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Bilin-dependent regulation of chlorophyll biosynthesis by GUN4

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Biosyntheses of chlorophyll and heme in oxygenic phototrophs share a common trunk pathway that diverges with insertion of magnesium or iron into the last common intermediate, protoporphyrin IX. Since both tetrapyrroles are pro-oxidants, it is essential that their metabolism is tightly regulated. Here, we establish that heme-derived linear tetrapyrroles (bilins) function to stimulate the enzymatic activity of magnesium chelatase (MgCh) via their interaction with GENOMES UNCOUPLED 4 (GUN4) in the model green alga *Chlamydomonas reinhardtii*. A key tetrapyrrole-binding component of MgCh found in all oxygenic photosynthetic species, *Cr*GUN4, also stabilizes the bilin-dependent accumulation of protoporphyrin IX-binding *Cr*CHLH1 subunit of MgCh in light-grown *C. reinhardtii* cells by preventing its photooxidative inactivation. Exogenous application of biliverdin IXα reverses the loss of *Cr*CHLH1 in the bilin-deficient heme oxygenase (*hmox1*) mutant, but not in the *gun4* mutant. We propose that these dual regulatory roles of GUN4: bilin complexes are responsible for the retention of bilin biosynthesis in all photosynthetic eukaryotes, which sustains chlorophyll biosynthesis in an illuminated oxic environment.

heme oxygenase | bilin reductase | reactive oxygen species | photosynthesis | phycocyanobilin

Chlorophylls and hemes comprise the core light-harvesting and energy-converting pigments in the photosynthetic apparatus of all oxygenic species and descendants of their nonoxygenic ancestors. Since both classes of tetrapyrroles are pro-oxidants, it is essential that their biosynthetic pathways are tightly regulated in aerobic environments (1, 2). Indeed, the chlorophyll pathway intermediates protoporphyrin IX (PPIX), MgPPIX, and protochlorophyllide (Pchlide) readily generate reactive oxygen species (ROS) via photosensitization of molecular oxygen upon light exposure, resulting in chlorophyll biosynthetic enzyme inactivation, reaction center turnover, antennae photobleaching, and cell death of photosynthetic tissues (3–5). Such photooxidative turnover does not occur in anoxygenic photosynthetic species, which perform photosynthesis in suboxic or anaerobic environments. Because of the light-lability of the photosynthetic apparatus, all oxygenic phototrophs therefore require protective systems to sustain chlorophyll synthesis in the presence of oxygen.

Metabolic regulation of biosynthetic pathways most commonly occurs at the first committed step of the pathway or at branch points that redirect metabolite flow to different products. In nearly every aerobic species examined, heme biosynthesis is regulated by feedback inhibition of the enzyme that yields 5-aminolevulinic acid (ALA), the first committed intermediate of its synthesis (6). Since chlorophyll and heme pathways share ALA as a common precursor, the regulation of ALA levels in photosynthetic species affects both pathways (6, 7). The most obvious targets for selective regulation of the individual pathways are the enzymes that insert iron or magnesium into PPIX, the last common intermediate of the two pathways (Fig. 1*A*).

Ferrochelatase (FeCh) catalyzes the insertion of ferrous iron into PPIX to generate heme, the prosthetic group of a wide range of hemoproteins, including key components of the photosynthetic electron transport system. Magnesium chelatase (MgCh) catalyzes the first step committed to the synthesis of chlorophyll (i.e., the ATP-dependent insertion of Mg^{2+} into PPIX to form MgPPIX) (8). In contrast with the single subunit of FeCh, MgCh consists of three subunits—CHLH, CHLI, and CHLD—all of which display transcriptional and posttranslational regulation in plants and algae (9, 10). Oxygenic phototrophs also evolved a novel tetrapyrrole-binding regulator of MgCh, GENOMES UNCOUPLED 4 (GUN4), which stimulates MgCh activity (11–14). Because of the chlorophyll-deficient phenotype of *gun4* mutants in plants, cyanobacteria, and algae especially under high light (11, 15, 16), GUN4 is essential to sustain chlorophyll synthesis in the presence of light and oxygen.

Consistent with its proposed role to facilitate delivery of PPIX substrate and release of MgPPIX product, GUN4 binds both tetrapyrroles and also interacts with the PPIX-binding CHLH subunit of MgCh (17–19). Porphyrin binding to GUN4 also promotes its interaction with CHLH and association with chloroplast membranes, the site of chlorophyll biosynthesis (20–22). Whereas the strong upregulation of GUN4 by light represents a plausible mechanism to redirect PPIX to the chlorophyll pathway (11), GUN4:PPIX complexes are highly photosensitizing, raising questions about its proposed role as a substrate/product chaperone (23). In the present study, we elucidate a GUN4-dependent regulatory mechanism of MgCh activity in the model green alga *Chlamydomonas reinhardtii* that leverages the ubiquitous presence of linear tetrapyrroles (bilins) in oxygenic phototrophs.

Significance

Enzymes of the chlorophyll biosynthetic pathway, which bind protoporphyrin and Mg-porphyrins, are susceptible to damage by singlet oxygen production in the presence of light and oxygen. These studies show that heme-derived linear tetrapyrroles (bilins) both stimulate and protect the protoporphyrin-binding CHLH subunit of Mg chelatase, the first committed enzyme of the chlorophyll synthesis, from self-sensitized photodamage and turnover via formation of nonphotosensitizing GENOMES UNCOUPLED 4 (GUN4):bilin:porphyrin adducts, which deliver protoporphyrin to CHLH. GUN4:bilin adducts likely evolved to sustain chlorophyll biosynthesis in an oxic world, accounting for retention of bilin synthesis in nearly all oxygenic photosynthetic species on Earth.

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The authors declare no competing interest.

