**INTRODUCTION:** Terrestrial cyanobacteria often occur in environments that receive strongly filtered light because of shading by plants or because of their associations with soil crusts, benthic mat communities, or dense cyanobacterial blooms. The light in such environments becomes highly enriched in far-red light (FRL) (wavelengths >700 nm). Cyanobacteria that are able to use FRL for photosynthesis have evolved a novel far-red light photoacclimation (FaRLiP) mechanism to gain a strong selective advantage over other cyanobacteria. The FaRLiP response involves extensive remodeling of photosystems I and II (PSI and PSII) and light-harvesting phycobilisome complexes. FaRLiP cells synthesize chlorophyll f (Chl f), Chl d, and FRL-absorbing phycobiliproteins under these conditions and thus can use FRL efficiently for oxygenic photosynthesis. A key element of the FaRLiP response is the FRL-specific expression of 17 genes that encode paralogs of core components of the three light-harvesting complexes produced during growth in white light.

**RATIONALE:** The ability to synthesize Chl f is a key element of the FaRLiP response, but the Chl f synthase had remained unknown. Transcription and phylogenetic profiling suggested that the gene(s) responsible for this activity were in the conserved FaRLiP gene cluster. This led us to focus on psbA4, a divergent member of the psbA gene family encoding so-called “super-rogue” PsbA, a paralog to the D1 core subunit of PSII. We used reverse genetics and heterologous expression to identify the Chl f synthase of two cyanobacteria capable of FaRLiP: *Chlorogloeopsis fritschii* PCC 9212 and *Synechococcus* sp. PCC 7335.

**RESULTS:** In both species, null mutants of psbA4 no longer synthesized Chl f and lacked FRL absorption and long-wavelength fluorescence emission, the key spectroscopic properties associated with Chl f. Heterologous expression of the psbA4 gene from *C. fritschii* PCC 9212 in the model non-FaRLiP cyanobacterium *Synechococcus* sp. PCC 7002 led to the synthesis of Chl f. These results showed that psbA4 (renamed *chlF*) encodes the Chl f synthase. Growth experiments using intervals of FRL and darkness showed that Chl f synthesis is light-dependent, which implies that ChlF is a photo-oxidoreductase that oxidizes Chl a (or Chlide a) instead of water.

**CONCLUSION:** ChlF may have evolved after gene duplication from PsbA of a water-oxidizing PSII complex by loss of the ligands for binding the Mn₄Ca₁O₅ cluster but by retaining catalytically useful chlorophylls, tyrosine Y₂, and plastoquinone binding. Alternatively, PsbA may have arisen by gene duplication from ChlF and then by gaining the capacity to bind the Mn₄Ca₁O₅ cluster. Because ChlF seems likely to function as a simple homodimer and belongs to the earliest diverging clade of PsbA sequences in phylogenetic analyses, Chl f synthase may have been the antecedent of water-oxidizing PSII. This hypothesis provides a simple explanation for the occurrence of multiple reaction centers in an ancestral cyanobacterial cell. Thus, a Chl a photo-oxidoreductase that initially evolved for enhanced use of FRL may explain the origin of oxygen-evolving PSII. From an applied perspective, knowing the identity of ChlF may provide a tractable route for introducing the capacity for FRL use into crop plants, greatly expanding the wavelength range that they can use to conduct photosynthesis.
Light-dependent chlorophyll f synthase is a highly divergent paralog of PsbA of photosystem II

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Chlorophyll f (Chl f) permits some cyanobacteria to expand the spectral range for photosynthesis by absorbing far-red light. We used reverse genetics and heterologous expression to identify the enzyme for Chl f synthesis. Null mutants of "super-rogue" psbA4 genes, divergent paralogs of psbA genes encoding the D1 core subunit of photosystem II, abolished Chl f synthesis in two cyanobacteria that grow in far-red light. Heterologous expression of the psbA4 gene, which we rename chlf, enables Chl f biosynthesis in Synechococcus sp. FCC 7002. Because the reaction requires light, Chl f synthase is probably a photo-oxidoreductase that employs catalytically useful Chl a molecules, tyrosine Y2, and plastoquinone (as does photosystem II) but lacks a Mn4Ca1O5 cluster. Introduction of Chl f biosynthesis into crop plants could expand their ability to use solar energy.

