Then, cells were resuspended in TAP-N medium to a density around 1×10^8 cells/mL and grown under LED blue light (455 ± 20 nm, ~30 μmol photons m^{-2} s^{-1}; Xuanmei Automation Technology Co, Nanjing, China) for 12~16 h to induce gametes. Equal amount of mt- and mt+ gametes were mixed and set under cool fluorescent white light (~30 μmol photons m^{-2} s^{-1}) to mate for 1~2 h. Cell mixture was transferred to 4% TAP-N plates and acclimated to darkness for 7 days. Vegetative cells were scraped away from the surface layer and zygotes attached to the plate were transferred to fresh TAP plates under dim light (~5 μmol photons m^{-2} s^{-1}) to germinate. Single clones were transferred to TAP plates containing 20 μg/mL paromomycin and 10 μg/mL

zeocin for selection. Colonies which survived on antibiotic plates and lacked *Cr*HMOX1 and *Cr*GUN4 proteins were selected as candidate *hmox1gun4* double mutants. To generate *CrGUN4* complemented strains, the genomic DNA of *CrGUN4* was introduced into pHSP70RbcS2-Hyg vector carrying a Strep Tag II and the resulting plasmid was linearized with KpnI and BamHI before electroporation into *gun4* or *hmox1gun4*. Single colonies surviving on TAP plates with 10 μg/mL hygromycin, which accumulated *Cr*GUN4-STII protein, were considered as *Cr*GUN4-complemented strains and were designated as *gun4::GUN4-STII* and *hmox1gun4::GUN4-STII*, respectively.

Expression of CrGUN4 apoprotein and CrGUN4:bilin complexes. *Cr*GUN4 (amino acids 46-260) from *C. reinhardtii* was PCR amplified using a cDNA library with forward (5′-CGCGGCAGCCATATGGCGATGCGCGTCACCGTCGC-3′) and reverse primers (5′-TGTCGACGGAGCTCGAATTCTTAGAACAGCGACTGTGTCCG-3′) designed with NdeI and EcoRI sites (underlined). The amplified PCR fragment was ligated into NdeI and EcoRI-digested pET-28a vector (Merck, Kenilworth, NJ, USA). The resulting construct was introduced into *E. coli* BL21-CodonPlus (DE3)-RIL for His-GUN4 apo-protein expression. His-GUN4 was induced with 0.3 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) at 16℃ over-night and the recombinant His-GUN4 was purified using high affinity Ni-NTA resin (GenScript, Nanjing, China). Briefly, cells were centrifuged at 8000 *g* for 10 min. Cell pellets were resuspended in lysis buffer (25 mM Tris-HCl, pH 7.5 and 150 mM NaCl), disrupted by high pressure homogenization and centrifuged at 10, 000 *g* for 20 min. Supernatant was incubated with Ni-NTA resin and the resin was washed using 40 mM imidazole (in lysis buffer) to remove nonspecifically bound proteins. His-GUN4 protein was eluted in 250 mM imidazole (in lysis buffer). For absorbance measurements, the eluted His-GUN4 was dialyzed in 1× PBS buffer and concentrated for further use. For ITC assay, the eluted His-GUN4 proteins were subsequently subjected to desalting, ion exchange, size-exclusion chromatography using a Superdex 200 Increase 10/300 column (GE Healthcare, Chicago, IN, USA) as described previously (3) and was finally stored in Tris buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl).

Alternatively, primers (5′-ACAGGAGGAATTAACCATGGGCAGCAGCCATCATC-3′ and 5′-CGGATCCCCTTCCTGCAGTTAGAACAGCGACTGTGTCCG-3′) were used to amplify open reading frame containing *Cr*GUN4 (amino acid 46-260) and His tag from pET28a-*Cr*GUN4 (46-260). PCR fragment was cloned into NcoI and PstI-digested pBAD vector (Thermo Fisher Scientific, Waltham, MA, USA). The resulting plasmid was transformed into LMG194/pPL-PCB or LMG194/pPL-BV, two *E. coli* strains engineered to synthesize PCB or BV, respectively (4). Expression and purification of His-tagged *Cr*GUN4 followed established procedures used for His-tagged cyanobacteriochromes (5). Purified *Cr*GUN4:bilin complexes from LMG194/pPL-PCB or LMG194/pPL-BV were designated as *Cr*GUN4:PCB and *Cr*GUN4:BV respectively, and these complexes were stored in 1×PBS buffer.