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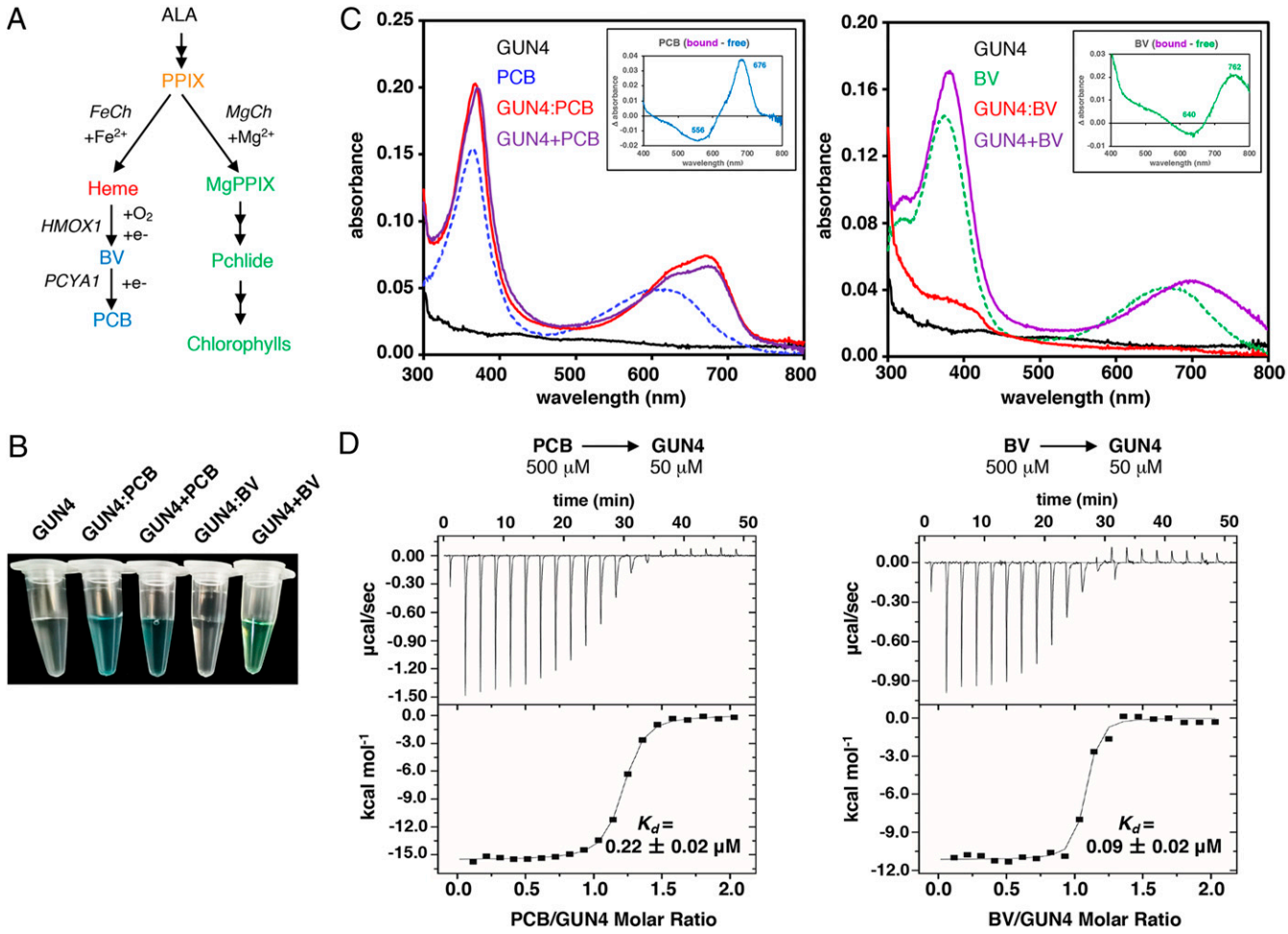

Fig. 1. *Cr*GUN4 interacts with bilins. (*A*) Tetrapyrrole pathways in *C. reinhardtii*. heme, protoheme IX (Heme B); PCYA1, phycocyanobilin:ferredoxin oxidoreductase. (*B*) Appearance of purified recombinant *Cr*GUN4 apoprotein (GUN4) and those coexpressed in the presence of PCB (GUN4:PCB) or BV (GUN4:BV) in *E. coli*. Also shown are purified *Cr*GUN4 apoprotein preparations assembled in vitro with equal molar concentrations of PCB (GUN4+PCB) or BV (GUN4+BV). (*C*) Absorption spectra of free bilins in solution (PCB or BV, 20 μM), purified in vivo GUN4:Bilin adducts (20 μM), and GUN4+Bilin mixtures (20 μM) assembled in vitro. *Insets* show difference spectra of bound (GUN4+bilin) minus free bilins. (*D*) ITC analysis of ligands binding by titration of *Cr*GUN4 apoprotein with bilins (*Left*, PCB; *Right*, BV). (*Upper*) Heat response to injections; (*Lower*) Integrated heats of each injection. The K_d values were derived from nonlinear least-squares analysis of the integration of the heats to a single-site binding model. Values are means ± SD of three technical replicates.

Bilins are linear tetrapyrroles derived from heme by sequential enzymatic reactions of heme oxygenases (HMOX) and ferredoxin-dependent biliverdin reductases (FDBRs) that are found in all oxygenic species (24–26). HMOX are widely distributed oxygen-dependent enzymes that convert heme to biliverdin (BV) with the release of the heme iron and carbon monoxide (24, 27). In cyanobacteria and other eukaryotic algae, FDBR-derived phycobilins function as chromophore precursors of the light-harvesting phycobiliproteins and light-sensing phytochromes, which facilitate phototrophic growth and photoacclimation in fluctuating light environments (28). Although *C. reinhardtii* lacks both phytochromes and phycobiliproteins, bilins act as a plastid retrograde signal to modulate expression of a small nuclear gene network to detoxify ROS during the diurnal dark-to-light transitions (29). These and follow-up studies have established that bilins are also essential within plastids for proper accumulation of photosystem I (PSI) reaction centers and PSI antennae proteins in daylight (30). In view of the similar chlorophyll-deficient phenotypes of *hmox1* and *gun4* mutants, such studies strongly implicate bilins to function as regulators of chlorophyll biosynthesis in *C. reinhardtii*.

The present investigation was undertaken to dissect the molecular basis of bilin regulation of chlorophyll biosynthesis in *C. reinhardtii*. Our studies establish that *Cr*GUN4 binds bilins with high affinity in vitro, and that bilins stimulate MgCh activity by binding to *Cr*GUN4. Leveraging genetic and chemical complementation analyses, we also show that *Cr*GUN4 and bilins are both required to prevent the light-dependent loss of the MgCh catalytic subunit *Cr*CHLH1 in vivo. Our results support the conclusion that GUN4 evolution is critical to the maintenance of chlorophyll biosynthesis in oxygenic phototrophs by its ability to form complexes with bilins that not only stimulate MgCh activity, but also protect the PPIX-binding CHLH subunit from photooxidative damage and turnover in the presence of light.

