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1 **Xanthophyll carotenoids stabilise the association of cyanobacterial chlorophyll synthase with the**
2 **LHC-like protein HliD**

3

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24

25 **Abstract**

26 Chlorophyll synthase (ChlG) catalyses a terminal reaction in the chlorophyll biosynthesis pathway,
27 attachment of phytol or geranylgeraniol to the C17 propionate of chlorophyllide. Cyanobacterial ChlG
28 forms a stable complex with high light-inducible protein D (HliD), a small single-helix protein
29 homologous to the third transmembrane helix of plant light-harvesting complexes (LHCs). The ChlG-

30 HliD assembly binds chlorophyll, β -carotene, zeaxanthin and myxoxanthophyll and associates with the
31 YidC insertase, most likely to facilitate incorporation of chlorophyll into translated photosystem
32 apoproteins. HliD independently coordinates chlorophyll and β -carotene but the role of the
33 xanthophylls, which appear to be exclusive to the ChlG-HliD assembly, is unclear. Here we generated
34 mutants of *Synechocystis* sp. PCC 6803 lacking specific combinations of carotenoids or HliD in a
35 background with FLAG- or His-tagged ChlG. Immunoprecipitation experiments and analysis of isolated
36 membranes demonstrate that the absence of zeaxanthin and myxoxanthophyll significantly weakens
37 the interaction between HliD and ChlG. ChlG alone does not bind carotenoids and accumulation of the
38 chlorophyllide substrate in the absence of xanthophylls indicates that activity/stability of the 'naked'
39 enzyme is perturbed. In contrast, the interaction of HliD with a second partner, the photosystem II
40 assembly factor Ycf39, is preserved in the absence of xanthophylls. We propose that xanthophylls are
41 required for the stable association of ChlG and HliD, acting as a 'molecular glue' at the lateral
42 transmembrane interface between these proteins; roles for zeaxanthin and myxoxanthophyll in ChlG-
43 HliD complexation are discussed, as well as the possible presence of similar complexes between LHC-
44 like proteins and chlorophyll biosynthesis enzymes in plants.

45

46 **Introduction**

47 Carotenoids are isoprenoid pigments that are categorized into two main classes, xanthophylls, which
48 contain oxygen, and carotenes, which do not. In chlorophototrophs carotenoids are important for light
49 harvesting, photoprotection and structural stabilization of proteins and membranes (1, 2). For
50 example, the trimeric photosystem I (PSI) from *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*)
51 contains 72 carotenoids and is dependent upon β -carotene for trimerization (3-6). β -carotene is also
52 a cofactor in photosystem II (PSII) and is required for its assembly (7, 8), is present in the cytochrome
53 *b₆f* complex (9, 10) and photosynthetic complex I (11), and plays a role in the assembly of the
54 phycobilisome antenna complex (5). In plants, xanthophylls play important roles in structural
55 stabilization of light-harvesting complexes (LHCs), light harvesting and photoprotection, and act as
56 lipid-soluble antioxidants (12-14). However, the role of xanthophylls in cyanobacteria, where they do
57 not participate in light harvesting, is less obvious. There is a pool of free xanthophylls in cyanobacterial
58 membranes that modulates membrane rigidity/fluidity (15, 16) and it is widely accepted that these
59 pigments provide protection against photooxidative stress and reactive oxygen and nitrogen species,
60 especially under high light conditions (17, 18). Although not integral components of either
61 photosystem, xanthophylls stabilize oligomers of PSI and PSII (5, 19), while keto-xanthophylls are

62 specifically required for non-photochemical dissipation of excess energy by the orange carotenoid
63 protein (20).

64 Chlorophyll (Chl) synthase (ChlG) is an integral thylakoid membrane protein that catalyses the addition
65 of a geranylgeranyl or phytol tail to the chlorophyllide (Chlide) macrocycle as one of the terminal steps
66 of Chl biosynthesis (**Figure 1A**). In *Synechocystis*, tagged ChlG co-purifies with the carotenoids β -
67 carotene, zeaxanthin and myxoxanthophyll in a pigment-protein complex also containing Chl, high
68 light-inducible proteins (Hlips), the membrane insertase YidC and the PSII assembly factor Ycf39 (21,
69 22). ChlG binds tightly to HliD in a ChlG-HliD 'core', with YidC and Ycf39 present in sub-stoichiometric
70 amounts (21). Hlips are single helix transmembrane pigment-binding proteins that are thought to be
71 the ancestors of LHCs in eukaryotic phototrophs (23). *Synechocystis* contains four Hlips (HliA-D), which
72 appear to be primarily involved in Chl biosynthesis/recycling and biogenesis and photoprotection of
73 Chl-binding proteins (24), although their exact roles remain enigmatic. An Hlip domain is also found
74 fused to the C-terminus of cyanobacterial and plant ferrochelatases (25). YidC assists in the integration
75 of translated proteins into the membrane bilayer (26) and its association with ChlG is presumed to
76 facilitate insertion of Chl molecules into newly synthesized Chl-binding proteins (21). The function of
77 Ycf39 in the ChlG-HliD complex is unclear but it could play a regulatory role in re-modelling ChlG-Hlip
78 assemblies in response to stress (27); Ycf39 and HliD interact in a separate complex that promotes the
79 synthesis and assembly of the core PSII subunits D1 and D2 during exposure to high light (28),
80 conditions under which Ycf39 dissociates from the ChlG complex (27) and HliD appears to be partially
81 replaced by HliC (22).

82 The approximate molar ratio of pigments in the ChlG complex is Chl (6): zeaxanthin (2.1-2.7): β -
83 carotene (1): myxoxanthophyll (0.6-1) (21, 22). HliD likely binds to ChlG as a dimer as the predicted
84 structure of Hlips shows that they cannot bind pigments as monomers (29); purified HliD dimers bind
85 6 Chl *a* molecules and 2 β -carotenes (22). Ultrafast transient absorption spectroscopy indicates that
86 one of the β -carotenes in the HliD adopts a 'twisted' configuration that can quench excited states of
87 Chl, resulting in the safe dissipation of excitation energy as heat (22, 30, 31), leading to the suggestion
88 that HliD photoprotects Chl-binding proteins and Chl-biosynthesis enzymes (24, 28, 30, 31). Given that
89 neither isolated HliD nor the Ycf39-HliD complex binds xanthophylls (22; 28, 30), and specific removal
90 of Ycf39 does not alter the pigment composition of the larger ChlG complex (27), the presence of
91 zeaxanthin and myxoxanthophyll appears to be strictly dependent on interaction of ChlG and HliD, but
92 their functional roles are unknown.

93 To investigate the requirement of xanthophylls in the ChlG-HliD complex, we generated a series of
94 *Synechocystis* strains lacking either combinations of xanthophylls or HliD and performed

95 immunoprecipitations using tagged ChlG as bait. Our results demonstrate that zeaxanthin is required
96 for stable formation of the ChlG-HliD complex, both *in vitro* and *in vivo*. ChlG alone does not co-purify
97 with carotenoids and its function appears to be perturbed in the absence of the xanthophyll-mediated
98 association with HliD. Possible roles of HliD in Chl trafficking and photosystem assembly/repair, and
99 candidates which may perform analogous functions in higher phototrophs, are discussed.

100

101 **Materials and methods**

102 **Growth of *Synechocystis* and strain generation**

103 *Synechocystis* was grown at 30 °C with moderate light (30-50 μmol of photons $\text{m}^{-2}\text{s}^{-1}$) in BG11 medium
104 (32) supplemented with 10 mM TES (Sigma Aldrich)-KOH pH 8.2 (BG11-TES). Liquid cultures were
105 shaken at approximately 150 rpm. For growth on plates, BG11-TES was supplemented with 1.5% (w/v)
106 agar and 0.3% (w/v) sodium thiosulphate. Antibiotics were included where appropriate (as detailed
107 below). Cultures for purification of protein complexes were grown photoautotrophically with ~ 100
108 μmol photons $\text{m}^{-2}\text{s}^{-1}$ illumination in 8 L vessels which were mixed by bubbling with sterile air and
109 maintained at 30 °C using a temperature coil connected to a thermostat-controlled circulating water
110 bath.

111 All reported mutant strains were prepared in the *Synechocystis* WT-P (WT) substrain (33); the FLAG-
112 *chlG* Δ *chlG* (FG/ Δ G) strain generated in this background has been reported previously (27). To
113 generate a strain producing 10xhistidine-tagged ChlG, the *NdeI*-*BglII* fragment encoding the 3xFLAG-
114 tagged *chlG* in pPD-NFLAG::*chlG* (21) was replaced with sequence encoding 10xhistidine-tagged *chlG*
115 (synthesised as a gBLOCK by Integrated DNA Technologies; see **Table S1** for sequence) and the
116 resulting allele exchange construct (pAH97) was introduced into WT *Synechocystis* with selection and
117 segregation on kanamycin, as detailed below. The zeocin resistance mutagenesis construct described
118 by Chidgey et al. (21) was used to delete the native *chlG* gene (slr0056) from the His-*chlG* strain,
119 generating the strain His-*chlG* Δ *chlG* (HG/ Δ G).

