suppression of the lethality of high light to a quadruple hli mutant by the inactivation of the regulatory protein pfsR in Synechocystis PCC 6803*

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A regulatory gene, designated pfsR (photosynthesis, Fe homeostasis and stress-response regulator), was discovered by a genetic screen in Synechocystis PCC 6803. Deletion of the gene from a high light-sensitive strain lacking four hli genes (4Xhli) restored viability to the parental strain under high light conditions. The quintuple mutant pfsR-/4Xhli retained photosystem-II and oxygen evolution capacity at levels similar to the wild-type levels under high light conditions. The transcripts of the two hfr genes (encoding bacterioferritin) were found to be constitutively up-regulated, whereas the transcripts of holl gene (encoding a heme oxygenase) were greatly down-regulated in high light upon deletion of pfsR. Under intermediate high intensity light, the pfsR deletion strains accumulated carotenoids and chlorophyll a to a significantly higher level than their corresponding parental strains. An exacerbated, transient increase in oxygen evolution during the early hours of high light acclimation and a somewhat increased steady-state level of photosystem-II-mediated oxygen evolution observed in the 4Xhli strain were brought back to the wild-type levels upon deletion of pfsR from the strain. The pfsR deletion mutants were found to be less sensitive to iron limitation under low light conditions and to suffer less lipid peroxidation following exposure to high light. Therefore, inactivation of PfsR resulted in tighter control of iron availability, which in turn reduced oxidative stress during photosynthesis in high light. These studies have revealed a critical role of PfsR in regulation of iron homeostasis and stress response.

Both microalgae and vascular plants have evolved mechanisms for photo-acclimation that enable them to tolerate the absorption of excess excitation energy and limited amounts of reactive oxygen (3–6). Acclimation mechanisms include (but are by no means limited to) changes in the composition of light-harvesting and/or reaction center pigment-protein complexes (7), dissipation of excess excitation energy as heat within the pigment bed of the antennae or reaction center core of photosystem (PS)II (8–11), synthesis of enzymes with antioxidant function such as superoxide dismutase (12–14), catalase (15–17), and peroxidases (18–21), and enhanced accumulation of soluble antioxidants such as carotenoids, tocopherol, and glutathione that can act as quenchers of triplet chlorophyll and singlet oxygen (22, 23).

The sequestration of free iron, which has been recognized in non-photosynthetic organisms as an antioxidant strategy (24, 25), could very likely be a vital photo-acclimation strategy in photosynthetic organisms. The photosynthetic complexes, PS-I, PS-II, and cytochrome b6f complexes all contain iron atoms in the forms of heme, nonheme iron, and/or iron-sulfur clusters (26). It is expected that damages to photosynthetic complexes under stress conditions would result in significant release of iron (II) from these complexes. Ferrous iron could catalyze the Fenton reaction, converting hydroperoxides to highly reactive hydroxyl radicals (2, 27). Therefore, control of iron flux or iron homeostasis would be of vital importance for photosynthetic organisms to acclimate to high light (HL) and other stress conditions.

Synthesis of stress-associated proteins also occurs when photosynthetic organisms are exposed to HL. These proteins are often important for the acclimation of cells to HL. The hli genes (also called scp genes) (28, 29), encoding the high light inducible polypeptides (HLIP) with similarity to the light harvesting chlorophyll a/b-binding proteins of plants, were shown to be important for the survival of cyanobacteria under high light conditions (30). On the Synechocystis PCC 6803 genome, there are four hli genes encoding HLIPs. A fifth similar gene sequence is fused to the hemH gene (or scpA) to encode the C terminus of the ferrochelatase (29, 30). The levels of the four HLIPs increase...
PfsR Deletion Suppresses HLIP Defect