Results

***Cr*GUN4 Interacts with Bilins.** Arabidopsis GUN4 has been shown to bind several tetrapyrrole molecules, including heme, PPIX, and MgPPIX (20). For this reason, we first tested whether *Cr*GUN4 could interact with bilins by coexpressing *Cr*GUN4 with cyanobacterial phycocyanobilin (PCB) and BV biosynthesis expression plasmids in *Escherichia coli* (31). As controls for PCB and BV binding, we respectively coexpressed the *Dolichomastix tenuilepis* phytochrome (*Dten*PHY1) and the *Deinococcus radiodurans* bacteriophytochrome (*Dr*BphP) with PCB and BV plasmids. Cell

pellets from PCB cultures of *Cr*GUN4 were blue colored, like those of *Dten*PHY1 (*SI Appendix*, Fig. S1*A*), and affinity-purified *Cr*GUN4 retained the blue color revealing that PCB was tightly bound to the *Cr*GUN4 protein (Fig. 1*B*). In contrast, unlike cell pellets from BV cultures of *Dr*BphP, *Cr*GUN4 BV cultures were not visibly green colored (*SI Appendix*, Fig. S1*A*), nor was affinity-purified *Cr*GUN4 derived therefrom (Fig. 1*B*). The absorption spectrum of the purified *Cr*GUN4:PCB adduct, as well as that assembled in vitro by mixing apo*Cr*GUN4 with PCB (*Cr*GUN4+PCB), exhibited two maxima at 368~370 and 670~674 nm (Fig. 1 *C*, *Left*). Addition of BV to affinity-purified *Cr*GUN4 (*Cr*GUN4+BV) yielded a green-colored species with absorption maxima at 380~382 and 691~705 nm (Fig. 1 *C*, *Right*). By comparison with spectra of PCB and BV in solution, the two absorption maxima of both bilins were significantly red-shifted upon binding to *Cr*GUN4 as documented by the bound- minus free-pigment difference spectra (Fig. 1 *C*, *Insets*).

Denaturation studies revealed that both bilins were noncovalently bound to *Cr*GUN4, consistent with the loss of Zn-dependent fluorescence of the *Cr*GUN4 protein after SDS/PAGE (*SI Appendix*, Fig. S1*B*) and with the identity of the absorption spectra of the free pigments and the denatured complexes (*SI Appendix*, Fig. S1 *C* and *D*). This contrasts with the retention of zinc-dependent fluorescence of *Dten*PHY1 following electrophoresis, as evidence of covalent PCB attachment (*SI Appendix*, Fig. S1*B*). We also examined the photochemistry of the *Cr*GUN4:PCB adduct. After irradiation with red light (650 ± 20 nm, ~38 μE for 5 min), only very slight photobleaching occurred and no stable photoproduct was observed (*SI Appendix*, Fig. S2*A*). The fluorescence spectrum of the *Cr*GUN4:PCB adduct was nearly indistinguishable from that of PCB in solution (*SI Appendix*, Fig. S2*B*), suggesting that the *Cr*GUN4:PCB adduct was not detectably fluorescent. Based on these studies, we conclude that *Cr*GUN4:bilin adducts are noncovalent, nonphotoactive and nonfluorescent.

We next performed spectrophotometric titrations and isothermal titration calorimetry (ITC) measurements to examine the interaction of bilins with *Cr*GUN4 (Fig. 1*D*). For these studies, we used both PCB and its biosynthetic precursor BV since both bilins are naturally produced in *C. reinhardtii* plastids (29). For spectrophotometric titrations, we monitored the absorption of premade solutions of PCB or BV to which we added incremental amounts of purified *Cr*GUN4 apoprotein up to 2× molar excess. These measurements showed that both bilins interact with *Cr*GUN4 as revealed by an ~16~28% increase in extinction coefficients and small red shifts of both absorption maxima upon binding (*SI Appendix*, Fig. S2 *C* and *E*). Such shifts appeared to saturate at equimolar concentrations for PCB as shown by the difference spectra (*SI Appendix*, Fig. S2*D*), thereby implicating formation of a stoichiometric *Cr*GUN4:PCB adduct. However, the difference spectra of *Cr*GUN4:BV suggests that BV may form higher-order complexes with *Cr*GUN4 (*SI Appendix*, Fig. S2*F*). By ITC measurements, both bilins gave well-behaved binding isotherms to *Cr*GUN4, revealing dissociation constants (K_d) of 0.22 ± 0.02 μM and 0.09 ± 0.02 μM, for PCB and BV, respectively, that are consistent with 1:1 binding stoichiometry (Fig. 1*D*).

The Effect of Bilins on PPIX Binding to *Cr*GUN4. In view of the strong bilin affinity of *Cr*GUN4, we next investigated the influence of bilins on PPIX binding to *Cr*GUN4. To do so, we first incubated 20 μM *Cr*GUN4 apoprotein with equimolar concentrations of PCB or PPIX followed by addition of the other pigment at the same concentration. A sharp new peak at 408 nm appeared upon mixing PPIX with *Cr*GUN4 (Fig. 2*A*, compare red dash with solid orange traces), that was coincident with loss of absorption of "free" PPIX in solution at 362 nm. This spectral shift is consistent with the disruption of PPIX dimers, the predominant form of PPIX in aqueous solution (32, 33). Other spectral changes seen in the long wavelength region between 450 and 750 nm indicate formation of a protein-bound PPIX adduct. Upon addition of PCB to this mixture, most of the 408-nm peak disappeared and significant changes also were seen in the region between 450 and 800 nm (Fig. 2*A*, solid blue trace). Such spectral changes are consistent with formation of the PCB adduct of *Cr*GUN4 and release of free PPIX since the difference spectrum constructed by subtracting the spectrum of a mixture of PPIX and PCB in solution (Fig. 2*A*, dashed turquoise trace) from that of the ternary mixture spectrum (Fig. 2*A*, solid blue trace) was nearly identical to that constructed by subtracting the spectrum of PCB in solution from that of the *Cr*GUN4:PCB adduct (compare *Insets* in Figs. 1 *C*, *Left*, and 2*A*).

The spectrum of the mixture of equimolar PPIX and *Cr*GUN4 (Fig. 2*A*, solid orange curve) could be resolved into two components corresponding to 60% bound PPIX (i.e., 12 μM *Cr*GUN4:PPIX adduct) and 40% free PPIX (i.e., 8 μM PPIX). For comparative purposes, the spectra of equimolar solutions of free PPIX and the *Cr*GUN4:PPIX adduct (minus free PPIX) clearly documents the changes in the PPIX environment upon binding to *Cr*GUN4 (Fig. 2*B*). As shown in Fig. 2*C*, addition of *Cr*GUN4 to a solution of 20 μM PPIX led to a 40- to 60-fold increase in fluorescence at 630 and 690 nm upon formation of the *Cr*GUN4:PPIX adduct. Moreover, addition of PCB to this mixture reversed this fluorescence increase (Fig. 2*C*). Together, these data indicate that the fluorescence emission of the *Cr*GUN4:PPIX adduct is quenched upon addition of PCB, reflecting a new electronic environment for PPIX.