120 To generate a *crtR* null mutant, a linear mutagenesis construct was generated by OLE-PCR to replace
121 the central 555 bp of the 939 bp gene (sll1468) with an erythromycin resistance cassette by allele
122 exchange. Similar constructs were generated to replace 568 bp of the 912 bp *cruF* gene (sll0814) with
123 the *aadA* gene (streptomycin resistance) from pCDFDuet-1 (Novagen), 710 bp of the 1629 bp *crtO*
124 gene (slr0088) with the chloramphenicol acetyl transferase (*cat*) from pACYC184 (NEB) or 722 bp of
125 the 1185 bp *cruG* gene (sll1004) with *aadA*. The erythromycin resistance mutagenesis construct
126 described by Xu et al. (34) was used to delete *hliD* (ssr1789). Linear DNA constructs were introduced

127 to *Synechocystis* by natural transformation and transformants were selected on BG11 agar with 7.5
128 $\mu\text{g ml}^{-1}$ kanamycin, 7.5 $\mu\text{g ml}^{-1}$ erythromycin, 5 $\mu\text{g ml}^{-1}$ streptomycin, 12.5 $\mu\text{g ml}^{-1}$ chloramphenicol or
129 2.5 $\mu\text{g ml}^{-1}$ zeocin, as appropriate. Segregation of genome copies was achieved by sequential plating
130 with increasing antibiotic concentration up to 20 $\mu\text{g ml}^{-1}$ (for zeocin), 30 $\mu\text{g ml}^{-1}$ (for
131 erythromycin/streptomycin/kanamycin) and 50 $\mu\text{g ml}^{-1}$ (for chloramphenicol) and was confirmed by
132 colony PCR. All primers used to generate constructs and screen *Synechocystis* strains/mutants are
133 provided in **Table S1**.

134

135 **Purification of FLAG-tagged and His-tagged ChIG**

136 FLAG-immunoprecipitations were performed as reported in our previous work (21, 27). *Synechocystis*
137 cultures were grown to an OD_{750} of ~ 0.7 - 1.0 , harvested by centrifugation (17 700 $\times g$, 4 °C, 20 min),
138 resuspended in binding buffer (25 mM sodium phosphate pH 7.4, 10 mM MgCl_2 and 50 mM NaCl, 10%
139 (w/v) glycerol and EDTA-free Protease Inhibitor [Roche]) and lysed in a Mini-Beadbeater-16. The lysed
140 cells were collected from atop the glass beads and thylakoid membranes were pelleted by
141 centrifugation (48 400 $\times g$, 4 °C, 30 min) and solubilised by incubation with 1.5% (w/v) n-dodecyl- β -D-
142 maltoside (β -DDM; Anatrace) at 4 °C for 1 h. Following centrifugation (48 400 $\times g$, 4 °C, 30 min) to pellet
143 insoluble debris, the solubilised thylakoid fraction (the supernatant) was diluted 2-fold and applied to
144 a 300 μL anti-FLAG-M2 agarose (Sigma-Aldrich) column equilibrated in wash buffer (binding buffer
145 with 0.04% (w/v) β -DDM). The resin was washed with 20 resin volumes of wash buffer to remove
146 contaminating proteins and the FLAG-tagged bait protein and associated interaction partners were
147 eluted in 400 μL of the same buffer containing 187.5 $\mu\text{g mL}^{-1}$ 3xFLAG peptide (Sigma-Aldrich). The
148 mixture was filtered through a 0.22 μm spin column (Sigma-Aldrich) to separate the resin from the
149 eluted protein. Eluates were analysed immediately or stored at -80 °C.

150 For purification of His-ChIG (H.ChIG), solubilised thylakoids were applied three times to a Ni^{2+} NTA
151 Agarose (Qiagen) immobilised metal affinity chromatography column that had been pre-equilibrated
152 in binding buffer (as above) supplemented with 5 mM imidazole. Approximately 250 μL of resin was
153 used per 8 L of cell culture from which the membrane fraction was isolated. The column was washed
154 with 20 column volumes of binding buffer followed by washes with binding buffer containing
155 progressively higher (20, 50 and 80 mM) imidazole concentrations, each containing 0.04% (w/v) β -
156 DDM. H.ChIG was eluted by incubation of the resin with 400 μL of elution buffer (binding buffer
157 containing 400 mM imidazole and 0.04% (w/v) β -DDM) for 1 h with gentle agitation at 4 °C before the
158 solution was filtered through a 0.22 μm spin column, separating the resin from the eluted protein.
159 Eluates were analysed immediately or stored at -80 °C.

160

161 **Pigment analysis by reverse phase high performance liquid chromatography (RP-HPLC)**

162 Pigments were extracted from cell pellets or FLAG-/His-eluates in 100% methanol and separated by
163 RP-HPLC on an Agilent 1200 HPLC system using a Discovery HS C₁₈ column (5 µm, 250 × 4.6 mm)
164 according to the slightly modified method of that of Largarde and Vermaas (35) described in Proctor
165 et al. (27). Absorbance was monitored at 450 nm and 665 nm and carotenoid species and Chl *a* were
166 identified by their known absorption spectra (**Figure S1**) and retention time (**Figures S2** and reference
167 27).

168

169 **Quantitative proteomic analysis**

170 Proteins were extracted from the FLAG eluates by precipitation using a 2-D clean-up kit (GE
171 Healthcare) and processed according to Hitchcock et al. (36) to generate tryptic peptides. Analysis was
172 performed by nano-flow reverse phase chromatography coupled to a mass spectrometer using system
173 parameters described by MacGregor-Chatwin et al. (37) with the exception that peptides were
174 resolved with a 75 min gradient in this study. Proteins were identified and quantified using MaxQuant
175 v.1.5.3.30 (38) to search a *Synechocystis* proteome database
176 (<http://genome.microbedb.jp/cyanobase/>).

177

178 **Protein electrophoresis and immunoblotting**

179 Protein electrophoresis was performed as reported in our previous work (21, 27). Proteins in the FLAG-
180 and His-eluates were separated by SDS-PAGE on Invitrogen 12% Bis-Tris NuPage gels (Thermo Fisher
181 Scientific) and visualised by staining with Coomassie Brilliant Blue (Bio-Rad). For blue-native (BN)-
182 PAGE, solubilised thylakoid membrane protein complexes, prepared as outlined above, were
183 separated on 8-16% BN-gels (25) to resolve ChlG-HliD and Ycf39-HliD complexes. Protein complexes
184 were further resolved by incubating the BN-gel strip in 2% (w/v) SDS and 1% (w/v) dithiothreitol for
185 30 min at room temperature followed by separation of individual protein components in the second
186 dimension by SDS-PAGE in a denaturing 12 to 20% (w/v) polyacrylamide gel containing 7 M urea. For
187 immunoblotting, proteins were transferred onto polyvinylidene fluoride membranes (Thermo Fisher
188 Scientific) and incubated with specific primary antibodies against the 3xFLAG tag (Sigma-Aldrich), His₆-
189 tag (Merck), HliD (Agrisera AS10-1615), ChlG (described in reference 21) or Ycf39 (21) followed by an
190 appropriate secondary antibody (anti-rat for 3xFLAG, anti-mouse for His₆ and anti-rabbit for HliD, ChlG

191 and Ycf39) conjugated with horseradish peroxidase (Sigma-Aldrich) to allow detection using the
192 WESTAR ETA C 2.0 chemiluminescent substrate (Cyanagen) with an Amersham Imager 600 (GE
193 Healthcare).

194

195 **Quantification of Chl and Chl precursors**

196 Chl content was determined spectrophotometrically following extraction from cell pellets (from 1 mL
197 of culture at $OD_{750} \approx 0.4$) with 100% methanol according to Porra et al. (39). Chl precursors were
198 extracted from cell pellets (from 2 mL of culture at $OD_{750nm} \approx 0.4$) of WT and mutant *Synechocystis*
199 strains (five biological replicates per strain) and analysed by RP-HPLC with two fluorescence detectors,
200 as described previously (40). Equivalent peaks were integrated, summed, and calculated as a
201 percentage of the WT values, which were set as 100%.