Terrestrial cyanobacteria are an underappreciated component of global primary productivity (GPP). About 55% of GPP is estimated to occur on land (1), and of the 45% of GPP that occurs in the oceans, oxygen-evolving Prochlorococcus and Synechococcus spp. are responsible for ~50% of the total (2, 3). Given that the estimates for total marine and terrestrial cyanobacterial biomass are similar (4), terrestrial cyanobacteria could account for a substantial proportion of GPP and nitrogen fixation (4, 5). Terrestrial cyanobacteria often occur in environments that receive strongly filtered light because of shading by plants or because of their associations with soil crusts, benthic mat communities, or dense cyanobacterial blooms (6). The light available in such environments becomes highly enriched in far-red light (FRL), specifically wavelengths greater than 700 nm (6). Therefore, cyanobacteria that are able to use FRL for photosynthesis gain a strong selective advantage over organisms that are unable to do so (6–8).

Cyanobacteria that grow in FRL undergo an extensive photoacclimation process known as far-red light photoacclimation (FaRLiP) (7). FaRLiP involves the FRL-dependent expression of a conserved cluster of 20 genes (fig. S1). Seventeen of these genes are paralogs of genes encoding core subunits of photosynthetic complexes that are expressed in white light (WL). The expression of the paralogous genes in FRL leads to extensive remodeling of the cores of the major light-absorbing complexes of cyanobacteria: phycobilisomes (9), photosystem I (PSI) (10), and PSI (6–8, 11). About 10% of the chlorophyll a (Chl a) molecules are replaced by Chl f in the paralogous PSI and PSII complexes produced in FRL (7, 8), and allophycocyanin B variants absorbing beyond 700 nm are also synthesized during FaRLiP (7, 8, 12, 13). Together, these changes allow cells to use FRL for growth (7, 8). The FaRLiP gene cluster also contains three regulatory genes—rfpA, rfpB, and rfpC—which form a signaling cascade that controls the expression of the FaRLiP genes (14). RfpA is a knotless red light (RL)/FRL-responsive phytochrome (RfpA) with a histidine kinase domain; RfpC is a small CheY-like response regulator that probably acts as a phosphate shuttle; and RfpB is a response regulator with a winged-helix DNA-binding domain and two CheY-like receiver domains (7, 14). Null mutations for any one of these three rfp genes cannot grow in FRL, cannot express the genes in the FaRLiP gene cluster, and cannot synthesize Chl f (14).

Chl f (i.e., 2-formyl Chl a) was only recently discovered (15–17) and is synthesized when cells capable of FaRLiP are grown in FRL (6–8, 18–20). Chl f absorbs maximally at ~707 nm in 100% methanol (21, 22), and it is presumably made by reaction(s) extending from Chl a or chlorophyllide a, the immediate precursor of Chl a (Fig. 1) (17, 23). Chl f could possibly be introduced into crop plants to extend their light harvesting into the far red (700 to 800 nm) and thereby improve their photosynthetic light use efficiency (24–26). However, the enzyme(s) responsible for Chl f synthesis have not been identified, and the complexity of Chl f synthesis remains unknown.

Identification of the gene encoding Chl f synthase by reverse genetics

To identify the Chl f synthase, the enzyme responsible for the synthesis of Chl f, we used reverse genetics in two cyanobacteria capable of FaRLiP—Chlorogloeopsis fritschi (PCC 9212 and Synechococcus sp. FCC 7335—and heterologous gene expression in the model cyanobacterium Synechococcus sp. PCC 7002 (hereafter Synechococcus 7002). Several observations led us to focus on psbA4, which encodes a so-called “super-rogue” paralog (sr-PsbA) of PsbA, the D1 core subunit of PSII (27).