To test whether the "released" PPIX remained bound to the newly formed *Cr*GUN4:PCB adduct, we performed side-by-side experiments to generate the *Cr*GUN4 adducts of PPIX and PCB, which were then adsorbed to a Ni^{2+}-NTA matrix. For one-half of each matrix, the bound *Cr*GUN4:PPIX and *Cr*GUN4:PCB adducts were incubated on-column with excess PCB and PPIX, respectively, while the other half were used as controls. After extensive washing, the His-tagged proteins were eluted with 250 mM imidazole. The absorption spectra revealed that both porphyrin and PCB remained bound in the ternary mixtures and that the order of addition did not significantly change the absorption spectra, excepting for light scatter (*SI Appendix*, Fig. S3*A*, compare orange and purple traces). Comparison of the spectrum of PPIX dissolved in 250 mM imidazole (*SI Appendix*, Fig. S3*A*, dashed red trace) with that of repurified *Cr*GUN4:PPIX (*SI Appendix*, Fig. S3*A*, solid red trace), shows that the electronic environment of PPIX is altered upon binding to *Cr*GUN4 (*SI Appendix*, Fig. S3*A*). Finally, as shown in *SI Appendix*, Fig. S3*B*, the calculated spectrum of the bound PPIX in the *Cr*GUN4:PCB:PPIX ternary complex (*SI Appendix*, Fig. S3*B*, solid dark blue trace) could be resolved by subtraction of the spectrum of the *Cr*GUN4:PCB complex from that of the ternary complex (*SI Appendix*, Fig. S3*B*, solid orange trace). Taken together, these results show that a tightly bound ternary complex of both tetrapyrroles is produced upon mixing *Cr*GUN4 with PCB and PPIX in either mixing order, so release of free PPIX into a more solvated environment, elicited by PCB binding, is unlikely in light of experiments in *SI Appendix*, Fig. S3. The similarity of the absorption spectra of free PPIX in solution and bound PPIX in the ternary complex suggests that bound PPIX is present as a dimer similar to that seen in aqueous solutions at these concentrations (32, 33).

***Cr*GUN4 Stimulation of MgCh Activity Is Enhanced by Bilins In Vitro.** It is well established that GUN4 promotes chlorophyll biosynthesis by directly activating MgCh enzymatic activity (11, 17). To explore the impact of bilins on MgCh activity, we next performed in vitro biochemical assays to quantify the production of MgPPIX. A complex enzyme with three subunits, MgCh activity is typically assessed by measuring MgPPIX formation by fluorescence (34, 35) as a function of the concentration of the porphyrin-binding subunit CHLH (9, 36, 37). *C. reinhardtii* also possesses two *CHLH* genes, only one of which, *CrCHLH1*, is highly expressed (30). However, MgCh activity saturates due to the fixed and limiting amount of the other two subunits, CHLI and CHLD. In the absence of *Cr*GUN4, *Cr*CHLH1-dependent MgCh activity is quite low (11). As shown in Fig. 3*A*,

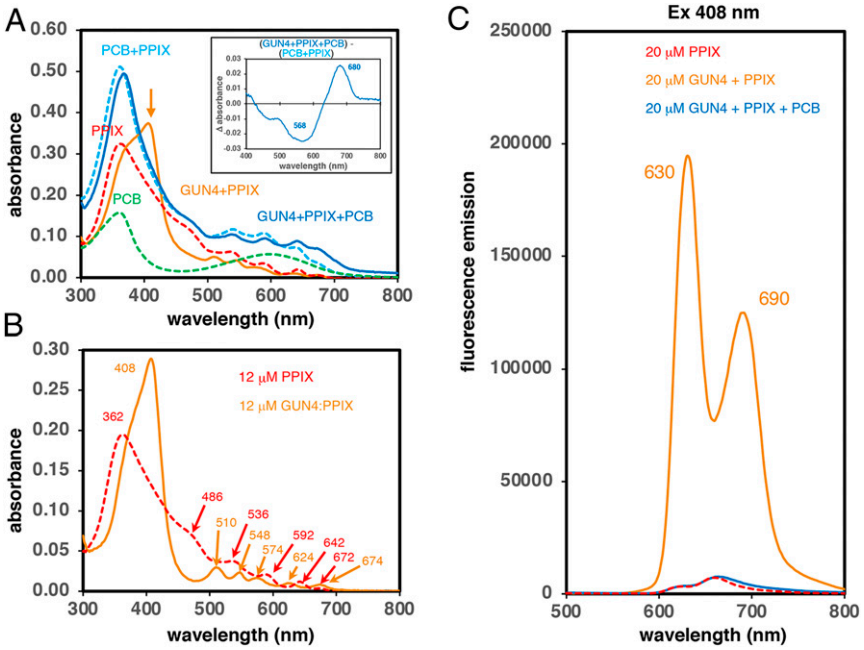

Fig. 2. PCB addition quenches the strong fluorescence of PPIX adducts of *Cr*GUN4. (*A*) Absorption spectra of 20 μM free PCB, PPIX, and PCB+PPIX in solution at pH 7.5 are compared with those of binary (*Cr*GUN4+PPIX) and ternary (*Cr*GUN4+PPIX+PCB) mixtures at pH 7.5. *Inset* shows the difference spectrum constructed by subtracting the spectrum of free PCB+PPIX mixture from that of the ternary (*Cr*GUN4+PPIX+PCB) mixture at pH 7.5. The orange arrow indicates the 408-nm peak in the *Cr*GUN4+PPIX sample. See *SI Appendix, Materials and Methods* for details. (*B*) Comparative absorption spectra of 12 μM PPIX and that "calculated" for the *Cr*GUN4:PPIX adduct at pH 7.5 by subtraction of an 8 μM PPIX spectrum from that of 20 μM *Cr*GUN4+PPIX at pH 7.5. Peak wavelengths in nanometers are shown. (*C*) Comparative fluorescence emission spectra (ex 408 nm) of 20 μM free PPIX, *Cr*GUN4+PPIX and *Cr*GUN4+PPIX+PCB at pH 7.5.

*Cr*CHLH1 displayed sigmoidal kinetics in the presence of excess *Cr*GUN4 with an $S_{0.5}$ of 206 nM (95% CI 193 to 223 nM) and a Hill coefficient of 3.0 (95% CI 2.6 to 3.4) in both the absence and presence of PCB ($P = 0.8$). Whereas the presence of *Cr*GUN4 alone afforded modest ~2-fold stimulation, the effect of PCB on MgCh activity was substantial with V_{max} increasing ~20-fold in the presence of PCB (Fig. 3*A*) (V_{max} = 2.6 nM MgPPIX min^{-1} per nanomolar CHLD [95% CI 2.5 to 2.8] with PCB versus 0.13 nM MgPPIX min^{-1} per nanomolar CHLD [95% CI 0.06 to 0.19] without PCB).

In the presence of PCB, the stimulation of MgCh activity by *Cr*GUN4 saturated at a concentration of *Cr*GUN4 similar with that of PCB in the assay (Fig. 3*B*). These results implicated a stoichiometric *Cr*GUN4:PCB complex to be responsible for MgCh activation, a conclusion consistent with the submicromolar affinity of PCB to *Cr*GUN4 determined by ITC (Fig. 1*D*). Comparative studies with BV or PCB further revealed that, at saturation, both bilins equally stimulated *Cr*GUN4-dependent MgCh activity (Fig. 3*C*). While both bilins yielded the same V_{max} of 1.46 nM MgPPIX min^{-1} per nanomolar CHLD (95% CI 1.30 to 1.67), BV afforded a K_m of 2,310 nM (95% CI 1,854 to 2,902 nM) that was 2× larger than PCB's K_m of 1,036 nM (95% CI 819 to 1,387 nM). This suggests that the *Cr*GUN4:BV complex is less potent an activator of MgCh than the *Cr*GUN4:PCB complex. However, the stimulation of MgCh activity is more complicated than a simple bilin:GUN4 complex activation implies, since we observed a single saturation curve for MgCh activity plotted versus PCB concentration for two different *Cr*GUN4:PCB stoichiometries (Fig. 3*D*).