202

203 **Results**

204 **Generation of strains with altered xanthophyll content**

205 Photosynthetic carotenoids are C_{40} molecules synthesized from 8 C_5 isoprene units (41). *Synechocystis*
206 accumulates four major carotenoid species, β -carotene, zeaxanthin, myxoxanthophyll and
207 echinenone, and produces others in lesser amounts, including synechoxanthin, 3'-hydroxy-
208 echinenone and β -cryptoxanthin (35, 42); the molecular structures of the major carotenoids are
209 presented in **Figure 1B**. An overview of carotenoid biosynthesis from all-*trans*-lycopene, the last
210 common precursor of all the mature carotenoids synthesized by *Synechocystis*, is given in **Figure S3**.
211 Briefly, lycopene is cyclized at one or both of its ψ -ends producing γ -carotene (one β -ionone ring) or
212 β -carotene (two β -rings); the myxoxanthophyll biosynthesis pathway branches from γ -carotene,
213 whereas the other carotenoids are produced by modification of β -carotene. The carotenoid contents
214 of WT *Synechocystis* and a strain containing an N-terminally 3xFLAG-tagged ChlG but lacking the native
215 *chlG* (FG/ Δ G) were analysed by RP-HPLC and the four expected major carotenoid species were
216 identified (**Figure S2**).

217 Mutants unable to synthesize β -carotene display severe growth phenotypes because it is required for
218 assembly of PSII (4, 7), but xanthophylls are dispensable for photoautotrophic growth under our low-
219 stress laboratory conditions (35, 42, 43). Mutants lacking xanthophyll biosynthesis genes were
220 generated in the WT and FG/ Δ G strains (**Table 1; Figure S4**), resulting in identical carotenoid
221 deficiencies in both backgrounds (**Figure S2**; summarised in **Table 2**).

222 Deletion of the *crtR* gene, which encodes the β -carotene hydroxylase acting at the 3/3' positions of
223 the ionone rings of β -carotene (7), prevented biosynthesis of both zeaxanthin and myxoxanthophyll
224 and resulted in the appearance of a new carotenoid species, previously identified as dehydroxy-
225 myxoxanthophyll (myxoxanthophyll missing the hydroxyl group on the β -ring (35, 44)). It is not
226 possible to generate a knockout strain that produces myxoxanthophyll but not zeaxanthin owing to
227 the shared requirement of CrtR for synthesis of both carotenoids, however, myxoxanthophyll
228 biosynthesis is specifically halted at the first dedicated step, 1',2'-hydroxylation of lycopene/ γ -
229 carotene, in the absence of the C-1'-hydroxylase CruF (**Figure S3**) (45, 46). Deletion of *cruF* generated
230 a mutant that did not contain any myxoxanthophyll or myxoxanthophyll-specific precursors but
231 otherwise had a normal carotenoid quota; *cruF* deletion in the Δ *crtR* strain resulted in a strain that
232 lacks 3-dehydroxy-myxoxanthophyll as well as zeaxanthin and myxoxanthophyll, accumulating only β -
233 carotene and echinenone in significant amounts (**Figure S2**). The only other confirmed enzyme in
234 myxoxanthophyll biosynthesis in cyanobacteria is the 2'-O-glycosyltransferase CruG, which adds a
235 sugar moiety to the carotenoid backbone (**Figure S3**; 45). Deletion of the *Synechocystis cruG*
236 homologue from the WT and Δ *crtR* strains resulted in accumulation of myxol or 3-dehydroxy-myxol,
237 respectively (**Figure S2**). Finally, we constructed a Δ *crtO* mutant lacking the FAD-dependent β -ionone
238 ring ketolase, which produces myxoxanthophyll and zeaxanthin but is unable to synthesise the keto-
239 carotenoids echinenone and 3'-hydroxy-echinenone (43).

240

241 **The *in vitro* interaction of ChIG and HliD requires zeaxanthin**

242 Parallel immunoprecipitations of FLAG-ChIG (F.ChIG) from the FG/ Δ G strain and the carotenoid
243 mutants were performed to investigate the effect of xanthophyll deficiency on the ChIG-HliD
244 interaction *in vitro* (**Figure 2**). Retrieval of F.ChIG from each strain was confirmed by SDS-PAGE (**Figure**
245 **2A**) and immunoblotting with FLAG and ChIG specific primary antibodies (**Figure 2B**). F.ChIG from the
246 FG/ Δ G strain co-eluted with both HliD and Ycf39 as observed previously (21, 27); note that F.ChIG from
247 strains lacking *crtR* migrated slightly further than expected on SDS-PAGE gels, which is discussed
248 below.

249 Only a residual immunoblot signal for HliD was observed in the FG/ Δ G/ Δ *crtR* and FG/ Δ G/ Δ *crtR*/ Δ *cruF*
250 eluates, accompanied by the loss of signal for Ycf39; the level of HliD was comparable in thylakoids
251 isolated from each of the strains (**Figure 2C**), ruling out the possibility of any pleiotropic effect on the
252 production of HliD associated with deletion of *crtR*. Consistent with the absence of HliD, direct
253 measurement of the absorbance spectra of the eluates from the FG/ Δ G/ Δ *crtR* and FG/ Δ G/ Δ *crtR*/ Δ *cruF*
254 strains revealed a drastic reduction in pigmentation compared to the visibly orange eluate from the

255 FG/ Δ G strain (**Figure 2D**). Qualitative analysis of the pigments in the eluates by RP-HPLC confirmed
256 Chl, β -carotene myxoxanthophyll and zeaxanthin were present in the complex isolated from the
257 FG/ Δ G strain (**Figure 2E**). Small amounts of dehydroxy-myxoxanthophyll, Chl and β -carotene were
258 present in the FG/ Δ G/ Δ *crtR* elution; the Chl and β -carotene likely originate from trimeric PSI which
259 contaminates FLAG-tag pulldowns (21).

260 In contrast to those from strains lacking *crtR*, FLAG-immunoprecipitation eluates from the
261 FG/ Δ G/ Δ *cruF* mutant were visibly orange and spectrally similar to those from the FG/ Δ G parent strain
262 (**Figure 2D**). Immunoblot analysis of the FG/ Δ G/ Δ *cruF* eluate gave clear signals for both HliD and Ycf39
263 and zeaxanthin was identified by RP-HPLC analysis of the extracted pigments (**Figure 2B, E**). Removing
264 the sugar group from myxoxanthophyll (producing myxol) or dehydroxy-myxoxanthophyll (producing
265 dehydroxy-myxol) by deletion of *cruG* from the FG/ Δ G and FG/ Δ G/ Δ *crtR* backgrounds, respectively,
266 did not alter the results compared to those of the respective parent strain (**Figure S5**). Finally, deletion
267 of *crtO* did not affect the composition of the F.ChlG complex (**Figure S6**), confirming that keto-
268 carotenoids are not involved in the ChlG-HliD interaction.

269 Quantitative proteomic analysis by mass-spectrometry was also used to compare the levels of HliD
270 and Ycf39 co-isolated with F.ChlG from the different carotenoid mutants (**Figure 2F**). When normalised
271 to the amount of bait protein, the absence of myxoxanthophyll (FG/ Δ G/ Δ *cruF*) did not significantly
272 change the amount of HliD co-purified with F.ChlG compared to the FG/ Δ G control (P = 0.35).
273 However, the absence of zeaxanthin (in the FG/ Δ G/ Δ *crtR* strain) or both zeaxanthin and
274 myxoxanthophyll (in FG/ Δ G/ Δ *crtR*/ Δ *cruF*) drastically reduced the level of HliD to 2-3% of the control
275 level (**Table S2**). These effects on the HliD:F.ChlG stoichiometry were mirrored by that of Ycf39:F.ChlG,
276 with no significant change after elimination of myxoxanthophyll (P = 0.67) and average decreases to
277 10-20% of the control level in the zeaxanthin-less strains.

278

279 **The protein tag does not affect formation of the ChlG complex**

280 As stated above, F.ChlG isolated from strains lacking *crtR* migrated slightly faster on SDS-PAGE gels
281 (**Figure 2A**), suggesting the protein was somehow smaller. Sequencing of the *psbAII* locus in these
282 FG/ Δ G/ Δ *crtR* strains confirmed the loss of one of the 3 \times FLAG-epitopes, leaving a 2 \times FLAG-tag in frame
283 with the *chlG* gene (**Figure S7**). Using a freshly isolated FG/ Δ G/ Δ *crtR* mutant confirmed to have the
284 3 \times FLAG-tag and performing parallel co-immunoprecipitations alongside a strain with a 2 \times FLAG-tagged
285 enzyme showed that the length of the tag did not affect the protein or pigment profiles of the eluates
286 (**Figure S8**).

287 The 3xFLAG-tag is relatively long (24 amino acids) and highly positively charged. To confirm that this
288 extra amino-acid sequence does not generate artefacts regarding the interaction with xanthophylls,
289 the experiments were repeated with an N-terminally 10xHis-tagged ChlG (H.ChlG; **Figure S4** and **S9**).
290 Purification of the His-tagged enzyme by immobilised nickel-affinity chromatography resulted in a
291 visibly pigmented eluate with very similar absorbance properties to the FLAG-immunoprecipitation
292 complex (**Figure S10C**). Analysis of the eluate by SDS-PAGE and immunoblotting showed a prominent
293 band corresponding to H.ChlG (**Figure S10A-B**); immunoblots also confirmed the presence of HliD
294 (**Figure S10B**), and zeaxanthin and myxoxanthophyll were identified by RP-HPLC (**Figure S10D**).
295 Consistent with the results with the FLAG-tagged enzyme (**Figure 2**), HliD does not co-elute with
296 H.ChlG in the absence of zeaxanthin and myxoxanthophyll (**Figure S10A-B**) and the eluate contained
297 very low levels of pigments (**Figure S10C-D**).