A sr-psbA4 gene is found in each FaRLiP gene cluster (fig. S1) (7, 8), and psbA4 expression is under the control of the RfpABC sensor kinase/response regulator system (14). Transcription profiling of FaRLiP strains (e.g., (7, 14)) revealed striking increases in relative transcript abundance for hundreds of genes when cells were shifted from WL into FRL. However, phylogenetic profiling of ~600 genes whose relative transcript abundances more than doubled in FRL in Leptolyngbya sp. JSC-1 (7)—including monoxygenases, oxidoreductases, and other enzymes that might be logical candidates to be Chl f synthase—showed that only the genes of the FaRLiP cluster are universally present in cyanobacteria capable of performing FaRLiP. These observations strongly suggested that the gene(s) responsible for Chl f synthesis must be present in the FaRLiP gene cluster. sr-PsbA4 is predicted to be structurally similar to the PsbA core subunit of PSII reaction centers (27, 28), but sr-PsbA4 sequences collectively lack key residues required for binding the Mn4CaO5 cluster for water oxidation (27, 28) (Fig. 2, B and C, and fig. S2). Additionally, sr-PsbA4 has some important differences in the vicinity of its putative plastoquinone binding site (8, 27, 28) (Fig. S2). However, sr-PsbA4 has retained tyrosine Y2 and the ligands for binding Chl a (Fig. 2 and fig. S2). The structural and functional changes imposed by the inability to bind the Mn4CaO5 cluster strongly suggested that sr-PsbA4 has a function other than being a core subunit of PSII.

Using a conjugation-based DNA transfer system (16), we constructed null mutants for psbA4 genes in two cyanobacteria capable of FaRLiP (8): C. fritschi (PCC 9212 and Synechococcus sp. FCC 7335 (fig. S0)). Neither psbA4 mutant was able to synthesize Chl f when the mutant cells were grown in FRL. The characteristic long-wavelength absorption at ~710 nm (Fig. 3A) and fluorescence emission at ~740 to 750 nm (Fig. 3B) associated with PS complexes containing Chl f was completely missing for the two psbA4 mutants (Fig. 3, C and D, and fig. S4D). High-performance liquid chromatography (HPLC) analyses of pigments extracted from cells of the psbA4 null mutants grown in FRL confirmed the absence of Chl f and showed that only Chl a and Chl d were synthesized (Fig. 4 and fig. S5). Reverse transcription polymerase chain reaction (RT-PCR) analyses showed that this was not due to a loss of transcriptional control by RfpABC. Transcripts of selected FaRLiP

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genes exhibited normal RL/FRL-dependent regulation (Fig. 5). Psaa2 and Psab2 were detected at low levels in PSI trimers isolated from cells of the *psba4* mutant of *C. fritschi* PCC 9212 grown in FRL by proteomic analysis (table S1). In the absence of Chl f in the *psba4* mutant, it seems that FaRLiP subunits accumulate to much lower levels than in wild-type cells grown in FRL (table S1).

**Heterologous expression of the sr-psba4 gene in *Synechococcus 7002***

To ascertain directly whether sr-PsbA4 is the Chl f synthase, we expressed the *C. fritschi* PCC 9212 *psba4* gene heterologously under the control of the strong *Synechocystis* sp. PCC 6803 cpeBA promoter from plasmid pAQA1 in the model cyanobacterium *Synechococcus* 7002 (29). *Synechococcus* 7002 does not naturally synthesize Chl f and is unable to grow in FRL (7). Cells were grown at low irradiance in WL (15 μmol photons m\(^{-2}\) s\(^{-1}\)) at room temperature in hopes of minimizing sr-PsbA4 turnover and preventing photo-oxidation of any Chl f that might be formed, because no known Chl f-binding proteins are present in this heterologous system. Reversed-phase HPLC analysis of pigments extracted from wild-type *Synechococcus* 7002 cells showed only Chl a, as expected (Fig. 6A). However, HPLC analysis showed that pigments extracted from the *Synechococcus* 7002 strain expressing *psba4* contained both Chl a and Chl f (Fig. 6A and fig. S6). Although only a small amount of Chl f was produced (~0.059% of total Chl), this pigment had the same retention time as the known Chl f (Fig. 7). The absorption spectrum of the pigment eluting at 14.5 min in Fig. 7 was identical to the spectra shown in Fig. 6B. (Note that retention times are different because an HPLC protocol was used that differed from that in Fig. 6A; see materials and methods for details.)