CrCHLH1 Protein Levels Are Reduced in *hmox1* and *gun4* Mutants. *C. reinhardtii hmox1* and *gun4* mutants are both defective in light-dependent growth and chlorophyll accumulation (29, 38). We therefore performed a side-by-side comparison of photoautotrophic (no acetate) and mixotrophic (with acetate) growth of both mutants under various light fluence rates, along with 4A+ WT and both complemented lines as controls (*ho1*C2, *gun4::GUN4-STII* #1~3) (Fig. 4 and *SI Appendix*, Fig. S4). Significant inhibition of mixotrophic growth of *hmox1* was observed under light intensity around 60 μmol photons m^{-2} s^{-1}, a phenotype that was more exaggerated under photoautotrophic growth conditions, as reported previously (29). In contrast, *gun4* displayed a more severe photosynthetic growth deficiency and failed to survive even under ~30 μmol photons m^{-2} s^{-1} light. Genetically complemented lines of both mutants demonstrated similar growth phenotype as 4A+ and *ho1*C2 control lines (Fig. 4*A*).

Previous studies have established that transcripts for tetrapyrrole biosynthetic enzymes in *C. reinhardtii* were mostly upregulated in the presence of light and that their levels were either comparable in *hmox1* or even elevated in *gun4* relative to parental WT strains (14, 30). To further investigate how bilins and *Cr*GUN4 affect accumulation of tetrapyrrole biosynthetic enzymes at the protein level in both light-grown and dark-adapted cultures, we leveraged available and newly developed antibodies to key enzymes of the *C. reinhardtii* tetrapyrrole pathway. Immunoblot analysis of total cellular protein revealed that levels of *Cr*GSA1, *Cr*CHLD, *Cr*CHLI1, *Cr*CHLI2, and *Cr*LPOR in both *hmox1* and *gun4* mutants were comparable to those found in 4A+ WT and the genetically complemented control lines (Fig. 4*B*). In striking contrast, the catalytic subunit of MgCh was dramatically reduced in both *hmox1* and *gun4* mutants and was restored in complemented lines, indicating that *Cr*HMOX1 and *Cr*GUN4 are both necessary for *Cr*CHLH1 protein accumulation in vivo (Fig. 4*B*).

CrGUN4 Is Required for BV Rescue of *Cr*CHLH1 and Chlorophyll Accumulation in *hmox1* Backgrounds. We previously showed that bilin-deficient *hmox1* mutants could be rescued by feeding exogenous BV (29, 30). We therefore analyzed whether exogenous BV could rescue *Cr*CHLH1 polypeptide accumulation in *hmox1* and *gun4*. In the presence of 20 μM BV, *Cr*CHLH1 levels were significantly restored in *hmox1* in both dark-adapted and light-treated cultures (Fig. 4*C*). In contrast, BV feeding did not restore

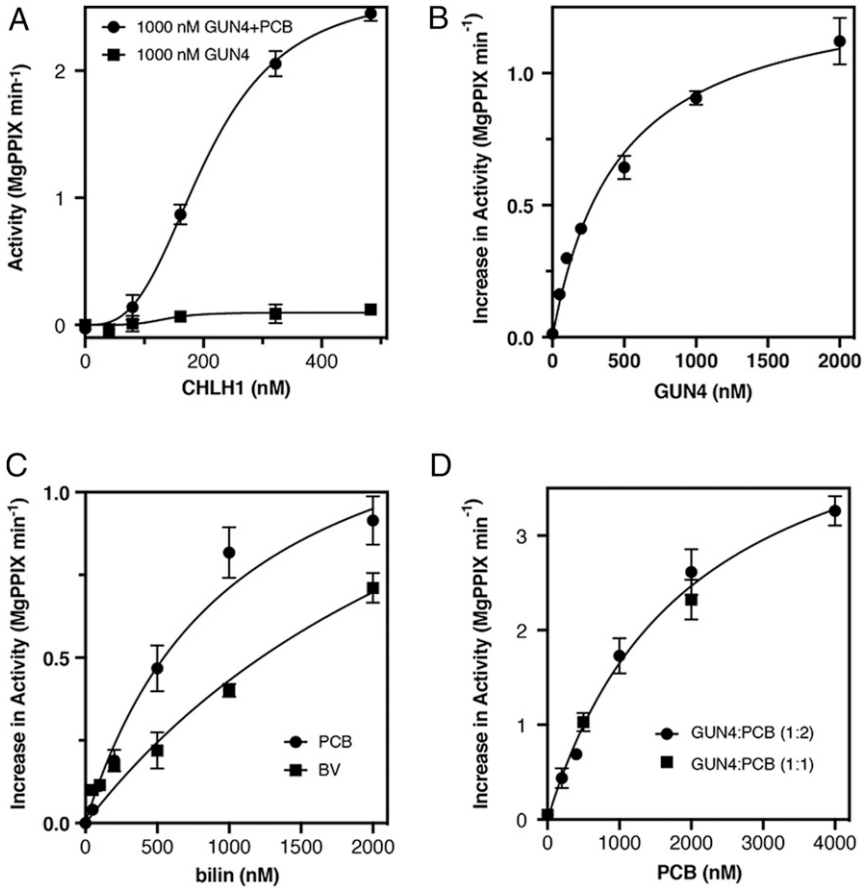

Fig. 3. *Cr*GUN4 stimulation of MgCh turnover is enhanced by bilins. (*A*) Comparative kinetics of MgCh activity varying CHLH1 in the presence of GUN4 alone or GUN4 with PCB. [PPIX] = 2 μM. (*B*) GUN4 stimulation of MgCh at 2,000 nM PCB, 500 nM CHLH1, and 1 μM PPIX, activity at 0 nM GUN4 = 0.013 ± 0.007 MgPPIX min^{-1}. (*C*) Stimulation of MgCh activity at increasing concentration of bilins (PCB and BV) in the presence of GUN4; 500 nM CHLH1 and 1,000 nM PPIX, activity at 0 nM bilin = 0.037 ± 0.009 MgPPIX min^{-1}. (*D*) Effect of increasing PCB concentration on MgCh at two different GUN4:PCB ratios with 1,000 nM CHLH1, activity at 0 nM PCB = 0.050 ± 0.018 MgPPIX min^{-1}. Values are means ± SD of three technical replicates in all panels.

*Cr*CHLH1 accumulation in the *gun4* mutant (Fig. 4*C*). These results demonstrate that the presence of bilins and *Cr*GUN4 are both required for *Cr*CHLH1 protein stability and accumulation.