298

299 **Association of xanthophylls with ChlG is dependent on HliD**

300 Previous studies have shown that *Synechocystis* $\Delta hliD$ mutants do not display a growth phenotype or
301 altered accumulation of photosystems under standard low-stress laboratory growth conditions (47-
302 49). However, deletion of *hliD* did decrease the level of ChlG, resulting in a concomitant six-fold
303 increase in the level of its substrate Chlide *a* (21). We generated an independent *hliD* deletion in the
304 FG/ ΔG background (**Figures S2** and **S4**) in order to determine the inherent pigment binding properties
305 of isolated F.ChlG in the absence of HliD. In agreement with previous reports, there was a marked
306 reduction in the level of F.ChlG isolated from FG/ ΔG / $\Delta hliD$ using the same amount of starting material
307 (solubilised thylakoid membranes), although it was possible to isolate Coomassie-stainable
308 quantities of the protein (**Figure 3A**). As expected, Ycf39 was not detectable by immunoblot (**Figure**
309 **3B**) and the eluate lacked Chl and carotenoids, evident from both the absorbance spectra (**Figure 3C**)
310 and RP-HPLC analysis of extracted pigments (**Figure 3D**), confirming that ChlG alone does not bind
311 carotenoids. Consistent with the result obtained with the FLAG-tagged enzyme, isolation of H.ChlG
312 from a $\Delta hliD$ background also resulted in a decreased level of ChlG (**Figure S10A-B**) and an immobilised
313 nickel-affinity chromatography eluate that lacked pigments (**Figure S10C-D**).

314

315 **Restoration of the ChlG-xanthophyll-HliD interaction in isolated membranes**

316 The results presented above indicate that xanthophylls are required to maintain the interaction
317 between ChlG and HliD, and that ChlG does not co-purify with carotenoids in the absence of HliD. To
318 determine whether the interaction could be restored in isolated membranes, solubilised membranes

319 from the FG/ Δ G/ Δ hliD strain, which synthesizes the normal complement of carotenoids but lacks HliD,
320 were incubated with those from the FG/ Δ G/ Δ crtR/ Δ cruF strain, which lacks zeaxanthin and
321 myxoxanthophyll but produces HliD (schematically illustrated in **Figure 4A**). F.ChlG was subsequently
322 isolated from the individual or mixed membrane samples by immunoprecipitation (**Figure 4B**). Unlike
323 eluates from either individual sample, immunoblotting detected both HliD and Ycf39 in the elution
324 from the mixed membranes (**Figure 4C**). Although considerably less pigmented than that from the
325 FG/ Δ G strain, the eluate from the mixed sample was visibly coloured and the absorbance spectra
326 revealed a small but clear increase in pigmentation compared to the eluates from the two mutant
327 strains (**Figure 4D**). Zeaxanthin and myxoxanthophyll were both present in the complex isolated from
328 the combined membranes (**Figure 4E**), confirming that the ChlG-HliD interaction can reform upon
329 provision of xanthophylls. Re-formation of the ChlG-HliD complex was also achieved in an analogous
330 experiment with membranes from the equivalent H.ChlG strains (**Figure S11**).

331

332 **ChlG-HliD complexes in thylakoid membranes are affected by xanthophyll deficiency**

333 Isolation of FLAG- or His-tagged ChlG complexes requires washing steps that might disrupt interactions
334 with weakly-binding proteins. To further ascertain the effects of the loss of xanthophylls on the ChlG-
335 HliD complex we used an alternative approach, separating solubilised thylakoid membrane proteins
336 by two-dimensional BN/SDS-PAGE followed by immunoblotting (**Figure 5**). Most of the detectable
337 ChlG in WT membranes appears to co-migrate with HliD in a ~100 KDa complex; this complex likely
338 forms the major fraction of our co-immunoprecipitated F.ChlG and it is likely to be composed of ChlG
339 associated with several copies of HliD (21). A larger ChlG-HliD complex is also detected; we refer to
340 the smaller ChlG-HliD assembly as complex 1 and the larger one as complex 2, as indicated above the
341 figure. Small amounts of free ChlG and HliD, and the Ycf39-HliD complex (28), which migrates slightly
342 faster than the ChlG-HliD complex 1, are also clearly observed.

343 Repeating the analysis with membranes of the Δ crtR/ Δ cruF double mutant lacking both zeaxanthin
344 and myxoxanthophyll, the ratio between the ChlG-HliD complex 1 and unattached ChlG differed from
345 the WT sample, with much less ChlG present in the complex with HliD and more in a free form. Along
346 with the clear reduction in ChlG-HliD complex 1, the larger complex 2 is almost completely absent in
347 the Δ crtR/ Δ cruF mutant. In contrast, the Ycf39-HliD interaction is unaffected by removal of
348 xanthophylls. This 2D-BN/SDS-PAGE analysis supports the proposed requirement for xanthophylls for
349 the stability of the ChlG-HliD complexes in the thylakoid membranes of *Synechocystis*.

350

351 Xanthophyll mediated association of ChlG and HliD promotes ChlG function

352 As discussed above, deletion of *hliD* results in a decreased cellular level of ChlG and accumulation of
353 its substrate, Chlide (21). To determine the effect of the absence of xanthophylls on Chl biosynthesis
354 the levels of Chl and its biosynthetic intermediates (see **Figure S12** for an overview of the Chl
355 biosynthesis pathway) were compared in WT, $\Delta crtR$, $\Delta cruF$ and $\Delta crtR/\Delta cruF$ cells (**Figure 6**). Under
356 standard growth conditions the whole cell absorbance spectra were similar for all four strains except
357 for the region where carotenoids absorb (450-500 nm; **Figure 6A**). The Chl level of the $\Delta cruF$ (4.8 ± 0.3
358 $\mu\text{g Chl per ml of } 1 \text{ OD}_{750}$ unit cells) and $\Delta crtR/\Delta cruF$ ($5.2 \pm 0.1 \mu\text{g ml}^{-1}$) strains were not significantly
359 different to that of the WT ($5.1 \pm 0.2 \mu\text{g ml}^{-1}$), but the $\Delta crtR$ mutant did display a small but significant
360 ($P = 0.04$) decrease in Chl ($4.6 \pm 0.2 \mu\text{g ml}^{-1}$) (**Figure 6B**).

361 Interestingly, the level of the ChlG substrate monovinyl (MV)-Chlide was 6-7 times higher in the $\Delta crtR$
362 and $\Delta crtR/\Delta cruF$ mutants compared to the WT (set as 100%) (**Figure 6C**), indicating that ChlG activity
363 is affected by the loss of zeaxanthin. Similarly, divinyl (DV)-Chlide, the substrate of the preceding
364 enzyme in the pathway, 8-vinyl reductase (8VR), also accumulated ~ 4 -fold in both strains compared
365 to the WT, suggesting the 8VR reaction is also affected, either directly or by the build-up of MV-Chlide.
366 In contrast, the $\Delta cruF$ strain contained both Chlide species at levels comparable to the WT. The only
367 other statistically significant differences in precursor levels were the decrease in magnesium-
368 protoporphyrin IX in the $\Delta crtR$ mutant and the increase in magnesium protoporphyrin monomethyl
369 ester in $\Delta cruF$. The reason(s) for these small variations in earlier pathway intermediates are likely due
370 to pleiotropic effects resulting from the loss of xanthophylls in these strains.

371

372 Discussion

373 We have shown that zeaxanthin stabilises the ChlG-HliD interaction in *Synechocystis* membranes. The
374 alternating single-double carbon-carbon bonds in the polyene chain of carotenoids makes them much
375 more rigid than other hydrophobic molecules, such as comparatively flexible lipids, and a scaffolding
376 role for carotenoids in LHC proteins in plants has been proposed previously (reviewed by 13). Although
377 HliD and ChlG still associate to a minor extent in the absence of both zeaxanthin and myxoxanthophyll,
378 this complex appears to be considerably less stable than in WT cells since the HliD protein is almost
379 completely absent in F.ChlG immunoprecipitation eluates from a $\Delta crtR$ mutant. Therefore, neither β -
380 carotene, echinenone nor dehydroxy-myxoxanthophyll (myxoxanthophyll with an unhydroxylated β -
381 ring which accumulates in the $\Delta crtR$ mutant (35, 44)) can substitute for zeaxanthin/myxoxanthophyll
382 in stabilising the ChlG-HliD interaction. These results suggest that the hydroxyl groups on the β ring(s)

383 of zeaxanthin and myxoxanthophyll, which allow xanthophylls to be held perpendicular to the
384 membrane through strong hydrogen bonds, enhancing their structural function (13), are essential for
385 their interaction with ChlG and HliD. Another Hlip (HliA/B) pigment-protein complex has also been
386 reported to contain zeaxanthin and myxoxanthophyll (50), while zeaxanthin binds to the conserved C-
387 terminal transmembrane Chl *a/b* binding (CAB) domains of dimeric ferrochelatase (25); thus,
388 xanthophylls may play similar roles in other Hlip-/CAB-domain protein assemblies in *Synechocystis*.