We also performed an alternative experiment designed to test whether light is required for the synthesis of Chl f (Fig. 8). Wild-type *C. fritschi* PCC 9212 was grown in continuous FRL or FRL interrupted by a dark period of 12 hours. At time zero, *C. fritschi* PCC 9212 wild-type cells that had been grown in a medium containing 5 mM fructose, a metabolizable carbon/energy source, were inoculated into two cultures, which were transferred to FRL for 24 hours to induce the expression of the FaRLiP gene cluster. One of the cultures was left in continuous FRL (Fig. 8A); the other was placed in darkness for 12 hours and then returned to FRL for another 12 hours (Fig. 8B). The Chl f content of the control cells, which could be detected by the increasing 740-nm low-temperature fluorescence emission from Chl f, increased throughout the FRL incubation. In the other sample, Chl f synthesis stopped when cells were placed in darkness, but Chl f synthesis resumed when cells were returned to FRL (Fig. 8B).

The data from both of these experimental approaches demonstrate that light is required for the synthesis of Chl f by ChlF/sr-PsbA4. Thus, we conclude that ChlF is a photo-oxidoreductase. Chl f synthase is the second light-dependent enzyme of Chl biosynthesis, the other being light-dependent protochlorophyllide oxidoreductase (POR) (32).

**Implications of Chl f synthase (ChlF) for evolution of water oxidation and PSI**

Within the context of PSI evolution, there are two ways to view the result that ChlF/PsbA4 is an enzyme that probably photo-oxidizes Chl a (or Chlde a) to produce Chl f (or Chlde f). In the first scenario, ChlF may have evolved from the PsbA (D1) subunit of a water-oxidizing PSI complex by loss of the ligands for binding the Mn-Ca-O\(_2\) cluster but retention of catalytically useful chlorophylls, tyrosine Y\(_Z\), and plastoquinone binding after gene duplication and divergence. Although this is certainly a reasonable possibility, phylogenetic analyses do not support this hypothesis...
An alternate possibility is perhaps more appealing: ChlF/PsbA4 could be ancestral to PsbA of PSII, and PsbA could have arisen by gene duplication and divergence to bind a Mn₄CaO₅ cluster to catalyze water oxidation, thus providing an explanation for the origin of PSII (fig. S8) (28). Because the selection pressure to expand the wavelength range of photosynthesis is largely responsible for the many different Chls and antenna complexes extant today, this scheme provides an explanation for the origin of ChlF for the reaction that it catalyzes, and for the occurrence of a presumably homodimeric type-2 reaction center in an ancestral cyanobacterium that may also have had a PSI-like reaction center (33, 34).

Supporting this second possibility further, phylogenetic analyses show that ChlF/sr-PsbA, together with a highly unusual PsbA-like sequence from Gloeobacter kilaueensis, are the earliest diverging members of the PsbA family (fig. S8) (8, 27, 28). We hypothesize that ChlF functions as a homodimer, because introduction of psbA4/chlF into Synechococcus 7002 was sufficient to enable Chl f synthesis. All currently known photochemical reaction centers are either homodimers or heterodimers (33, 34). Furthermore, because ChlF is apparently a photo-oxidoreductase and oxidation of the substrate probably occurs by a radical mechanism [assuming a one-photon, one-electron mechanism as in PSII (39)], dioxygen may not be required for the synthesis of Chl f. Thus, it is possible that Chl f synthase appeared before the atmosphere became oxic (i.e., before PSII had evolved). These implications could mean that ChlF is a long-sought transitional intermediate in the evolution of oxygenic photosynthesis: a simple, homodimeric, type 2 reaction center that might have first evolved in anoxygenic ancestors of modern cyanobacteria (fig. S8) (29, 34, 35). Subsequent gene duplication events [two are required to form the heterodimers found in PSII (35)] along with divergence to acquire the ligands to bind the Mn₄CaO₅ cluster, could have ultimately led from an enzyme that could oxidize Chl (or Chlide) to an enzyme that could oxidize water.