To test the role of bilins in *Cr*CHLH1 accumulation and chlorophyll biosynthesis in vivo, we constructed the *hmox1gun4* double mutant by crossing *hmox1* and *gun4* (*SI Appendix*, Fig. S4*A*). Compared with *hmox1* and *gun4* single mutants, the *hmox1gun4* double mutant, like *gun4*, was similarly sensitive to light, and proved viable only under heterotrophic growth conditions in the dark or under dim light (< 30 μmol photons m^{-2} s^{-1}) (Fig. 5*A*). The double mutant also exhibited a more pronounced yellow-brown color than *gun4*. Expression of the WT *CrGUN4* allele containing an introduced Strep tag II (*GUN4-STII*) partially complemented the photosynthetic growth deficiency of the *hmox1gun4* double mutant, restoring it to that similar to the *hmox1* single mutant (Fig. 5*A*).

We then measured chlorophyll content in dark-adapted mixotrophic cultures before and after 12-h light exposure that were supplemented with or without 50 μM BV. In contrast with 4A+ WT where chlorophyll levels increased approximately twofold after illumination and in the *gun4* complemented line *gun4::GUN4-STII* (*SI Appendix*, Fig. S4*B*), light-dependent chlorophyll accumulation in *hmox1*, *gun4*, *hmox1gun4*, and *hmox1gun4::GUN4-STII* lines did not occur (Fig. 5*B*). Consistent with *hmox1* rescue by BV ((29)), BV supplementation also rescued light-dependent chlorophyll accumulation in *hmox1gun4::GUN4-STII*. This recovery was not observed in *gun4* or in the *hmox1gun4* double mutant, thus indicating that light-dependent chlorophyll accumulation requires both bilins and *Cr*GUN4 (Fig. 5*B*).

Discussion

Bilins are linear tetrapyrroles derived from heme that play a retrograde signaling function in *C. reinhardtii* when exported out of the chloroplast (28, 29). The targets of bilins in *C. reinhardtii*, which lacks cytosolic phytochromes, is presently unknown; however, their export promotes accumulation of a small set of transcripts of genes implicated in oxygen metabolism (29). Since bilins themselves are the product of oxygen metabolism, it makes sense that their production would be a proxy for the presence of light and oxygen. In this investigation, we establish that bilins also perform additional roles within chloroplasts to sustain chlorophyll synthesis.

Bilins Perform Dual Roles within Chloroplasts to Maintain Robust Chlorophyll Biosynthesis in the Presence of Light and Oxygen. Previous studies have established that Arabidopsis GUN4 stimulates MgCh activity by binding PPIX or MgPPIX, the respective substrate and product of MgCh (11). Our studies establish that *Cr*GUN4 can also bind bilins, the presence of which leads to >20-fold enhancement of MgCh turnover beyond that with *Cr*GUN4 alone. While the mechanism of this enhancement requires further study, we conclude that the *Cr*GUN4:PCB:PPIX ternary complex is responsible for this enhancement possibly by promoting the rate of *Cr*CHLH1 loading with its PPIX substrate. We hypothesize that