in response to HL, low temperature, and nutrient limitation (30), whereas the levels of the ferrochelatase are reduced under these conditions.3 In addition, the HLIP-like extension of the ferrochelatase did not functionally complement HLIPs under HL conditions, and the mutant (4Xhli) lacking all four hli genes (hliA-D) dies in HL, whereas any of the triple mutants survives under the same conditions (30). These results suggest that the regulation of hli genes is different from that of the hemH gene and support that HliA-D and the ferrochelatase might perform rather distinct functions (29), at least in HL. The functions of HLIPs so far are unclear. However, they are likely to perform multiple functions (even though these polypeptides could functionally complement each other) because multiple functions (even though these polypeptides could functionally complement each other) because hli genes in *Synechocystis* PCC 6803 are found to be differentially expressed in the cells and distinctly localized in fast protein liquid chromatography (30). In addition, groups of hli genes in the HL-adapted *Prochlorococcus* strain MED4 are found to be differentially expressed under phosphorus starvation or HL stress, suggesting that groups of hli genes contribute to specific stress responses (31). HLIPs may enable cyanobacteria to cope with the production of reactive oxygen species, possibly by regulating tetrapyrrole biosynthesis as a function of the demand for chlorophyll (32, 33) or by promoting the dissipation of the excess light energy absorbed (34).

In this study, we used a suppressor screen (35), in an attempt to elucidate the functions of HLIPs and the mechanisms of HL survival. The genetic screen has resulted in the discovery of the regulatory gene *pfsR* (photosynthesis, Fe homeostasis and stress response regulator), corresponding to the open reading frame sll1392 in *Synechocystis* PCC 6803. The *pfsR* gene was inactivated from the wild-type and 4Xhli strain, generating the mutant strain (30). The strain lacks four HLIPs and dies upon exposure to HL. This permits screening of suppressors, mutants with secondary mutations that counteract the effects of the primary mutations (inactivation of *hli* genes), by isolation of survivor colonies in HL. The 4Xhli was mutagenized with N-methyl-N'-nitrosoguanidine (36). Aliquots of mutagenized cells were spread onto BG-11 plates containing appropriate antibiotics and incubated at 20 μmol photon m⁻² s⁻¹ for 3 days. The plates were then exposed to HL for 48 h to bleach cells before being returned to LL. Colonies of survivors typically appeared within 10 days. These putative suppressors were re-tested for HL tolerance. A total of six stable suppressors, named “pfs1–6” (the naming reflects our wishes to isolate mutants involve in photosynthesis, Fe homeostasis and stress responses), were obtained. These suppressors grow well in LL with growth rates that are not significantly different from that of the wild type. A single mutant, designated pfs1, was successfully complemented (see below).

**Experimental Procedures**

*Culture Growth Conditions and Light and Iron Treatments—Synechocystis* PCC 6803 strain was cultivated in BG-11 medium with 10 mM TES, pH 8.2, at 30 °C. The culture was bubbled with air under LL (50 μmol photon m⁻² s⁻¹) or intermediate HL (300 μmol photon m⁻² s⁻¹) or HL (500 μmol photon m⁻² s⁻¹) conditions. Photon flux densities were measured with LI-250 Quantum-meter (LI-COR Biosciences). For HL or intermediate HL experiments, the cells reaching mid-logarithmic growth phase (OD₇₃₀ ~ 0.6) were diluted to OD₇₃₀ ~ 0.1 and exposed to light at 300 μmol photon m⁻² s⁻¹ or higher for various lengths of time as indicated. The cultures in glass tubes were placed in a thermo-stated glass chamber filled with water at 30 °C. For testing the sensitivity of iron, cells were diluted to an OD₇₃₀ of ~0.2 and continued to grow in three different iron conditions. The low iron medium used was BG-11 medium without supplement of ferric ammonium citrate, and the medium was treated with 50 μM dipyridyl, an iron chelator. Normal and high iron medium were BG-11 medium supplemented with 30 μM and 300 μM ferric ammonium citrate, respectively. The growth curve was determined by measuring OD₇₃₀.