Bilins and PPIX preparations for enzyme assays and binding studies. For *C. reinhardtii* cell feeding experiments, about 13 mg biliverdin IXα (Frontier Scientific, Logan, UT, USA) powder was dissolved in 1 mL methanol and centrifuged at 12, 000 *g* for 5 min to remove undissolved powder, and the supernatant was retained as BV stock solution. For absorbance spectra measurement and *in vitro* MgCh enzymatic activity assays, BV IXα and phycocyanobilin (PCB) powder were dissolved in DMSO. For isothermal titration calorimetry (ITC) assays, a small amount of BV IXα (BV) and PCB powder were firstly dissolved in about 5 μL 1 M NaOH and diluted in 500 μL reaction buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl). After centrifugation at 13, 000 *g* for 5 min, supernatants were retained as stock solution. The above stock solutions were diluted ~2000 folds in 5% (v/v) HCl/methanol for absorption measurement at 377/696 nm (BV) and 374/690 nm (PCB). Concentrations of BV stock solution was calculated using the formula: 2000 × A_{377}/66.2 (mM) or 2000 × A_{696}/30.8 (mM). Concentration of PCB stock solution was calculated using the formula: 2000 × A_{374}/47.9 (mM) or 2000 × A_{690}/37.9 (mM). PPIX (Frontier Scientific, Logan, UT, USA) stock solution was prepared as described previously (3). Briefly, a small amount of PPIX powder was dissolved in about 5 μL 1 M NaOH, diluted in 500 μL reaction buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl) and then centrifuged at 13, 000 *g* for 5 min. The supernatant was diluted in 5% (v/v) HCl for absorption measurement at 407.5 nm and the

concentration of PPIX was calculated using molar extinction coefficient of 278,000 M^{-1} cm^{-1}. An average of three technical replicates were used to estimate the *Kd* values.

In vitro MgCh enzymatic activity assays. Mature proteins (without chloroplast transit peptides) of *C. reinhardtii* *Cr*CHLD, *Cr*CHLH1, *Cr*CHLI1 and *Cr*CHLI2 were purified as described previously (3). Above MgCh subunits and *Cr*GUN4 (amino acids 46-260) were used for *in vitro* MgCh assays in reaction buffer (50 mM Tricine-NaOH, pH 8.0, 10% v/v glycerol, 15 mM $MgCl_2$ and 4 mM ATP) containing 2 μM PPIX with minor modifications of the previously published method (3) as detailed below. Immediately prior to assay, BV and PCB (Frontier Scientific, Logan, UT, USA) were each dissolved in DMSO to yield 2 mM stock solutions. When bilins were added to the assay, a 1:1 molar ratio mixture of *Cr*GUN4 and bilins (20 μM final concentration each) was pre-incubated at room temperature for 20 min. In parallel, the *Cr*CHLI1-CHLI2-CHLD complex was prepared with a final concentration of each component at 1 μM in assay buffer and incubated at room temperature for 20 min. Assay mixtures (20 μL or 50 μL) were prepared at final concentrations of 50 nM *Cr*CHLI1-CHLI2-CHLD complex, 1 μM *Cr*GUN4 or *Cr*GUN4:Bilin complex, and additional 50 nM *Cr*CHLI1 in a microtiter plate in assay buffer at 30℃. The 20 μL reactions were prepared using an ECHO 550 acoustic liquid handler (Labcyte, San Jose, CA, USA). Assays were initiated by adding PPIX and *Cr*CHLH1 to the concentrations as indicated. Fluorescence was measured in a PHERAstar plate reader (BMG Labtech, Hopkinton, MA, USA) every 30 s over a 30 min period using excitation at 420 nm and emission at 600 nm to determine the maximal rate (MgPPIX min^{-1}) calibrated by comparing to a MgPPIX standard curve prepared in reaction buffer containing 1 mg/mL bovine serum albumin. All turnover rates are shown as MgPPIX min^{-1} based on concentration of CHLD in the assay, i.e. moles MgPPIX min^{-1} per mole CHLD in the assay.