In either case, the likely selective pressure for the synthesis of Chl f would have been to extend photosynthetic light harvesting into the far-red spectral region. The ability to use FRL or near-infrared light for photosynthesis provides a strong selective advantage to those organisms that can extend their light-harvesting range beyond the wavelengths absorbed by Chl a (7). Examples include the homodimeric type 1 reaction centers of Chloracidobacterium thermophilum (36) and green sulfur bacteria (37), both of which bind Chl a and bacteriochlorophyll a (BChl a). Similarly, the unusual cyanobacterium Acaryochloris marina has evolved to use Chl d, another FRL-absorbing Chl (see Fig. 1), together with Chl a to extend its photosynthetic light harvesting into the far-red region (36, 39). It is increasingly clear that Chl d is the central “hub” compound of Chl biosynthesis, and that the biosynthetic pathway repeatedly diverged away from this molecule (or from one of its precursors, 3,8-divinyl-protochlorophyllide) to extend light harvesting into the far red and near-infrared (23, 40). These findings additionally provide strong support for the Granick hypothesis concerning the evolution of heme and Chl biosynthesis, namely that pathways evolved forward as organisms evolved (see discussion in (40)).

By using domain-swapping and site-directed mutagenesis methods, it may be possible to recapitulate evolution to determine which amino acid residues must be changed in PsbA to obtain a protein that no longer binds a Mn₄CaO₅ cluster but can synthesize Chl f (or more interestingly, the

Fig. 2. Structural model of photosystem II core components from Thermosynechococcus vulcanus (PDB 3WU2). (A) PsbA (chain A) is shown in ribbon format in tan. Other components: green, three Chl a molecules and one pheophytin a molecule; teal, plastoquinone Q₉; orange, non-heme iron atom; magenta, tyrosine Y₇; yellow, β-carotene; lilac, green, and red, the water-oxidizing Mn₄CaO₅ cluster; aqua, amino acid side chain ligands to the Mn₄CaO₅ cluster; blue, second-tier amino acids in the vicinity of the cluster. (B) Enlargement of the Mn₄CaO₅ cluster that shows a histidine residue (pink) that may be involved in proton-coupled electron transfer in PSII. Components of the cluster: lilac, manganese atoms; green, calcium atom; red, bridging oxygen atoms; aqua, ligands to the Mn₄CaO₅ cluster. (C) Model of the sr-PsbA4 Mn₄CaO₅ cluster-binding site of C. fritschii PCC 9212. Only one ligand, a glutamate residue (aqua), and the C-terminal carboxyl group ligands of a nonconserved threonine residue are retained. Amino acids that replace the conserved ligands to the Mn₄CaO₅ cluster are shown in orange. These include a second tyrosine residue found in 7 of 13 PsbA4 sequences. Note that tyrosine Y7 and the associated histidine residue (magenta and pink, respectively) are conserved. The color coding used in this figure matches that in the multiple sequence alignment file shown in fig. S2. Images were created with the UCSF Chimera program (51); residues were substituted with conformations of highest probability using the Dunbrack backbone-dependent rotamer library (52).

Fig. 3. The psbA4 mutant of C. fritschii PCC 9212 lacks far-red absorbance and fluorescence emission. (A to D) Whole-cell absorption spectra [(A) and (C)] and low-temperature fluorescence emission spectra [(B) and (D)] of wild-type [(A) and (B)] and psbA4 mutant cells [(C) and (D)] of C. fritschii PCC 9212 grown in WL (solid lines) or FRL (dashed lines). The arrows in (A) and (B) point to long-wavelength absorption and fluorescence features attributable to Chl f.
converse). Such approaches could provide an experimental framework for studying the origins of PSII, water oxidation, and thus oxygenic photosynthesis. Finally, because Chl f biosynthesis is the product of a single gene product, it may now be feasible to introduce the capacity for Chl f biosynthesis into plants to extend the wavelength range for their photosynthesis into the far-red region of the solar spectrum (24–26).