*Mutagenesis and Screening for Suppressors—*The parental strain for mutagenesis was the *Synechocystis* PCC 6803 4Xhli strain (30). The strain lacks four HLIPs and dies upon exposure to HL. This permits screening of suppressors, mutants with secondary mutations that counteract the effects of the primary mutations (inactivation of *hli* genes), by isolation of survivor colonies in HL. The 4Xhli was mutagenized with N-methyl-N’-nitrosoguanidine (36). Aliquots of mutagenized cells were spread onto BG-11 plates containing appropriate antibiotics and incubated at 20 μmol photon m⁻² s⁻¹ for 3 days. The plates were then exposed to HL for 48 h to bleach cells before being returned to LL. Colonies of survivors typically appeared within 10 days. These putative suppressors were re-tested for HL tolerance. A total of six stable suppressors, named “pfs1–6” (the naming reflects our wishes to isolate mutants involve in photosynthesis, Fe homeostasis and stress responses), were obtained. These suppressors grow well in LL with growth rates that are not significantly different from that of the wild type. A single mutant, designated pfs1, was successfully complemented (see below).

**Complementation of the pfs1 Mutant**—Molecular techniques were performed using standard protocols (37). To identify the point mutations in the pfs1 suppressor, genomic DNA was isolated from pfs1 and mechanically sheared to ~4–7-kb fragments by agitation in the presence of 0.1-mm glass beads. The DNA fragments were then cloned in pGEM-T vector after the ends were fixed by TaqDNA polymerase, according to the manufacturer’s recommendation (Promega). The genomic DNA library was transformed into the 4Xhli mutant, and transformants were selected for ampicillin resistance, which results when a plasmid integrates into the chromosome by single homologous recombination. Several complements that tolerate HL exposure were obtained. Two colonies were randomly selected for chromosomal DNA extraction. The genomic regions flanking the inserted plasmid DNA, which delimits the putative complementing fragments, were determined by direct sequencing using the universal forward and reverse primers that bind at the multiple cloning site of pGEM-T, as previously reported (38).

**DNA Manipulation and Mutant Construction**—The plasmid in which the *pfsR* gene (sll1392) was inactivated was generated by ligating a 420-bp fragment of *pfsR* gene (from start codon to 420 bp into the gene), a ~1.5 kb erythromycin-resistant cartridge, and a 426-bp PCR fragment generated to sequences downstream of *pfsR* gene (113 to 539-bp downstream of the stop codon), all in proper orientation. The primers used to amplify the *pfsR* gene fragment are: 5’-ATGTTAATCCATCCACCCATG-3’ and 5’-TcaGACCGCATGAGCAGAAACCAAGGC-3’; the two lowercase letters indicate insertions added for creation of a XbaI site. The primers used to amplify the *pfsR* downstream sequences are 5’-CCAGGGGAATTCTCCTCCAGT-3’ and 5’-TAgg(A)Gcc(G)Ctcataata-ATGACCC-3’. The lowercase letters in the latter primer indicate mutations introduced for creation of an Apal restriction site. The original nucleotides are included in parentheses to the

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3 Q. He, personal observation.
left of the corresponding mutations. The resulting plasmid, pPFSR-Ery, carries a 294-bp deletion (182-bp pfsR coding sequences plus 112-bp downstream sequences). The pPFSR-Ery plasmid was used to transform the Synechocystis PCC 6803 wild type and the 4Xhli strain with an ery′ cassette recycled by the method reported (32). The transformants were selected by screening for resistance to 10 μg of erythromycin/ml in BG-11 medium. Transformants were restreaked onto the same medium and segregation of the inactivated pfsR gene was monitored by PCR using genomic DNA of transformants and the two inner primers 5′-CCTCAAGGATCTTACCCCCTAGA-3′ and 5′-ATAGAGGTCTCTGATAGCGGTTTTTTTTT-3′ that recognize sequences upstream or downstream of the inserted ery′ cassette. The fragment sizes of pfsR and pfsR-ery′ amplified are 600 bp and 1.75 kb, respectively. Homoplasmic mutants were obtained, and the mutants generated by transformation of wild type and 4Xhli were designated as pfsR− and pfsR−/4Xhli, respectively.