Chlorophyll measurements. Chlorophyll extraction was performed as described previously (6). 4A+, *hmox1*, *gun4*, *hmox1 gun4* and *hmox1 gun4*::*GUN4-STII* were grown in cool white fluorescent light (~30 μmol photons m^{-2} s^{-1}) in TAP medium at room temperature to mid logarithmic phase. After dilution to 1~2×10^6 cells/mL in TAP medium supplemented with or without 50 μM BV, cells were either dark-adapted for 24 h or dark-adapted for 12 h followed by 12 h exposure to white

fluorescent light (~120 μmol photons m^{-2} s^{-1}). Aliquots of 1 mL cells were harvested and mixed with 1 mL DMF (dimethylformamide). Cell mixtures were vortexed for 1 min and centrifuged at 12,000 *g* for 5 min. Supernatants were used for absorption measurement at 663.8 nm and 646.8 nm. Chlorophyll content was calculated using the formula: Chl *b* = 20.78 × $A_{646.8}$- 4.88 × $A_{663.8}$; Chl *a* = 12 × $A_{663.8}$ - 3.11 × $A_{646.8}$; total Chl = 17.67 × $A_{646.8}$ + 7.12 $A_{663.8}$ (Porra, 2002).

Protein Extraction and Immunoblot Analysis. Total protein extraction from *C. reinhardtii* cells for immunoblot analyses were performed as described previously (1). An antibody against Strep tag II (PHY1926A, anti-rabbit, 1:2000 dilution) was obtained from PhytoAB (San Jose, CA, USA). Antiserum against *Cr*HMOX1 (anti-rabbit, 1:1000 dilution) was generated previously (1). Antisera against *Cr*GUN4 (Cre05.g246800), *Cr*CHLH1 (Cre07.g325500), *Cr*CHLI1 (Cre06.g306300), *Cr*CHLI2 (Cre12.g510800), *Cr*CHLD (Cre05.g242000), *Cr*LPOR (Cre01.g015350), *Cr*GluTR (Cre07.g342150) and *Cr*GSA1 (Cre03.g158000) were provided by PhytoAB. The secondary antibody conjugated to horseradish peroxidase (CWBio, Beijing, China; CW0102S, goat anti-mouse IgG; or CW0103S, goat anti-rabbit IgG) was used (1:10000 dilution) with the enhanced chemiluminescence detection kit (Clarity Western ECL Substrate; Bio-Rad, Hercules, CA, USA) for signal acquisition.

SDS PAGE and on-blot zinc-dependent fluorimaging and colorimetric staining. Purified proteins were analyzed by SDS-PAGE using standard procedures (7), followed by semi-dry transfer to PVDF membranes, and sequential zinc-dependent fluorescence visualization of bilin-bound proteins and staining with Coomassie Blue G-250 (8). Purified *Dolichomastix tenuilepis* phytochrome, *Dten*PHY1 (9), was used as a positive control for zinc-dependent fluorimaging.

Spectrophotometric analysis of CrGUN4:bilin complexes. For photoconversion analysis, affinity-purified *Cr*GUN4:PCB complex was diluted to 20 μM using 1×PBS buffer and dark-adapted for 30 min before spectral measurement. Dark-adapted *Cr*GUN4:PCB was subsequently irradiated by LED red light (650 ± 20 nm, ~ 38 μE; Xuanmei Automation Technology Co, Nanjing, China) for 5 min. A spectrophotometer was used to record absorbance changes from 300-800 nm (Mapada Instruments, Shanghai, China). For fluorescence test, *Cr*GUN4 apo-protein, *Cr*GUN4:PCB complex and PCB stock solution were all diluted to 20 μM (100 mM Tris-HCl, pH

7.5, 100 mM NaCl). Samples were respectively excited by 650 nm wavelength light and spectrum were recorded using a microplate reader (Tecan, Männedorf, Switzerland). For spectral titrations of bilins with various amount of *Cr*GUN4 protein, BV and PCB stock solution (2 mM in DMSO) were diluted to appropriate concentrations using 1×PBS buffer. Bilins (final concentration 20 μM) were incubated with *Cr*GUN4 apo-protein (final concentrations of 10 μM, 20 μM or 40 μM) at room temperature for 15 min. Absorbance changes were measured by a microplate reader (Tecan, Männedorf, Switzerland).