Materials and methods
Organisms and cultivation conditions
Cyanobacterial strains used in this study were obtained from the Pasteur Culture Collection (www.pasteur.fr/pcc_cyanobacteria) (41). Synechococcus 7002 was grown in medium A containing 10 mM nitrate (denoted as Medium A’) (42). This cyanobacterium was routinely grown under “standard conditions” (43); 38°C, sparging with 1% CO2 (v/v) in air, at 250 μmol photons m⁻² s⁻¹ provided by cool white fluorescent tubes. For some experiments, cells were grown at room temperature at an irradiance of ~15 μmol photons m⁻² s⁻¹.

Synechococcus sp. PCC 7335 was cultured in ASI-III growth medium (47) to which vitamin B₁₂ (final concentration 10 μg ml⁻¹) and Tris-HCl, pH 8.0 (final concentration 10 mM) were added. C. fritschii PCC 9212 was grown in the B-HEPES growth medium (44), a modified BG-11 medium containing 1.1 g liter⁻¹ HEPES (final concentration) with the pH adjusted to 8.0 with 2.0 M KOH. Cool white fluorescent bulbs provided continuous illumination at ~250 μmol photons m⁻² s⁻¹ (WL), and liquid cultures were sparged with 1% (v/v) CO₂ in air. Synechococcus sp. PCC 7335 was grown at a lower WL intensity of ~50 μmol photons m⁻² s⁻¹. In some experiments, C. fritschii PCC 9212 was grown mixotrophically by adding 5 mM fructose to the growth media. Far-red light was provided by Epitex, L720-06AU LEDs (Marubeni, Santa Clara, CA) with emission centered at 720 nm (26 to 30 LEDs (Marubeni, Santa Clara, CA) with emission centered at 720 nm (26 to 30 LEDs (Marubeni, Santa Clara, CA) with emission centered at 720 nm (26 to 30 LEDs). Light boards composed of 50 LEDs were provided by cool white fluorescent tubes. For some experiments, cells were grown at room temperature at an irradiance of ~15 μmol photons m⁻² s⁻¹.

**Construction of mutants and conjugation**

J. Zhao (Peking University) provided the biparental conjugation system (45), and the method used was very similar to that described in (14). The donor E. coli strain HB101 contained the conjugal plasmid pRL443 and the helper plasmid pRL623 (46, 47). Figure S3 shows maps of the scheme employed to delete the psbA4 gene of C. fritschii PCC 9212 and Synechococcus sp. PCC 7335. The psbA4 gene of C. fritschii was deleted and replaced by an ermC cassette conferring resistance to erythromycin, while the psbA4 gene of Synechococcus sp. PCC 7335 was partly deleted and replaced by an aphII cassette, conferring resistance to kanamycin. Upstream and downstream DNA fragments for each target psbA4 gene were cloned into the cargo plasmid pRL277 with an interposing DNA fragment encoding ermC or aphII to replace the target gene through a double-crossover recombination event. For each target gene, upstream and downstream DNA fragments with a size of ~2.5 to 3.0 kb were amplified by PCR using Phusion HF DNA polymerase (New England Biolabs). The primers for DNA fragment amplification of the upstream and downstream regions are listed in table S2.