389 The ChlG-HliD interaction was maintained in a $\Delta cruF$ mutant that produces zeaxanthin but not
390 myxoxanthophyll, indicating that zeaxanthin alone can mediate association of the two proteins.
391 However, reconstitution of the ChlG-HliD interaction in isolated membranes showed both
392 myxoxanthophyll and zeaxanthin were incorporated into the re-formed complex; thus, promiscuity
393 versus specificity of zeaxanthin and myxoxanthophyll binding sites in the ChlG-HliD complex requires
394 further study. Insight into any specific role of myxoxanthophyll requires a strain that produces
395 myxoxanthophyll in the absence of zeaxanthin, but this is not possible by deletion of native genes
396 because of the shared requirement of CrtR for biosynthesis of both carotenoids. We attempted to
397 generate such a strain using CrtR from the filamentous cyanobacteria *Nostoc* sp. PCC 7120 (alr4009),
398 which produces keto-myxoxanthophyll species but does not synthesise zeaxanthin (51-53). However,
399 our preliminary results indicate that neither myxoxanthophyll nor zeaxanthin biosynthesis was
400 restored when alr4009 was expressed in place of the native *crtR* or at the *psbAII* locus in the $\Delta crtR$
401 mutant (data not shown).

402 Isolation of native ChlG complexes from other chlorophototrophic organisms has not yet been
403 reported but given the high-level of sequence conservation of cyanobacterial ChlG enzymes (e.g., 84%
404 identity in *Synechocystis* and *Synechococcus* sp. PCC 7002) we predict that ChlG-xanthophyll-HliD
405 complexes will be conserved in cyanobacteria. In support of this, we previously reported that ChlG
406 from *Synechococcus* sp. PCC 7002, produced in *Synechocystis*, interacts with HliD, zeaxanthin and
407 myxoxanthophyll (27). The myxoxanthophyll species produced in *Synechocystis* is myxol-2'
408 dimethylfucoside, whereas *Synechococcus* sp. PCC 7002 produces myxol-2' fucoside, which lacks two
409 methyl groups on the sugar moiety (45, 54), indicating that the fucose group is not important for the
410 interaction of myxoxanthophyll with the complex, as found with the $\Delta cruG$ mutant here.

411 Although we cannot rule out that altered thylakoid membrane carotenoid content in the xanthophyll
412 mutants may affect the detergent-sensitivity of protein complexes, our data still supports a role for
413 xanthophylls in stabilising the interaction of ChlG and HliD. Destabilising this association by removal
414 of xanthophylls or deletion of HliD results in a significant build-up of Chlide and, in the latter case, the
415 accumulation of ChlG is also reduced. However, consistent with previous results, there are no major

416 phenotypic consequences relating to growth or chlorophyll biosynthesis upon the loss of HliD, at least
417 under our standard, low-stress laboratory growth conditions. It is possible that the ChlG-xanthophylls-
418 HliD complex is particularly important under specific stress-conditions. For example, the xanthophylls
419 could quench singlet oxygen produced during photo-oxidative stress; work is underway to elucidate
420 how xanthophyll-mediated recruitment of HliD affects Chl metabolism in cyanobacteria *in vivo*.

421 In contrast to cyanobacterial ChlG, the enzymes from *Arabidopsis thaliana* (*Arabidopsis*) and the green
422 alga *Chlamydomonas reinhardtii* do not associate with HliD or xanthophylls when heterologously
423 produced in *Synechocystis* (27). These phototrophs lack Hlips, and decreased sequence identity
424 between plant and cyanobacterial ChlG (e.g., 63% for *Arabidopsis* and *Synechocystis*) may explain why
425 the eukaryotic enzymes do not interact with HliD. Plants do, however, contain single helix LHC-like
426 proteins that are homologous to Hlips called One-Helix Proteins (OHPs) (**Figure S13**) (55). Like HliD and
427 HliC, which are predicted to form heterodimers in cyanobacteria (22, 28), *Arabidopsis* OHP1 and OHP2
428 have been shown to dimerise *in vivo* (56). Furthermore, OHPs bind the plant homolog of Ycf39
429 (HCF244) and are suggested to function in pigment delivery to newly synthesized PSII subunits (56-
430 58), as proposed for the cyanobacterial Ycf39-Hlip complex (28, 59). It is currently unclear whether
431 Ycf39-Hlip-dependent synthesis of PSII subunits relies on the additional interaction of Hlips with ChlG.
432 If so, then OHPs (and HCF244) may associate with ChlG in plants as well, although evidence of such a
433 complex is yet to be reported. Plants also synthesise LIL3 (55), a two transmembrane helix protein
434 with a proposed role in Chl biosynthesis; LIL3 interacts with the Chl biosynthesis enzymes
435 protochlorophyllide oxidoreductase (POR) and geranylgeranyl diphosphate reductase (ChlP), although
436 whether it forms a complex with ChlG is not clear and may be species-specific (60-62). Further work is
437 required to clarify the interactions between these LHC-like proteins and Chl biosynthesis enzymes in
438 plants, but it is feasible that zeaxanthin and/or other abundant plant xanthophylls, such as lutein, may
439 play stabilization roles in such complexes.

440

441 **Competing Interests:** The authors declare that there are no competing interests associated with the
442 manuscript.

443

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456

457 **Abbreviations**

458 β -DDM ,n-dodecyl- β -maltoside; BN-PAGE, blue native polyacrylamide gel electrophoresis; Chl(s),
459 chlorophyll(s); Chlide, chlorophyllide; ChlG, chlorophyll synthase; F.ChlG, FLAG-chlorophyll synthase;
460 H.ChlG, His-chlorophyll synthase; Hlips, high-light-inducible proteins; HliC, high-light-inducible protein
461 C; HliD, high light inducible protein D; LHCs, light-harvesting complexes; RP-HPLC, reverse phase high-
462 performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel
463 electrophoresis; PSI, photosystem I, PSII, photosystem II; WT, wild-type.

464

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Tables (1-2) and Figures (1-6)

Table 1. *Synechocystis* strains used in this study. Mutations were generated in the WT, FG/ Δ G and HG/ Δ G backgrounds, as detailed in the text.

Strain	Properties	Reference/source
Wild-type (WT)	Glucose tolerant WT-P substrain of <i>Synechocystis</i> sp. PCC 6803	33
FG/ Δ G	N-terminally 3xFLAG-tagged <i>Synechocystis</i> sp. PCC 6803 <i>chlG</i> inserted in place of <i>psbAII</i> and deletion of native <i>chlG</i> gene; kanamycin resistant (Kan ^R) and zeocin resistance (Zeo ^R)	27
HG/ Δ G	N-terminally 10xHis-tagged <i>Synechocystis</i> sp. PCC 6803 <i>chlG</i> inserted in place of <i>psbAII</i> and deletion of native <i>chlG</i> gene; Kan ^R and Zeo ^R	This study
Δ <i>crtR</i>	Deletion of <i>crtR</i> (sll1468); erythromycin resistant (Em ^R)	This study
Δ <i>cruF</i>	Deletion of <i>cruF</i> (sll0814); streptomycin resistant (Sm ^R)	This study
Δ <i>crtO</i>	Deletion of <i>crtO</i> (slr0088); chloramphenicol resistant (Cm ^R)	This study
Δ <i>cruG</i>	Deletion of <i>cruG</i> (sll1004); Sm ^R	This study
Δ <i>crtR</i> / Δ <i>cruF</i>	Deletion of <i>cruF</i> in Δ <i>crtR</i> background; Em ^R and Sm ^R	This study
Δ <i>crtR</i> / Δ <i>cruG</i>	Deletion of <i>cruG</i> in Δ <i>crtR</i> background; Em ^R and Sm ^R	This study
Δ <i>hliD</i>	Deletion of <i>hliD</i> (ssr1789); Em ^R	This study

Table 2. Carotenoid deficiencies of *Synechocystis* mutants lacking combinations of *crtR*, *cruF*, *cruG* and *crtO*.