The spectra deconvolution experiment in Fig. 2B was performed empirically and is based on the assumption of two components in the 20 μM *Cr*GUN4 + 20 μM PPIX sample - one with absorption spectrum similar to PPIX in buffer (red dashed spectrum in Fig. 2A). Subtraction of the spectrum corresponding to 8 μM PPIX (40% that of the red dashed spectrum in Fig. 2A) from that of the 20 μM *Cr*GUN4 + 20 μM PPIX (solid orange spectrum in Fig. 2A) was used to generate the 'deduced' spectrum of 12 μM *Cr*GUN4:PPIX species (solid orange spectrum in Fig. 2B).

Isothermal Titration Calorimetry (ITC) assays. For ITC assays, purified apoprotein of *Cr*GUN4 (amino acids 46-260) was dissolved in 200 μL reaction buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl; protein concentration is 50 μM) and was placed in the sample chamber of an Auto-iTC200 titration calorimeter (Malvern Panalytical, Malvern, UK) and held at 25 °C. Solutions of PCB or BV in 40 μL reaction buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl; bilin concentration is 500 μM) were used for titration. The first injection (0.4 μL) was followed by additional 19 injections of 2 μL each. The heat of dilution values for PCB or BV were measured by injecting the PCB or BV into buffer alone and subtracted from the experimental curves before data analysis. MicroCal ORIGIN software supplied with the instrument was used to determine the site-binding model that produced the best fit for the resulting data (OriginLab Corporation, Northampton, MA, USA).

***Binding and fluorescence quenching analysis of PPIX and Cr*GUN4 with or without PCB.** PCB and PPIX stock solutions and purified His-*Cr*GUN4 apoprotein were prepared in TBS buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl). Briefly, 20 μM free PPIX was mixed with or without equimolar concentrations of apo-*Cr*GUN4 protein at 25°C for 10 min and then further mixed with

or without 20 μM PCB at 25°C for 10 min. Absorption and fluorescence spectra (excited at 408 nm) of free PPIX, free PCB, PPIX+PCB, *Cr*GUN4+PPIX and *Cr*GUN4+PPIX+PCB adducts (final concentration 20 μM for each component) were determined using a microplate reader (Tecan, Männedorf, Switzerland).

On-column assembly and purification of CrGUN4 adducts with PCB and/or PPIX. PCB and PPIX stock and purified His-*Cr*GUN4 apoprotein solutions were prepared in TBS buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl). Briefly, 500 μL of 20 μM his-tagged *Cr*GUN4 protein was incubated with 500 μL of 100 μM PCB (or 100 μM PPIX) in TBS at 25℃ for 15 min. Ni-NTA resin (50 μL; GenScript, Nanjing, China) was used to capture each his-tagged adduct followed by 3-5 x 1 mL washes with TBS to remove excess unbound pigment. The washed resins were designated as *Cr*GUN4:PCB or *Cr*GUN4:PPIX. Each resin was then divided into two parts. One half of each resin was eluted with 250 μL of 250 mM imidazole in TBS to purify on-column assembled *Cr*GUN4:PCB and *Cr*GUN4:PPIX binary adducts. The other half of the *Cr*GUN4:PCB resin was incubated at 25℃ for 15 min with 250 μL of 100 μM PPIX and then washed 3-5 times to remove excess unbound PPIX. Similarly, half of the *Cr*GUN4:PPIX resin was incubated at 25℃ for 15 min with 250 μL of 100 μM PCB and then washed 3-5 times to remove unbound PCB. Each ternary mixture was eluted with 250 μL of 250 mM imidazole in TBS to purify the on-column assembled respective *Cr*GUN4:PCB:PPIX and *Cr*GUN4:PPIX:PCB ternary adducts. Absorption spectra of free PCB, PPIX and PCB+PPIX pigments, the binary adducts *Cr*GUN4:PCB and *Cr*GUN4:PPIX and ternary adducts *Cr*GUN4:PCB:PPIX and *Cr*GUN4:PPIX:PCB in 250 mM imidazole in TBS were determined using a microplate reader (Tecan, Männedorf, Switzerland). The final concentration of *Cr*GUN4 in the binary and ternary mixtures is ≲ 20 μM.