Cargo plasmids were transformed into the donor E. coli HB101 cells containing the conjugal plasmid pRL443 and the helper plasmid pRL623. The resulting E. coli HB101 strains were grown in 5 to 20 ml of Luria-Bertani (LB) medium supplemented with appropriate antibiotics and cultured at 37°C.
overnight. The *E. coli* cells were harvested by centrifugation at low speed, washed with fresh LB medium three times, and resuspended in fresh LB, B-HEPES medium, or ASN-III medium (100 to 200 μl). Meanwhile, freshly grown wild-type *C. fritschii* 9212 or *Synechococcus* sp. PCC 7335 cells (OD<sub>750 nm</sub> = 0.6 to 1.0; 500 μl to 2.0 ml) were centrifuged at low speed, washed with fresh B-HEPES or ASN-III medium three times, and finally resuspended in B-HEPES or ASN-III medium (100 to 200 μl). The *E. coli* and cyanobacterial cells were gently mixed in a sterile microcentrifuge tube and incubated at 30°C under low light for 4 to 6 hours, and the cell mixture was then spread onto a sterile nitrocellulose filter overlaid on a B-HEPES or ASN-III agar plate without antibiotics. The plate was incubated at 30°C under low light for another 18 to 36 hours, and then the filter was transferred to a B-HEPES agar plate containing 20 μg erythromycin ml<sup>-1</sup> or an ASN-III agar plate containing 100 μg kanamycin ml<sup>-1</sup>. Brown- or green-colored colonies appeared on the filter after about 4 weeks. Colonies were picked and streaked repeatedly on selective medium, and the purified transconjugant cells were ultimately transferred to liquid B-HEPES or ASN-III medium for analyses. Segregation of wild-type and mutant alleles of the target gene was analyzed by colony PCR.

### Pigment extraction, HPLC analyses, and mass spectrometry

Two methods, denoted MY1 and MY2, were used for pigment extraction and HPLC analysis. Method MY1 was performed as previously described (7, 8, 14). Pigments were extracted with acetone/methanol (7:2, v/v) from cells and analyzed by reversed-phase HPLC on an Agilent 1100 HPLC system (Agilent Technologies) with an analytical Discovery C18 column (4.6 mm × 25 cm) (Supelco, Sigma-Aldrich). The gradient elution program [B, minutes] using Solvent A (methanol/acetonitrile: H<sub>2</sub>O = 42:33:25) and Solvent B (methanol/acetonitrile:ethyl acetate = 50:20:30) was set as [30%, 0], [100%, 50], [100%, 58], [30%, 60] at a flow rate 1 ml min<sup>-1</sup>. Elution of pigments was monitored at 705 nm, the absorbance maximum of Chl f.

Method MY2, adapted from Armenta et al. (46), was performed for both analytical and semi-preparative purposes. Pigments were extracted using a previously published method (49) and separated with the same HPLC system using an analytical Discovery C18 column described above or a semi-preparative Discovery C18 column (10 mm × 25 cm) (Supelco, Sigma-Aldrich). Solvents A and B were 88:10:2 methanol/ethyl acetate/H<sub>2</sub>O (v/v/v) and 48:50:2 methanol/ethyl acetate/H<sub>2</sub>O (v/v/v), respectively. After 10 min of washing with Solvent A, Chls were eluted with a linear gradient of 0% to 20% solvent B over 20 min, increasing to 100% to wash the column. The analytical program was performed at 1 ml min<sup>-1</sup> and 3.5 ml min<sup>-1</sup> for analytical and semi-preparative purposes, respectively.

Mass spectrometric analysis of chlorophyll pigments collected by semi-preparative method MY2 was performed on a Waters Q-TOF Premier quadrupole/time-of-flight (TOF) mass spectrometer [Waters Corporation (Micromas Ltd.), Manchester, UK]. Operation of the mass spectrometer was performed using MassLynx software Version 4.1 (www.waters.com). Samples were introduced into the mass spectrometer using a Waters 2695 HPLC system. The samples were analyzed using flow injection analysis (FIA), in which the sample is injected into the mobile phase flow and passed directly into the mass spectrometer, where the analytes are ionized and detected. The mobile phase used was 90% acetonitrile (LC-MS grade) and 10% aqueous 0.1% formic acid. The flow rate was 0.15 ml min<sup>-1</sup>. The nitrogen drying gas temperature was set to 300°C at a flow of 6 liters min<sup>-1</sup>, and the capillary voltage was 2.8 kV. The mass spectrometer was set to scan from 100 to 1000 m/z in positive ion mode, using electrospray ionization (ESI). The MS analysis of Chls and chlorophyllins was performed in the Proteomics and Mass Spectrometry Core Facility, Huck Institutes of the Life Sciences, Pennsylvania State University.