Mutant	Xanthophyll deficiency^a	Intermediates accumulated
<i>ΔcrtR</i>	zeaxanthin, cryptoxanthin ^b , myxoxanthophyll, 3'-hydroxyechinenone ^b	dehydroxy-myxoxanthophyll
<i>ΔcruF</i>	myxoxanthophyll	N/A
<i>ΔcruG</i>	myxoxanthothyll	myxol
<i>ΔcrtO</i>	echinenone, 3'-hydroxyechinenone ^b , canthaxanthin ^b	N/A
<i>ΔcrtR ΔcruF</i>	zeaxanthin, cryptoxanthin ^b , myxoxanthophyll, 3'-hydroxyechinenone ^b	N/A
<i>ΔcrtR ΔcruG</i>	zeaxanthin, cryptoxanthin ^b , myxoxanthophyll, 3'-hydroxyechinenone ^b	dehydroxy-myxol

^aThe xanthophyll deficiency was the same in the WT, FG/ Δ G and HG/ Δ G backgrounds. ^bThese carotenoids were not detected by RP-HPLC in this study; deficiency is assumed based on literature, as detailed in the text and Figure S1.

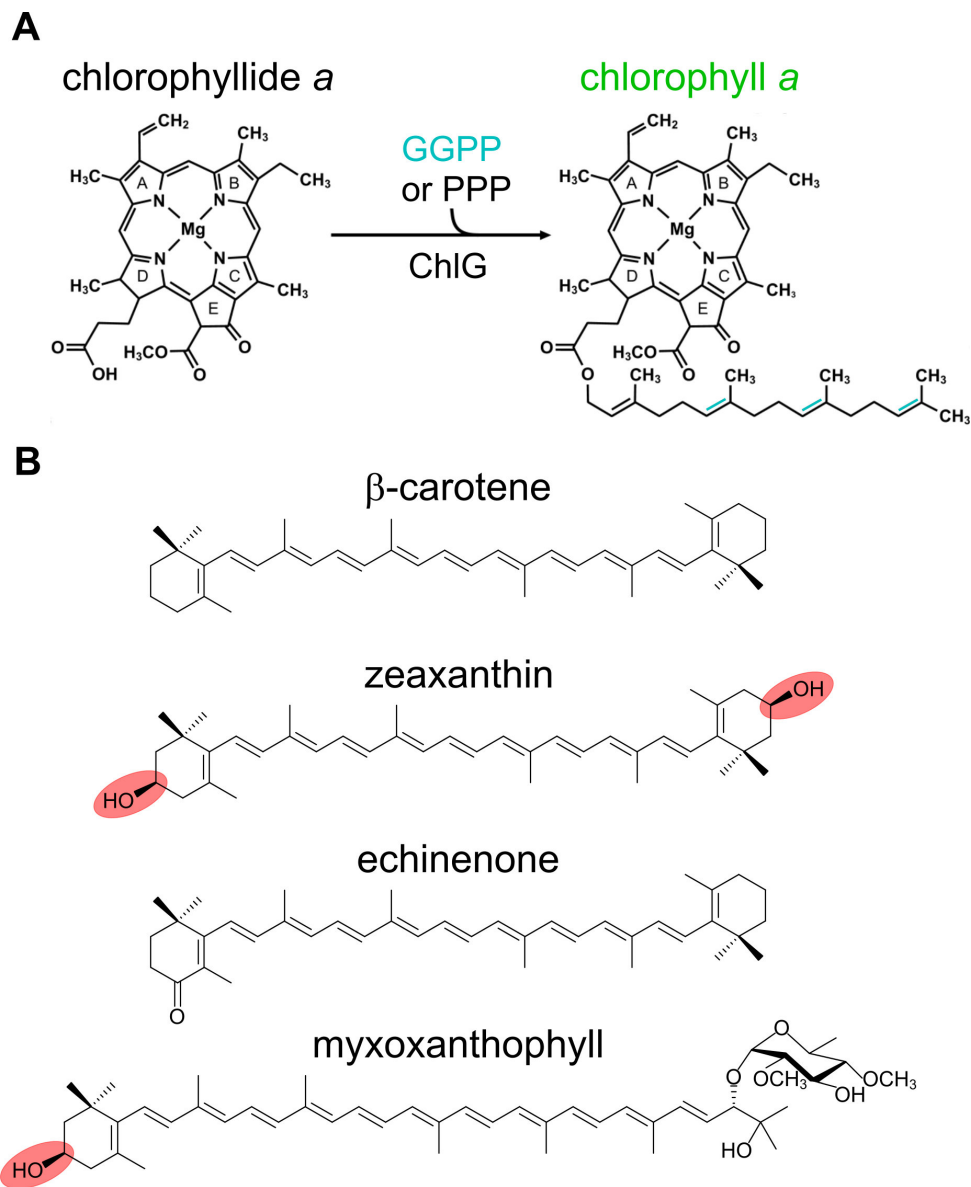


Figure 1. The reaction catalysed by chlorophyll synthase (ChlG) and the molecular structures of the four major carotenoids produced by *Synechocystis*. **(A)** ChlG esterifies C17 on ring D of chlorophyllide *a* with geranylgeranyl-pyrophosphate (GGPP) or phytyl pyrophosphate (PPP) to produce (GG-)chlorophyll *a*. **(B)** The molecular structures of the four major carotenoids produced by WT *Synechocystis*: β -carotene, zeaxanthin, echinenone and myxoxanthophyll. The hydroxyl groups on the β -rings of zeaxanthin and myxoxanthophyll are highlighted in red.

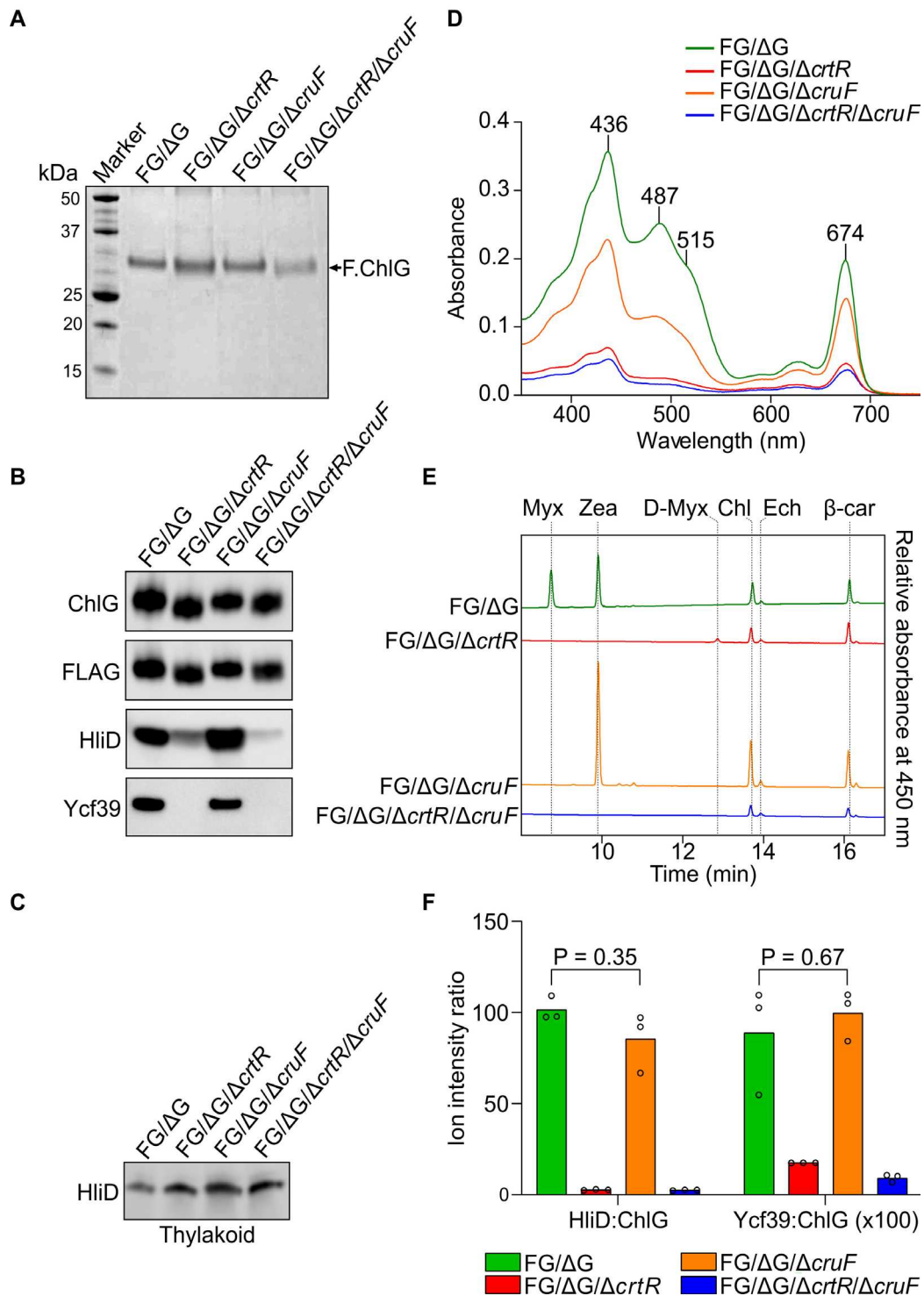


Figure 2. Analysis of pigment-protein complexes isolated by co-immunoprecipitation of F.ChlG from *Synechocystis xanthophyll* mutants. (A) Coomassie stained SDS-PAGE analysis of FLAG immunoprecipitation eluates obtained from the FLAG-*chlG* Δ *chlG* (FG/ Δ G), FLAG-*chlG* Δ *chlG* Δ *crtR* (FG/ Δ G/ Δ *crtR*), FLAG-*chlG* Δ *chlG* Δ *cruF* (FG/ Δ G/ Δ *cruF*) and FLAG-*chlG* Δ *chlG* Δ *crtR* Δ *cruF* (FG/ Δ G/ Δ *crtR*/ Δ *cruF*) strains of *Synechocystis*. (B) The presence of the FLAG-ChlG (F.ChlG) bait, HliD and Ycf39 was confirmed by immunoblotting with specific primary antibodies. (C) Anti-HliD