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Bilin adducts of *Cr*GUN4 are non-covalent. (**A**) Co-expression of *Cr*GUN4 in PCB-producing *E. coli* yields blue-colored cell pellets. This indicates that the expressed *Cr*GUN4 is capable of binding of PCB similar to that of the *Dolichomastix tenuilepis* phytochrome *Dten*PHY1 control. However, in contrast with the green color of *E. coli* cells co-expressing BV with the *Deinococcus radiodurans* bacterial phytochrome *Dr*BphP, cell pellets of *Cr*GUN4 were not significantly green. (**B**) Characterization of purified *Cr*GUN4:PCB and *Cr*GUN4:BV by Coomassie Blue (CB) staining (lower panel) or by zinc-dependent fluorescence (upper panel), before (-PCB, -BV) or after (+PCB, or +BV) incubation with additional bilins *in vitro*. All samples were denatured by 5×SDS protein loading buffer before electrophoresis. The red band indicates *Dten*PHY1 with covalently bound PCB as a positive control. (**C**) Normalized absorption spectra of free PCB and denatured *Cr*GUN4:PCB purified from PCB-producing *E. coli* and (**D**) free BV or *Cr*GUN4:BV

purified from BV-producing *E. coli*. All samples in panels C & D were prepared by 1:10 dilution in 7 M guanidinium chloride containing 1% (v/v) HCl.

Figure S2. *Cr*GUN4:PCB adduct formation saturates at 1:1 stoichiometry. (**A**) Absorbance spectra of dark-adapted and red-light (650 ± 20 nm)-irradiated *Cr*GUN4:PCB (20 μM) adducts are indistinguishable. Light exposure time was 5 min at a fluence rate of 38 μE m^{-2} s^{-1}. (**B**) Fluorescence emission spectra (ex 650 nm) of *Cr*GUN4:PCB (20 μM) and free PCB (20 μM) are quite weak and very similar. (**C**) Absorbance spectra of PCB (20 μM) with addition of increasing *Cr*GUN4 concentrations. (**D**) Difference spectra were derived by subtracting the 20 μM PCB spectrum from 20 μM PCB+*Cr*GUN4 (10 μM, 20 μM, or 40 μM) spectra. (**E**) Absorbance spectra of BV (20 μM) with increasing *Cr*GUN4 concentrations. (**F**) Difference spectra were derived by subtracting the 20 μM BV spectrum from 20 μM BV + *Cr*GUN4 (10 μM, 20 μM, or 40 μM) spectra.

Figure S3. On-column assembly & elution of ternary *Cr*GUN4:PCB:PPIX complexes. (**A**) Comparative absorption spectra of PPIX in solution (dashed red trace), and raw affinity-purified binary (*Cr*GUN4:PCB and *Cr*GUN4:PPIX) and ternary (*Cr*GUN4:PCB:PPIX and *Cr*GUN4:PPIX:PCB) adducts. (**B**) Resolution of the absorption spectrum of *Cr*GUN4-bound PPIX (solid dark blue trace) in the ternary *Cr*GUN4:PCB:PPIX adduct (solid orange trace) by subtraction of the spectrum of the binary *Cr*GUN4:PCB adduct (solid turquoise trace; which is 60% of that shown in panel A). Soret peak positions in nm are indicated as well as long wavelength peaks for the calculated spectrum of *Cr*GUN4-bound PPIX (solid dark blue trace). See Supplementary Methods for details.

Figure S4. *Cr*HMOX1 and *Cr*GUN4 protein levels and chlorophyll accumulation in *hmox1*, *gun4* and complemented lines. (**A**) Immunoblot analysis of GUN4, HMOX1 and GUN4-STII protein accumulation in respective strains. Similar amount of protein (~30 μg) was loaded for each sample. Cells were grown in constant cool white fluorescent light (~30 μmol photons m^{-2} s^{-1}). CB, Coomassie blue staining. (**B**) Chlorophyll accumulation of *gun4* and *gun4::GUN4-STII* complemented lines. Exponential-phase *C. reinhardtii* cells were either acclimated to darkness for 24 h (Dark) or grown in the dark for 12 h and then exposed to light (~120 μmol photons m^{-2} s^{-1}) for another 12 h (Light). Asterisks above pairs of bars indicate significant differences (Student's

t-test, $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$; n.s., not statistically significant). Values are means ± SD of three biological replicates.