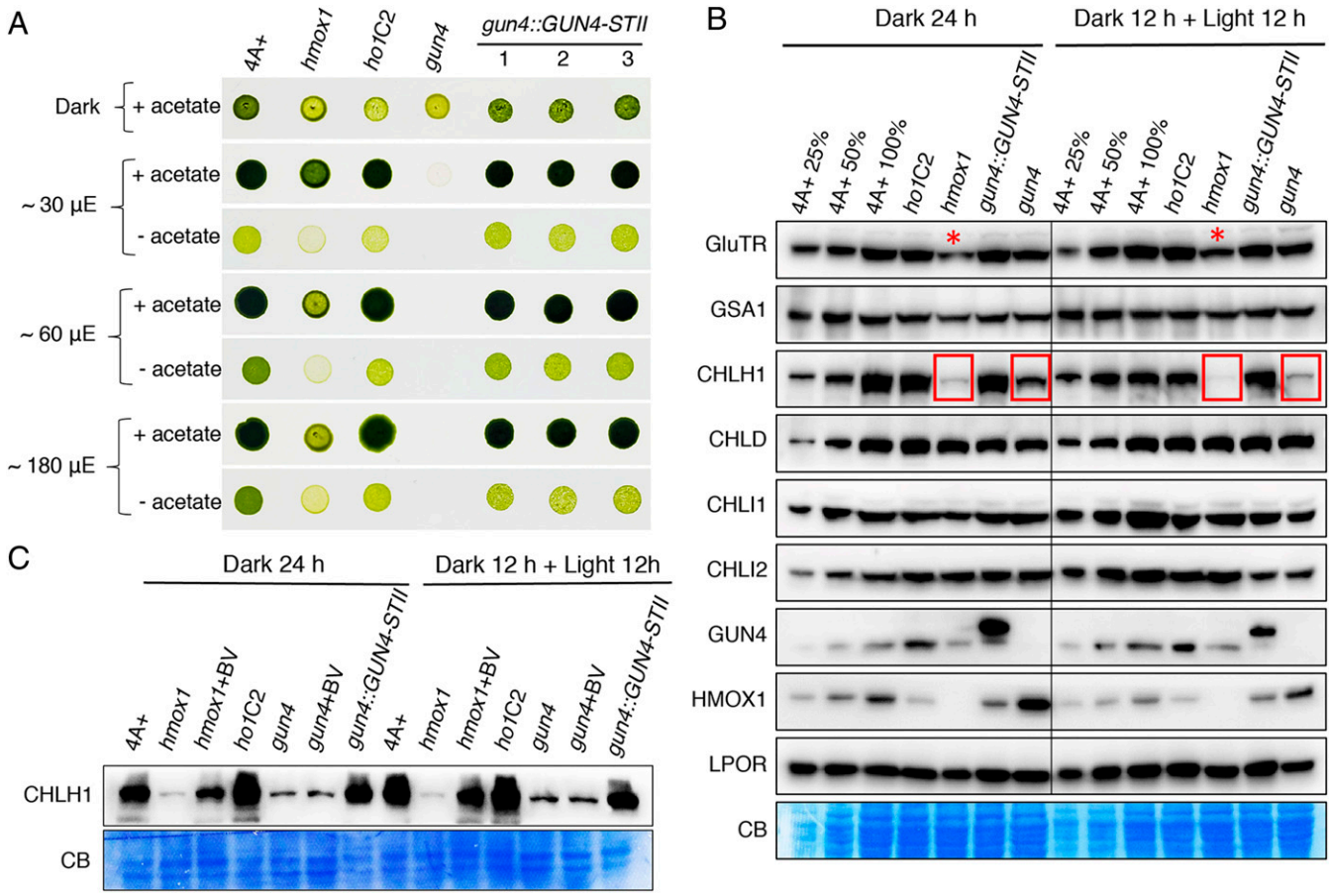

Fig. 4. *Cr*CHLH1 is deficient in *hmox1* and *gun4* mutants. (*A*) Comparative growth of 4A+, *hmox1*, *gun4*, cDNA complemented line *ho1*C2 and genomic DNA complemented lines *gun4::GUN4-STII* #1∼3 in the dark or under constant white fluorescent light. STII, twin-strep-tag. (*B*) Immunoblot analyses of representative tetrapyrrole biosynthetic enzymes using corresponding monospecific antibodies. *C. reinhardtii* cells grown in TAP medium under moderate light (∼30 µmol photons m^{-2} s^{-1}) were diluted to a density around ∼1 × 10^6 cells/mL. Half of the cell cultures were acclimated to darkness for 24 h (dark 24 h), whereas the other half were grown in the dark for 12 h and then exposed to light (∼30 µmol photons m^{-2} s^{-1}) for another 12 h (dark 12 h + light 12 h). Similar amount of protein (∼30 µg) was loaded for each sample. A dilution series (25% and 50%) of the 4A+ WT strain is included for both growth conditions. CB, Coomassie blue staining. Lanes with significant reduction in *Cr*CHLH1 or *Cr*GluTR proteins are indicated with red boxes and asterisks, respectively. (*C*) Rescue of CHLH1 polypeptide accumulation by BV feeding. Exponential-phase cell cultures grown in darkness were supplemented with or without 20 µM BV.

PCB remains bound throughout catalysis, thereby ensuring an adequate supply of chlorophyll to sustain phototrophic growth and to replace damaged photosystems.

Despite its strong transcriptional induction by light, the PPIX-binding *Cr*CHLH1 subunit of MgCh nearly disappears in the absence of either bilins or *Cr*GUN4. Our biochemical and genetic data establish that this phenomenon reflects the lack of *Cr*GUN4:PCB adducts because *Cr*CHLH1 is nearly absent in both *hmox1* and *gun4* mutants, and the presence of *Cr*GUN4 is required for BV rescue of the *Cr*CHLH1 deficiency in *hmox1* backgrounds. Hence, *Cr*GUN4:bilin complexes perform dual roles in *C. reinhardtii* chloroplasts, as activators of MgCh activity and as factors that stabilize *Cr*CHLH1 accumulation.

The reduced activity of MgCh in *hmox1* and *gun4* mutants is also expected to lead to overaccumulation of PPIX, a strongly photodynamic molecule that readily generates singlet oxygen ($^1O_2$), thereby promoting oxidative damage of the photosynthetic apparatus as has been observed in both mutants (30, 38). Presumably, triplet chlorophyll production in an anaerobic environment harmlessly decays, making GUN4 unnecessary in chlorophyll-containing anaerobic phototrophs. Our studies clearly show that bilin binding to the *Cr*GUN4:PPIX adduct effectively quenches the excited state of the latter, which would inhibit PPIX triplet state formation and subsequent $^1O_2$ generation. Based on this knowledge, we hypothesize that the interaction between the ternary *Cr*GUN4:PCB:PPIX complex and *Cr*CHLH1 facilitates transfer of PPIX substrate to *Cr*CHLH1 while also protecting the resulting *Cr*CHLH1:PPIX complex from photosensitized inactivation and turnover. We conclude that this protective function of bilins via GUN4 binding provides a solid rationale for the retention of bilin biosynthesis in all oxygenic phototrophs from cyanobacteria to all extant eukaryotic phototrophic species (26, 39). In future studies, it will be of great interest to evaluate whether bilins other than PCB can support a similar GUN4-dependent photoprotective role in other photosynthetic species to sustain MgCh activity in the presence of light and oxygen. Understanding the mechanism of damaged CHLH1 turnover in *C. reinhardtii* also remains an important objective of such studies.

Additional GUN4-Related Proteins Are Found in Cyanobacteria and Other Nonphotosynthetic Bacteria. The first committed step of chlorophyll biosynthesis is catalyzed by MgCh, which is present in both anoxygenic and oxygenic phototrophic organisms. In contrast with oxygenic phototrophs, anoxygenic photosynthetic bacteria, such as *Rhodobacter capsulatus*, lack GUN4 orthologs (11, 40). Instead, they possess BchJ, which functions as an MgPPIX chaperone