### Absorption and fluorescence spectroscopy

Room-temperature absorption spectra were recorded with a GENESIS 10 spectrophotometer (ThermoFisher Scientific) or a Cary 14 UV-Vis-NIR spectrophotometer modified for computer-controlled operation by OLIS Inc. (Bogart, GA). Absorption spectra of whole cells of *Synechococcus* sp. PCC 7335 and mutants derived from it were recorded in growth medium ASN-III. The absorption spectra of *C. fritschii* PCC 9212 and mutant strains derived from it were recorded by homogenizing, resuspending, and diluting cells in B-HEPES medium with 60% v/v sucrose (1:10 dilution). Before recording the absorption spectra, whole cells were homogenized as necessary, and
spectra were recorded through the opal glass side of the cuvettes to allow correction for scattering. Fluorescence emission spectra of whole cells at 77 K were measured with an SLM 8000C spectrophotometer, modified for computer-controlled operation by OLIS Inc. (Bogart, GA). Cells in exponential growth phase (OD750 nm = ~0.6 to 0.7) were adjusted to a concentration of ~0.5 OD750 nm and quickly frozen in liquid nitrogen. The excitation wavelength was set to 440 nm to excite Chl f preferentially. Isolation and quick-freezing in liquid nitrogen. The excitation wavelength was set to 440 nm to excite Chl f preferentially.

Expression of the psbA4 gene of C. fritschii PCC 9212 in Synechococcus 7002

To generate the expression vector for psbA4, a DNA fragment containing the psbA4 gene was amplified by PCR from chromosomal DNA of C. fritschii PCC 9212 using Phusion HF DNA polymerase. The primers used were 9212 psbA4 pAq1-1 (Neo1Ndel) and 9212 psbA4 pAq1-2 (BamHI) (table S2). After digestion with Ndel and BamHI, the psbA4 gene amplicon was cloned into the pAQuiEx-FP200/Gm shuttle vector (29). After verification by DNA sequencing, the psbA4 expression construct [pAQuiEx-9212 psbA4 (Gm)] was transformed into the cells of Synechococcus 7002. Transformants were selected on agar plates made with A’ medium amended with gentamicin. Transformants harboring the expression plasmid were confirmed by PCR analysis and DNA sequence analysis.

Isolation of PSI complexes and LC-MS/MS analysis

PSI complexes of the wild-type and psbA4 mutant strains of C. fritschii PCC 9212 were isolated from cells grown under WL and FRL conditions following the procedures described previously (7). In-solution digestion of proteins with trypsin and LC-MS/MS analysis were performed at the PARC Mass Spectrometer Facility at Washington University in St. Louis. Peptide mass spectra (m/z range 380 to 1500) were acquired at high mass resolving power (70,000 for ions with m/z > 200) with a Fourier Transform (FT) Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). The raw data from the LC-MS/MS analysis was directly loaded into PEAKS (v 7.0, Bioinformatics Solution Inc., Waterloo, Ontario, Canada) for performing database searches against the total proteome for C. fritschii PCC 9212, which was derived from the annotated genome sequence.

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Light-dependent chlorophyll f synthase is a highly divergent paralog of PsbA of photosystem II
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Sometimes, red light means grow
Some cyanobacteria are able to use the far-red end of the light spectrum by synthesizing chlorophyll f pigments. Introducing the protein responsible for chlorophyll f synthesis into crop plants could potentially expand the range of wavelengths that such plants use during photosynthesis and thereby increase their growth efficiency. Ho et al. identified chlorophyll f synthase (ChlF) in two cyanobacteria that are acclimatized to grow using far-red light. Introducing the ChlF-encoding gene into a model cyanobacterium allowed the organism to synthesize chlorophyll f. Similarities between ChlF and a core protein of photosystem II suggest that they have a close evolutionary relationship, and ChlF may even represent a more primitive photochemical reaction center.

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