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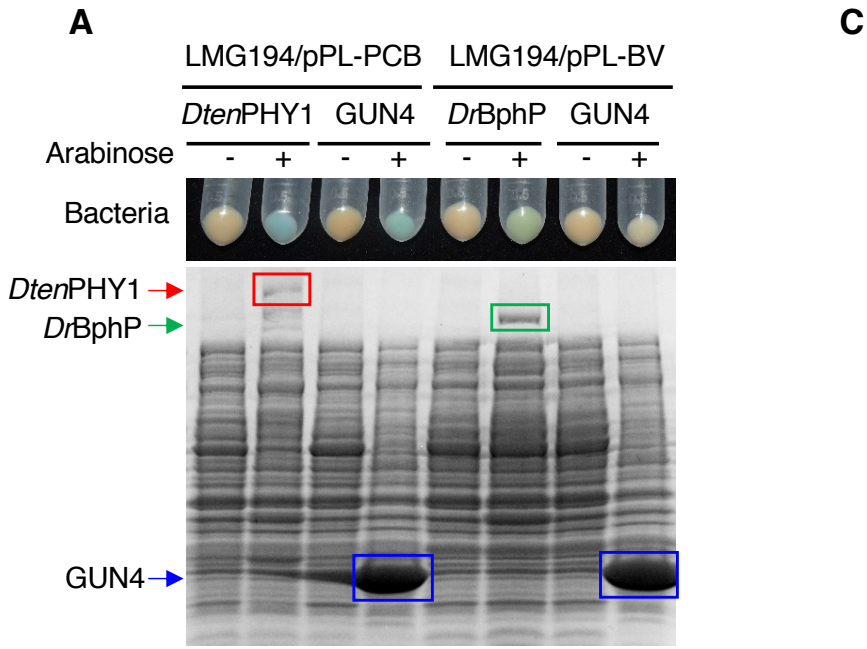