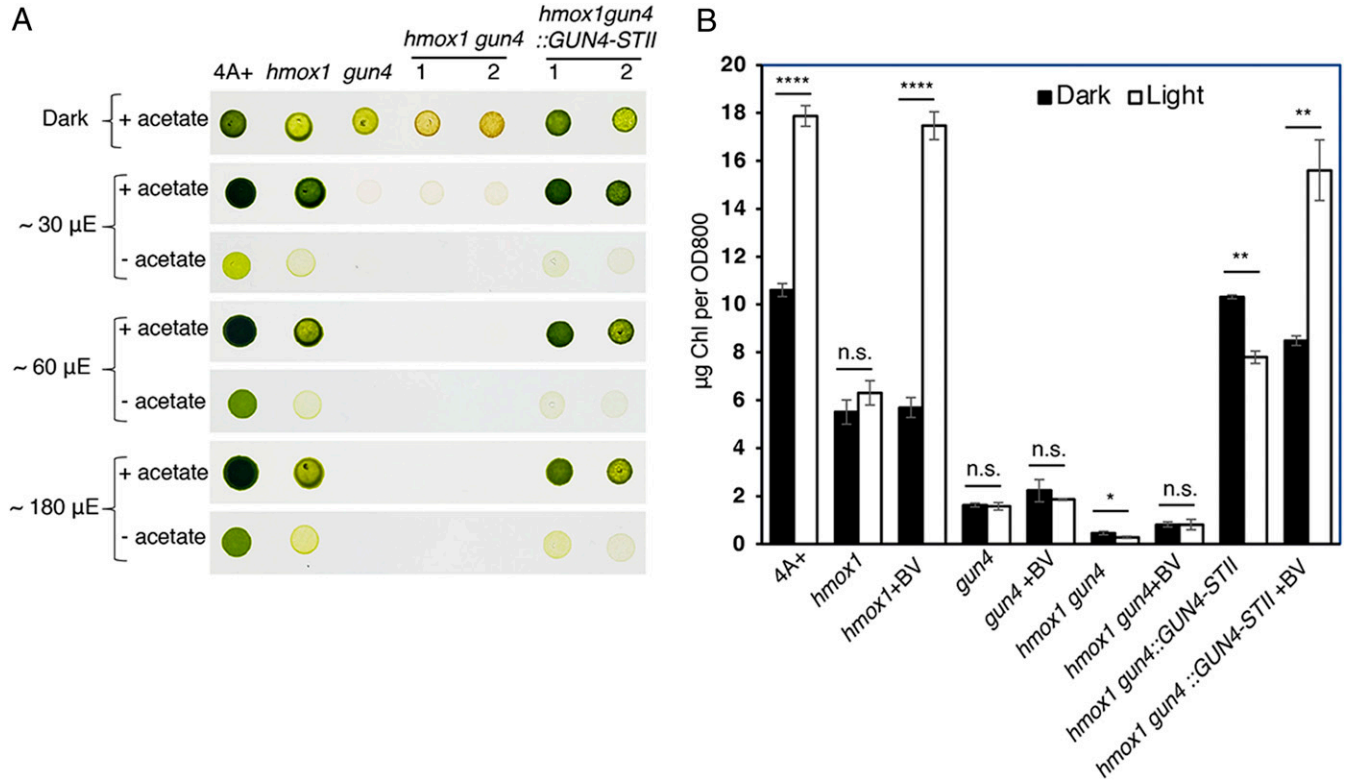

Fig. 5. BV rescue of *Cr*CHLH1 and chlorophyll accumulation in *hmox1* backgrounds requires *Cr*GUN4. (*A*) Comparative growth of 4A+, *hmox1*, *gun4*, *hmox1gun4*, and two independent genomic DNA complemented lines *hmox1gun4::GUN4-STII* in the dark or under constant white fluorescent light. STII, Strep-tag II. (*B*) Chlorophyll accumulation under different growth conditions. 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). Various mutant strains were supplemented with or without 50 µM BV. Asterisks above pairs of bars indicate significant differences (Student's *t* test, $*P < 0.05$, $**P < 0.01$, and $****P < 0.0001$; n.s., not statistically significant). Values are means ± SD of three biological replicates.

(41). It is notable that many cyanobacteria also possess other GUN4-related proteins that are associated with N-terminal extensions with unknown functions (16, 17). The biological roles of these and other GUN4-related proteins found in actinobacteria, alphaproteobacteria, and archaea (see the PFAM database, http://pfam.xfam.org/family/PF05419) are presently unknown. We speculate that these GUN4-related domains are bilin-binding regions that might also function to protect their translationally fused partners from ROS. In this regard, the presence of a gene that encodes a GUN4-CHLH chimera in the dinoflagellate *Symbiodinium microadriaticum* (locus OLP92483.1; http://refuge2020.reefgenomics.org) and transcriptome evidence in other dinoflagellates are consistent with this hypothesis (42).

Regulation of Chlorophyll Biosynthesis in Plants Differs from that in Chlorophytes. In *Arabidopsis thaliana*, regulation of tetrapyrrole biosynthesis occurs mainly through transcriptional and posttranslational control of glutamyl-tRNA reductase (GluTR), the first committed enzyme of heme and chlorophyll pathways (7, 43). Bilins strongly impact these processes as well via phytochrome-mediated stimulation of photosynthesis-associated nuclear gene expression, which affects both heme and chlorophyll pathways in plants (1). Heme affects the interaction between GluTR and the GluTR-binding protein (GBP) by exposing GluTR for Clp protease-mediated protein degradation (44), and dark-accumulated Pchlide also inhibits GluTR activity in plants (3, 45). Although GBP is widespread in plants, *C. reinhardtii* contains a GBP-like protein with only 23% homology to Arabidopsis GBP (44, 46).

The evidence that Pchlide does not accumulate in darkness in *C. reinhardtii* due to the presence of dark-operative protochlorophyllide oxidoreductase (47, 48), and that chlorophyte algae, such as *C. reinhardtii* lack phytochromes, underscores the differences between the regulation of chlorophyll synthesis in higher plants and chlorophytes. Indeed, the absence of *Cr*GUN4 leads to a significant accumulation of PPIX in *C. reinhardtii*, whereas the increase in PPIX was much less pronounced in the Arabidopsis *gun4* mutant (12, 38, 49). This suggests that plants possess distinct mechanisms to protect against photodynamic inactivation of enzymes of the chlorophyll biosynthetic pathway which remain to be identified.

Bilins Are Potent Antioxidants that Must Be Localized near the Site of $^1O_2$ Generation. In the *C. reinhardtii gun4* mutant, accumulation of PPIX accompanies transcriptional up-regulation of $^1O_2$-responsive gene glutathione peroxidase 5 (*GPX5*) during the dark-to-light transition, implicating a key role for *Cr*GUN4 in mitigating ROS-induced stress in membranes (14). While PPIX is generated in plastids, its membrane solubility allows it to rapidly distribute into cytoplasmic-facing membranes of the *C. reinhardtii* cells when overproduced. PPIX generation of $^1O_2$ in membranes is most likely to lead to lipid peroxidation, which is reversed by GPX5. Thus, the bilin-based retrograde signaling pathway is well suited to deal with cytosolic ROS stress that is triggered at dawn (29). *Cr*GUN4-dependent mitigation of ROS production in the chloroplast and this retrograde signaling pathway are connected by bilins. The interdependency of chlorophyll and bilin synthesis therefore appears to be fundamental for the survival of photosynthetic species in an

oxygen-rich environment. While this signaling pathway could be mediated by a bilin-dependent photoreceptor like phytochrome that was previously named chlorochrome (30), it is also possible that cytosolic proteins, which bind both bilins and released PPIX like GUN4, would even be more effective at preventing $^1O_2$ production in the first place. Such proteins have not been identified, but remain a potential innovation whose nature is likely to have been explored more than once.

Finally, $^1O_2$ is a potent oxidant of proteins, lipids, and DNA (50) but only when it is generated nearby due to its short intrinsic lifetime. $^1O_2$-mediated protein oxidation usually induces changes of biological properties (i.e., enzyme inactivation and proteolytic degradation) (51). We therefore propose that the reduced accumulation of CHLH1 in *hmox1* and *gun4* results from oxidative modification caused by PPIX-generated $^1O_2$ in situ. The decreased accumulation of CHLH1 was also observed in the PPIX-accumulating *gun4* mutant of cyanobacteria *Synechocystis* sp. PCC 6803, whereas CHLH protein abundance was not significantly changed in the Arabidopsis *gun4* mutant (12, 16). PPIX-bound GUN4 and CHLH were both reported to significantly generate $^1O_2$ and GUN4 was proposed to directly interact with a sensor to deliver the retrograde $^1O_2$ signal (23). Bilins might be essential to fine-tune the distribution of PPIX between GUN4 and CHLH to reduce the GUN4:PPIX-generated $^1O_2$. It will be interesting to test whether or not the $^1O_2$ derived from GUN4:PPIX is detoxified through the bilin-based retrograde signaling or even by bilin molecule itself (30).

Materials and Methods

Detailed materials and methods are provided in *SI Appendix*. This includes details about Chlamydomonas strains and their growth conditions, generation of *hmox1gun4* double mutant and complemented strains, and methods regarding recombinant enzyme production, MgCh enzyme activity assays, bilin and porphyrin preparations, protein extraction and immunoblot analysis, chlorophyll assays, pigment binding assays, and spectrophotometric measurements.

Data Availability. All study data are included in the article and *SI Appendix*.

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