For PCR analysis, Synechocystis PCC 6803 genomic DNA was prepared by a mini preparation procedure. Briefly, a loopful of cyanobacterial cells was suspended in 200 μl of Tris-EDTA buffer (pH 8.0) and transferred to a microcentrifuge tube with 200 μl of glass beads (0.1-mm diameter; Sigma). The cells were broken in a MiniBead Beater (Biospec Products) by two cycles of agitation at the low speed setting for 30 s; between each cycle, the cells were cooled on ice for 2–3 min. Lysates were extracted with phenol-chloroform, and the DNA was precipitated, washed, and dried according to standard procedures (37). The dry DNA pellet was dissolved in 40 μl of H2O of which 1 μl was used for each PCR.

RNA Isolation and Northern Blot—RNA was isolated from cells as previously described (39). For RNA blot hybridizations, 3 μg of total RNAs was separated by 1.2% agarose-formaldehyde gel and transferred onto Immobilon NY (Millipore). Ethidium bromide was included in the loading buffer, allowing for visualization of the tRNA bands and confirmation of equal loading of RNA samples after transfer. The gene-specific probes for hybridization were prepared by PCR using genomic DNA of transformants and the manufacturer’s instructions.

Oxygen Evolution and Fluorescence Measurements—Cells were grown to mid-logarithmic phase and harvested by centrifugation at 5,000 × g for 5 min. The cells were then resuspended to the concentration of 5 μg of chlorophyll per ml. O2 evolution was measured using a Clark-type electrode and illuminated with white light at 1000 μmol of photon m−2 s−1. Cultures were supplemented with 1 mM sodium bicarbonate for the determination of whole chain photosynthetic electron transport activities. PS-II-mediated electron transport activities were measured in the presence of 0.5 mM 2,6-dichloro-p-benzoquinone and 1 mM K3FeCN6 (41).

Fluorescence measurements were performed on a dual-modulation kinetic fluorometer (Model FL-3000, Photon Systems Instruments). Cultures (2 μg of chlorophyll/ml) were dark incubated for 10 min in the presence of 0.3 mM phenyl-p-benzoquinone and followed by a 1-min incubation in the presence of 40 μM DCMU (42). Relative PS-II content was determined as (Fmax - Fo)/Fo (41).

Lipid Peroxidation Assessment—Lipid peroxidation was assessed by measuring the amount of malondialdehyde (MDA) and 4-hydroxyalkenals (HAE), the decomposed products of polyunsaturated fatty acid peroxides. MDA and HAE were quantified using the BIOXYTECH® LPO-586™ kit (Oxis International Inc.) according to the manufacturer’s instructions. This assay is based on the reaction of a chromogenic reagent N-methyl-2-phenylindole with MDA and HAE at 45 °C to yield a stable chromophore with maximal absorbance at 586 nm.

Western Blot Analysis—Thylakoid membranes were prepared according to the method reported (39). Solubilization of thylakoid membranes and SDS-PAGE were performed as described by Peter and Thornber (43). Approximately 5 μg of membrane proteins were resolved by SDS-PAGE in a 16% polyacrylamide with 6 M urea. Polypeptides were electrophoretically transferred onto nitrocellulose membrane, and immunodetection of polypeptides was performed using commercial antibodies, as recommended by the suppliers of the antibodies (Santa Cruz Biotechnology; AgriSera).

Pigment Analysis—Cellular pigments were extracted in 90% methanol for qualitative analysis. For quantitative determination of the pigment content of cells, pigments were extracted in dimethylformamide. After centrifugation in a microcentrifuge at 4 °C for 10 min, the supernatant was taken to quantify the pigment content photometrically. The concentration of total colored carotenoids ([Carotenoid]) in a DMF extract was determined by the formula in Equation 1 (44).

\[
[\text{Carotenoid}]_{\mu g/ml} = (A_{461} - 0.046 \times OD_{664}) \times 4 \quad \text{(Eq 1)}
\]

The chlorophyll a concentration ([Chlorophyll a]) was measured from the DMF extract by the formula in Equation 2 (45).

\[
[\text{Chlorophyll a}]_{\mu g/ml} = 12.1 \times OD_{664} - 0.17 \times OD_{625} \quad \text{(Eq 2)}
\]

Statistics—The experiments reported here were carried out independently at least three times on different days. In experimental groups that were statistically analyzed for differences, Student’s t test was used, in which p < 0.05 was defined as statistically significant. The numerical data are expressed as means ± S.D.