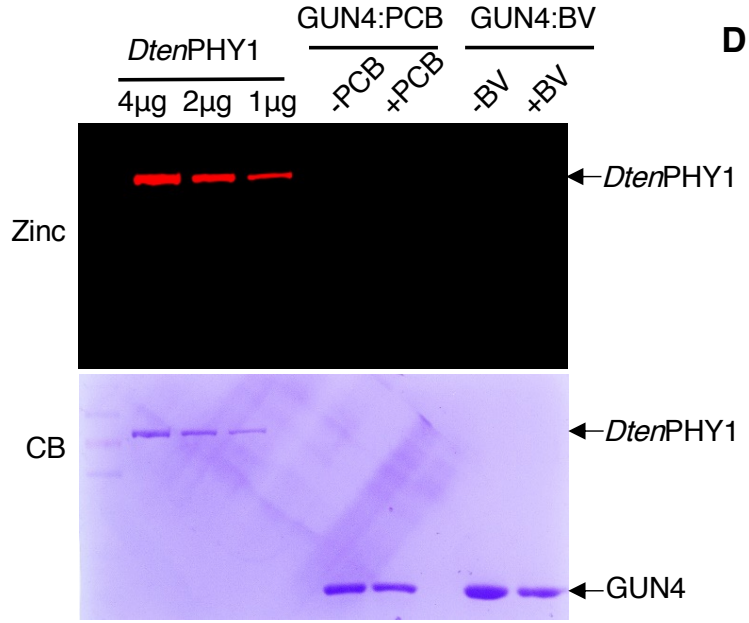

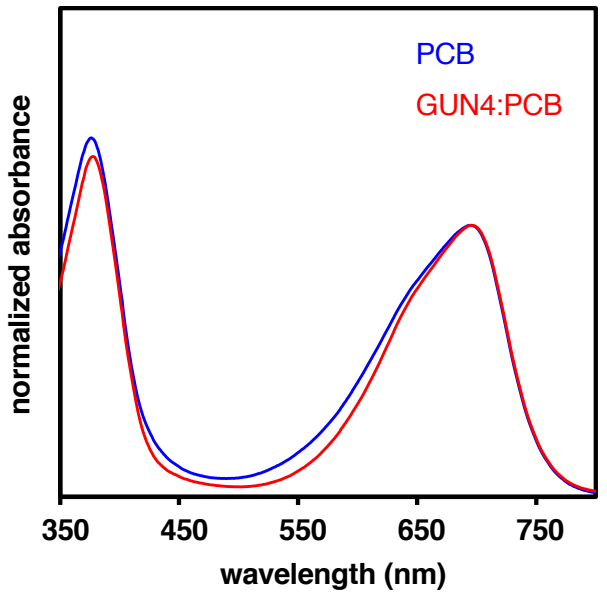

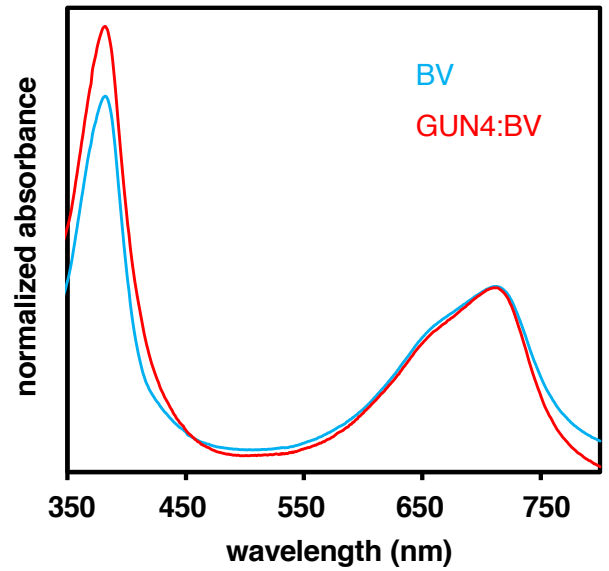

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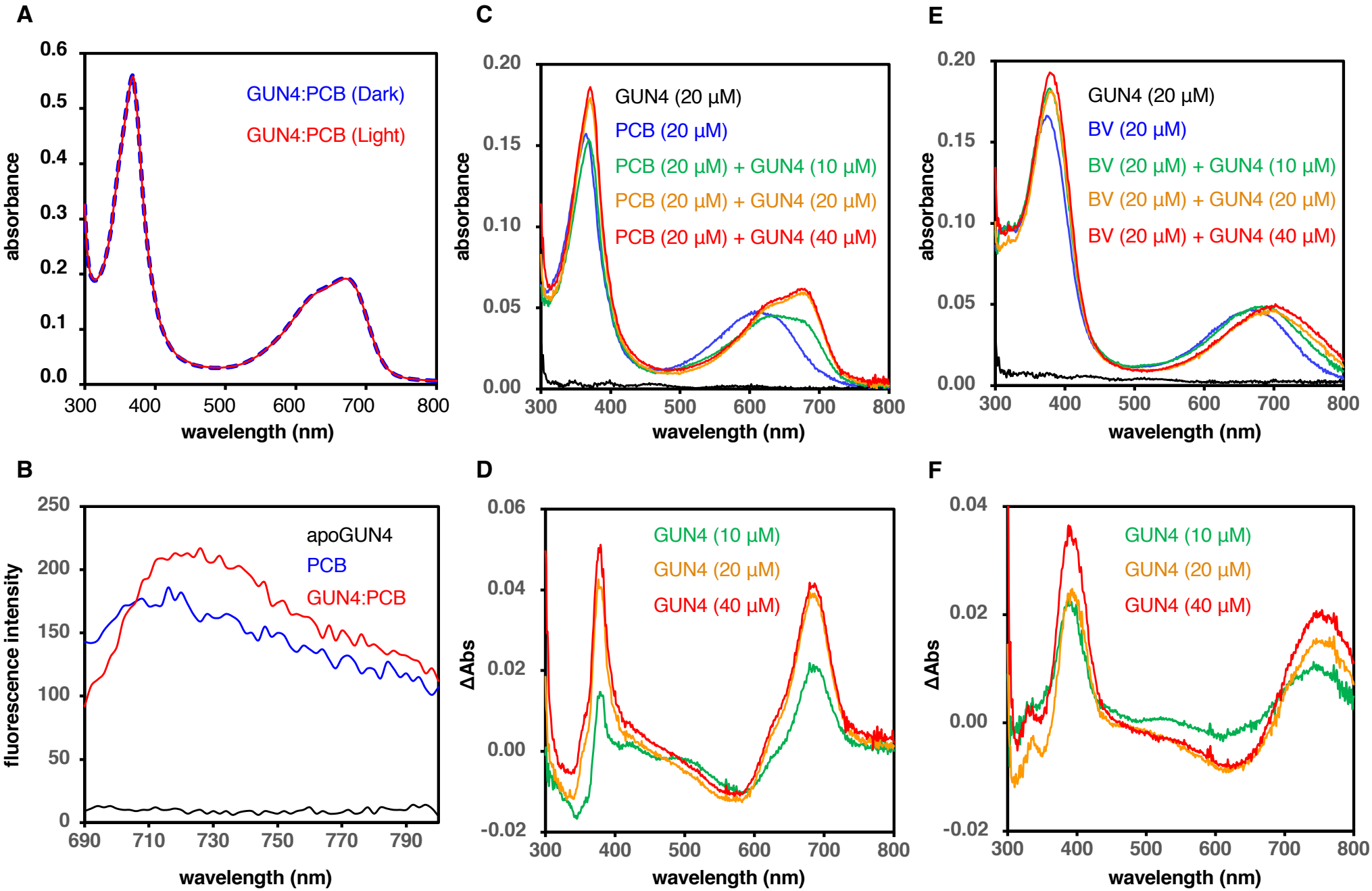