immunoblot of thylakoid membranes (30 μ g of chlorophyll was loaded in each lane) from WT and mutant strains. (D) Absorbance spectra of the FG/ Δ G (green), FG/ Δ G/ Δ crtR (red), FG/ Δ G/ Δ cruF (orange) and FG/ Δ G/ Δ crtR/ Δ cruF (blue) eluates. (E) Separation of pigments extracted from immunoprecipitation eluates by RP-HPLC analysis monitoring absorbance at 450 nm. Myxoxanthophyll (Myx), zeaxanthin (Zea), 3-dehydroxy-myxoxanthophyll (D-Myx), chlorophyll *a* (Chl), echinenone (Ech) and β -carotene (β -car). In (A-E) data are representative of at least three independent experiments. (F) Quantification of F.ChlG, HliD and Ycf39 in immunoprecipitation eluates by mass spectrometry. The ion intensities shown in Table S2 were used to determine the ratios of HliD and Ycf39 to F.ChlG. The results of three technical repeats are presented with P values derived from a Student's t-test (paired, 2-tails).

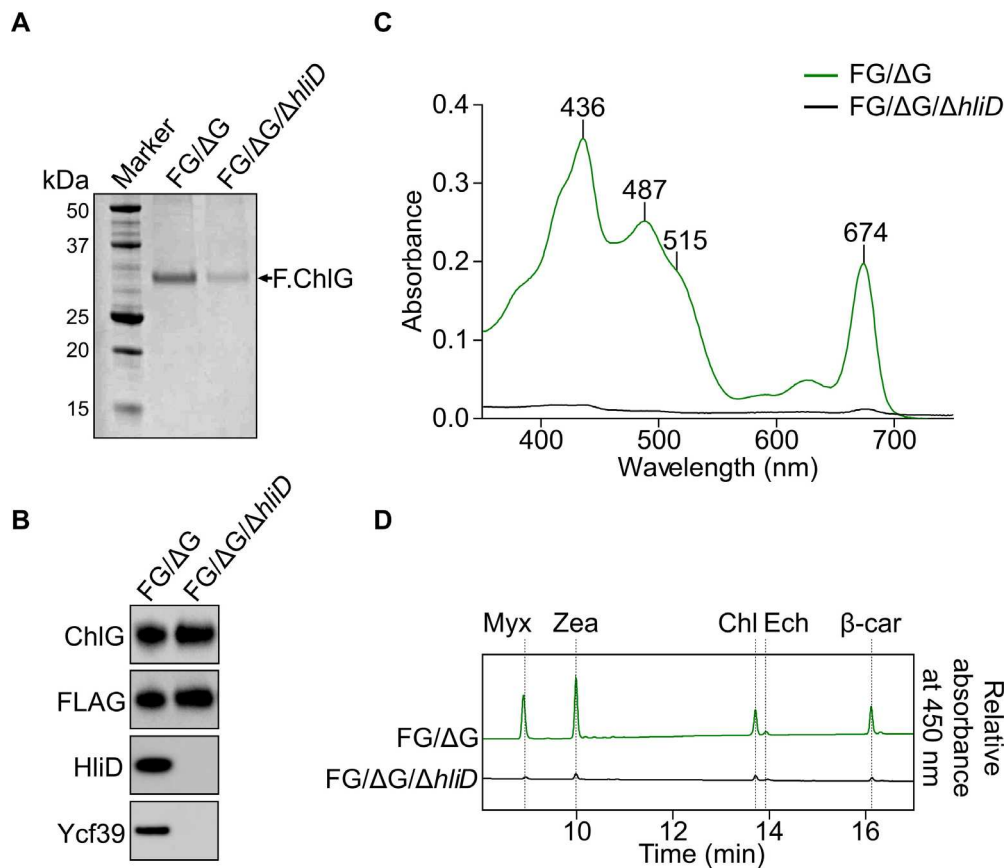


Figure 3. Analysis of pigment-protein complexes isolated by co-immunoprecipitation of F.ChlG from a *Synechocystis* $\Delta hliD$ mutant. (A) Coomassie stained SDS-PAGE analysis of FLAG immunoprecipitation eluates obtained from the FLAG-*chlG* $\Delta chlG$ (FG/ ΔG) and FLAG-*chlG* $\Delta chlG$ $\Delta hliD$ (FG/ ΔG / $\Delta hliD$) strains. (B) The presence of the FLAG-ChlG (F.ChlG) bait, HliD and Ycf39 was identified by immunoblotting with specific primary antibodies. The FG/ ΔG / $\Delta hliD$ protein concentration was normalised to that of FG/ ΔG . (C) Absorbance spectra of the FG/ ΔG (green) and FG/ ΔG / $\Delta hliD$ (black) eluates. (D) Separation of pigments extracted from immunoprecipitation eluates by RP-HPLC analysis monitoring absorbance at 450 nm. Myxoxanthophyll (Myx), zeaxanthin (Zea), 3-dehydroxy-myxoxanthophyll (D-Myx), chlorophyll *a* (Chl), echinenone (Ech) and β -carotene (β -car). Data are representative of at least three independent experiments.