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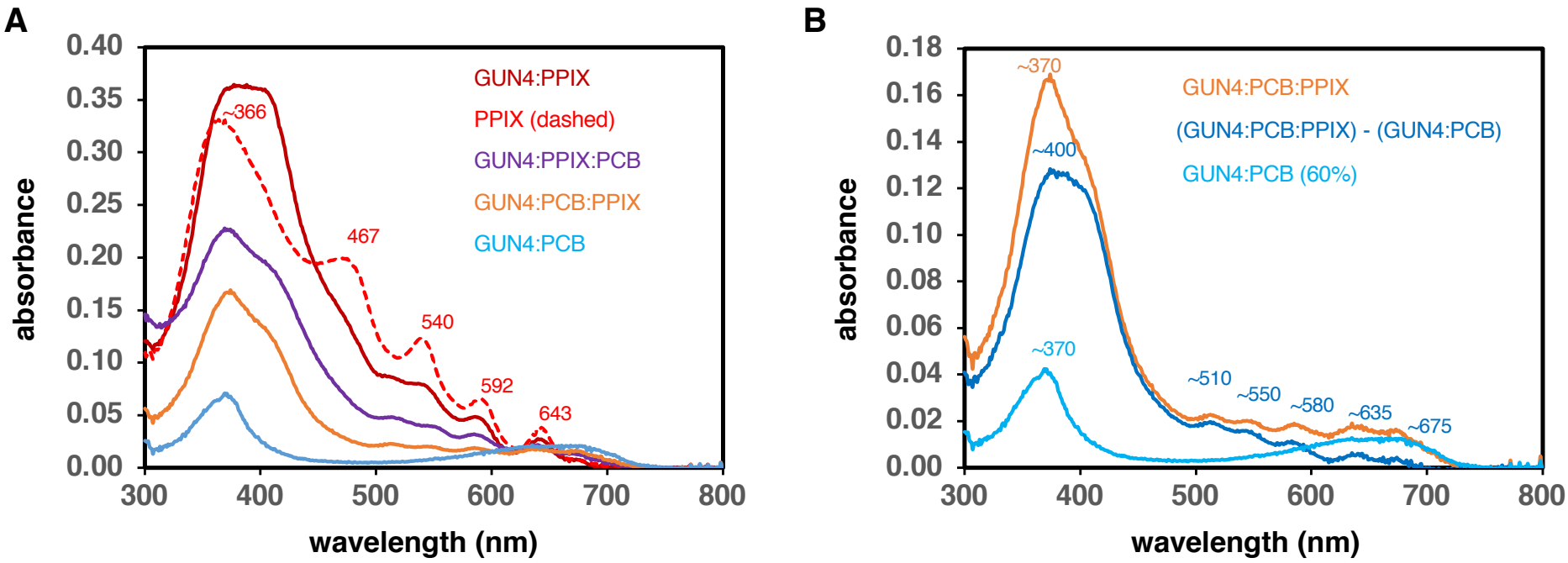

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