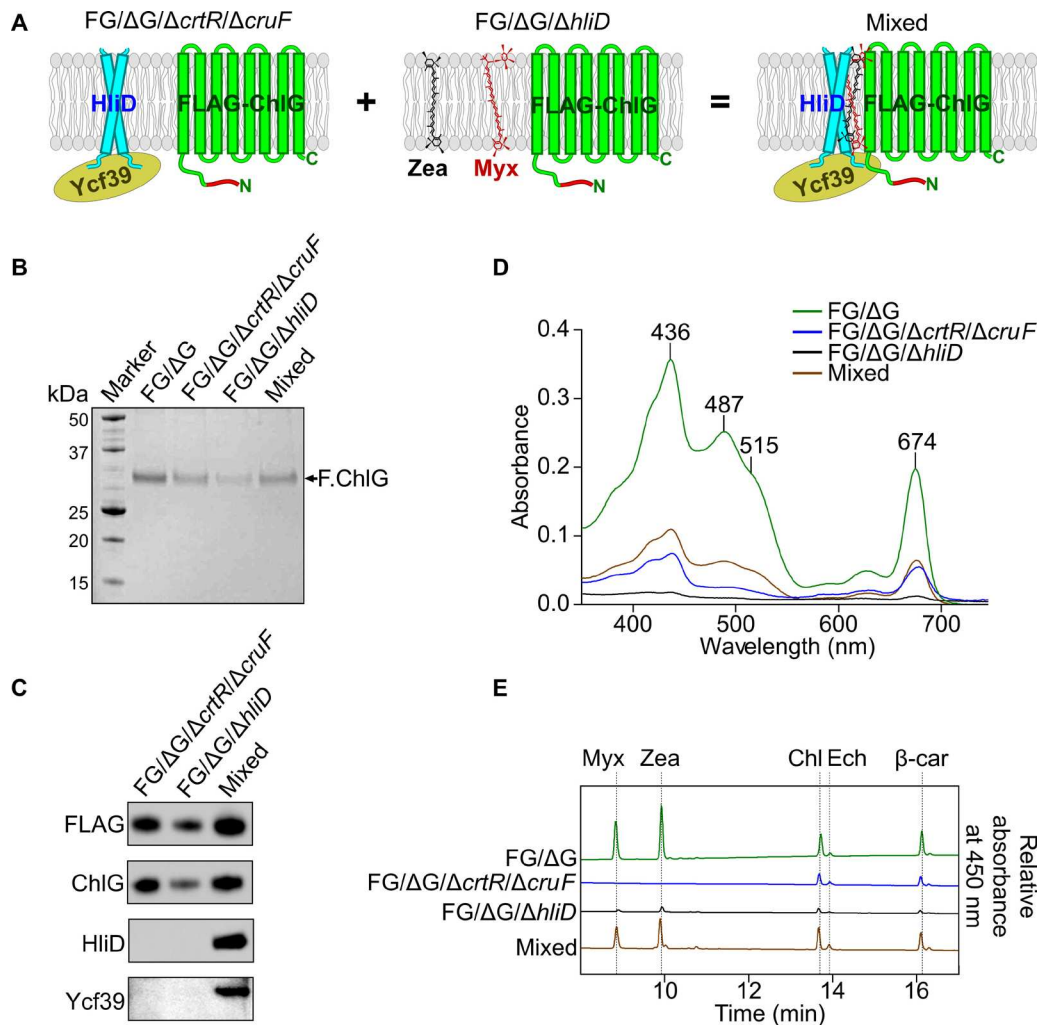


Figure 4. Reconstitution of F.ChlG with carotenoids and HliD in isolated membranes. (A) Solubilised thylakoid membranes from the FLAG-*chlG* $\Delta chlG$ strain lacking zeaxanthin and myxoxanthophyll (*FG/ΔG/ΔcrtR/ΔcruF*) but containing FLAG-ChlG (green) and HliD (blue) were mixed with membranes from the *FG/ΔG/ΔhliD* strain, which lacks HliD but contains myxoxanthophyll (Myx, red) and zeaxanthin (Zea, black). (B) Analysis of FLAG immunoprecipitants by SDS-PAGE and Coomassie blue staining; the mixed membranes are labelled as 'Mixed'. (C) Immunodetection of FLAG-ChlG (F.ChlG), HliD and Ycf39 in the immunoprecipitation eluates. HliD and Ycf39 are restored in the mixed membrane sample. (D) Absorbance spectra of each immunoprecipitation eluate. (E) Qualitative RP-HPLC identification of myxoxanthophyll (Myx), zeaxanthin (Zea), chlorophyll *a* (Chl) echinenone (Ech) and β-carotene (β-car). Data are representative of at least three independent experiments.

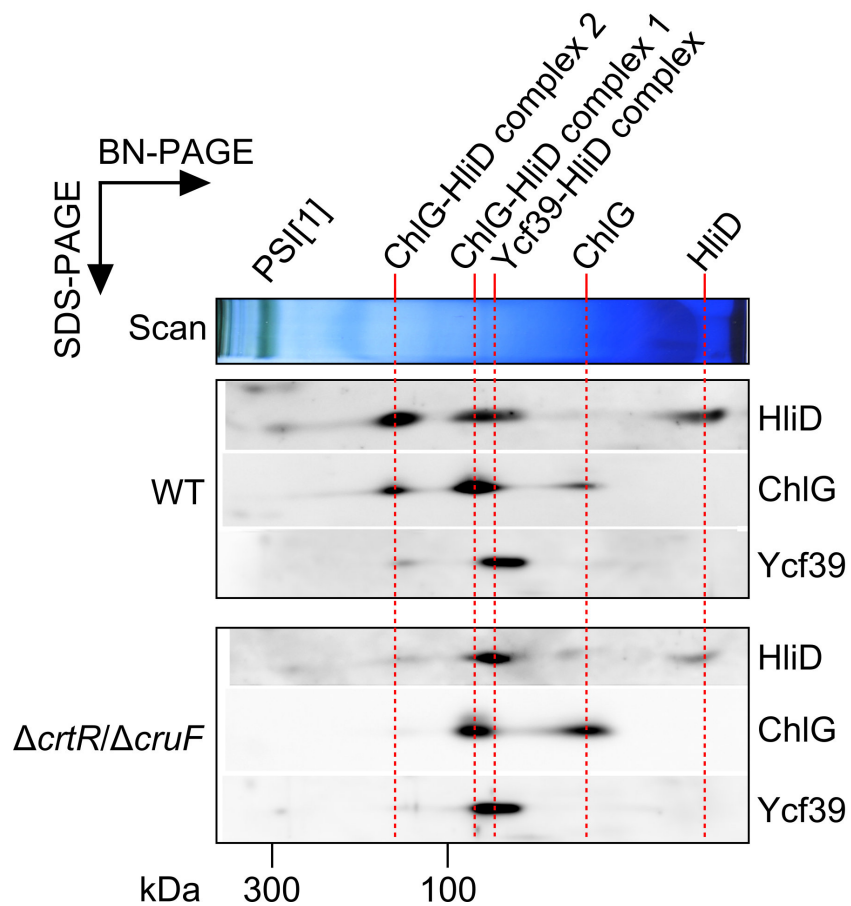


Figure 5. Formation of ChIG-HliD complexes is altered by the loss of zeaxanthin and myxoxanthophyll. Analysis of thylakoid membrane proteins by 2D-PAGE and immunoblotting. Thylakoid membranes were purified from WT and $\Delta crtR/\Delta cruF$ cells and separated by BN-PAGE in the first dimension and denaturing SDS-PAGE in the second dimension. Proteins were transferred to a PVDF membrane and immunodetection of ChIG, HliD and Ycf39 was performed using protein specific primary antibodies. Proteins and protein complexes assigned according to previous studies (28, 30) are indicated with red dashed lines and labelled above the gel slice/blots. The approximate molecular weight is indicated below the blots.

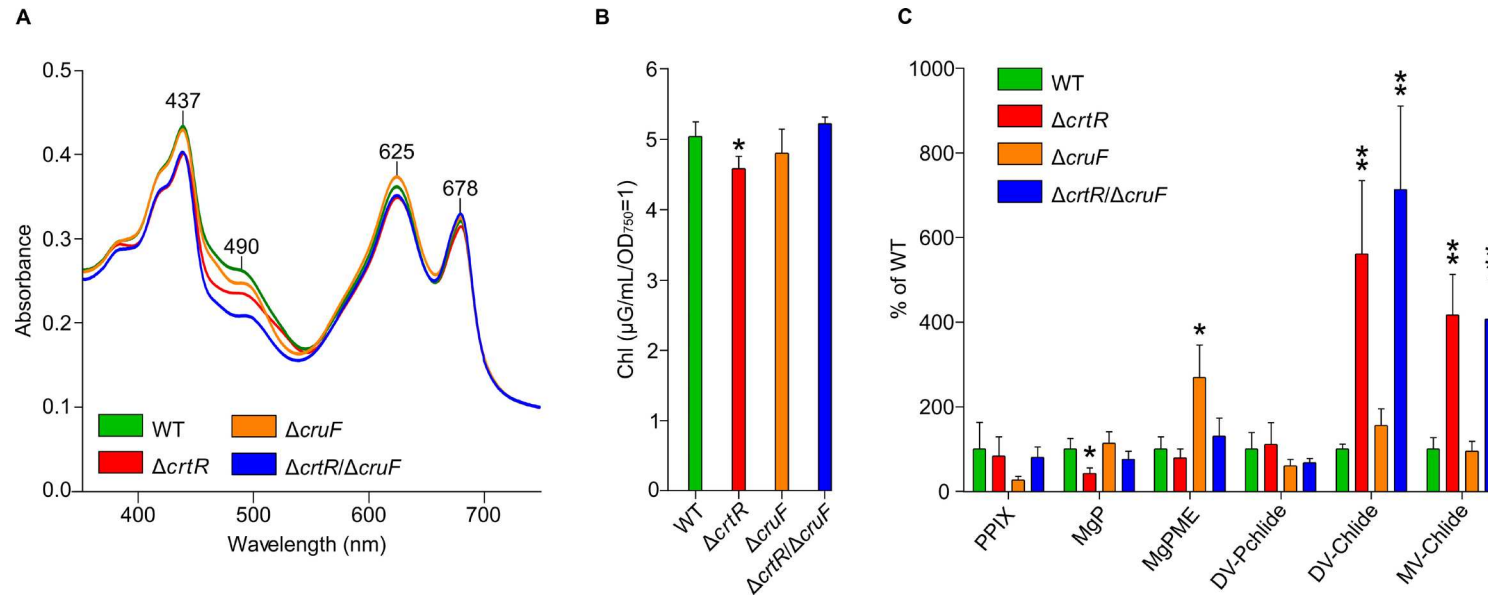


Figure 6. Quantification of Chl and Chl precursors in wild-type *Synechocystis* and xanthophyll deficient mutants. (A) Whole cell absorbance spectra of wild-type (WT, green), $\Delta crtR$ (red), $\Delta cruF$ (orange) and $\Delta crtR/\Delta cruF$ (blue). (B) Chl content of each strain. Error bars represent the standard deviation from the mean of 5 biological replicates. (C) Levels of Chl precursors extracted from $\Delta crtR$, $\Delta cruF$ and $\Delta crtR/\Delta cruF$ cells, relative to those in the WT, which was set at 100% for each pigment. PPIX = protoporphyrin IX; MgP = magnesium-protoporphyrin IX; MgPME = magnesium protoporphyrin monomethyl ester; DV-Pchlde = divinyl-protochlorophyllide; DV-Chlide = divinyl-chlorophyllide; MV-Chlide = monovinyl-chlorophyllide. Error bars represent the standard deviation from the mean of 5 biological replicates. In (B) and (C) statistical significance between the means for Chl/each precursor was determined using one-way analysis of variance (ANOVA); * = $p < 0.05$; ** = $p < 0.001$.