RESULTS

Identification of the Suppressor Mutation in the Suppressor pfs1—To identify the suppressor mutation in pfs1, the 4Xhli mutant was transformed with a recombinant genomic library of pfs1, and transformants were screened for complementation of the defect in HL tolerance as described under “Experimental Procedures.” Two complements (HL tolerant colonies) were randomly selected and propagated for direct genomic DNA sequencing using the universal forward and reverse primers. The sequence data revealed that the two overlapping genomic fragments from pfs1 were integrated into the genome of the two HL tolerant colonies, respectively. The overlap is a 4.6-kb fragment and contains three and a half open reading frames (ORF).
PfsR Deletion Suppresses HLIP Defect

We termed sll1392 as pfsR (photosynthesis, Fe homeostasis and stress response regulator). Analysis of the deduced PfsR protein sequence showed that it shares sequence similarity with the superfamily of TetR regulators (28–33% identity). It is presented in all other cyanobacterial genomes that have been sequenced. The PfsR protein contains a DNA-binding helix-turn-helix (HTH) motif. A large portion of the protein (from Tyr-37 to Tyr-135) shows high degrees of similarity (28% identities, 41% positives) with the C-terminal portion of the LRR (leucine-rich repeats) domain of the Arabidopsis COII protein, a key negative regulator of the jasmonate signaling network (46).

Characteristics of pfsR− Mutants in High Light—The pfsR gene was inactivated from the wild type and 4Xhli by targeted mutagenesis, and homoplasmic mutants were obtained (Fig. 2). Table 1 summarizes some of the characteristics of these mutants. Clearly, upon deletion of pfsR from 4Xhli, the quintuple mutant was capable of growth in HL. The growth rate of the quintuple mutant was similar to that of the pfsR− single mutant and was not much different from that of wild type in both LL and HL. The loss of oxygen evolution capability seen in the 4Xhli mutant in HL was alleviated. These results demonstrated that pfsR deletion suppressed the lethality of the 4Xhli mutant in HL. To investigate whether pfsR deletion helped to reduce oxidative stress, we estimated lipid peroxidation, a commonly accepted indicator of oxidative stress, in LL-grown or HL-treated cells by measuring the content of MDA and HAE. The results are shown in Table 2. The MDA + HAE levels are similar for all four strains in LL. Upon exposure of cells to HL for 36 h, we found that MDA + HAE levels increased by about 50% for the wild-type and pfsR− strains. In contrast, the lipid peroxidation level (as evaluated by measurement of MDA + HAE) was tripled in the 4Xhli strain as compared with low light level. This implies that the 4Xhli mutant suffered extensive membrane damage under very high intensity light (500 mol photon m−2 s−1), which could be the major reason for its HL sensitivity. The level of lipid peroxidation in 4Xhli is about twice as much as that in the quintuple mutant in HL. This indicates that the aggravated oxidative damage to 4Xhli cells in HL has been essentially assuaged by the deletion of pfsR.

Pigment Analysis—To better understand the relationship between the pfsR gene and the hli genes, we analyzed the pigment composition and photochemical activities of the mutants under low to intermediate HL intensities at 300 μmol photon m−2 s−1, under which conditions all strains survive but may experience various degrees of excess excitation.

FIGURE 2. Inactivation of the pfsR gene. A, PCR analysis of the pfsR gene in potential mutants in which a fragment of the pfsR gene was deleted and replaced with an erythromycin resistant cartridge (ery). Genomic DNA for PCR analysis was isolated from putative pfsR mutants (lanes 1 and 2) or from the pfsR−/4Xhli isolates (lanes 3–5). M, DNA size marker; WT, wild type. B, depiction of the plasmid construct used to generate the pfsR− strains. The plasmid carries a 294-bp deletion (182-bp pfsR coding sequences plus 112-bp downstream sequences). (Fig. 1). The three complete ORFs (sll1392-1394) and the fourth partial ORF (sll1395) are in the same direction. sll1392 encodes a hypothetical transcription factor, while the other three sll1393 (gqA), sll1394 (msrA), and sll1395 (pfbD) encode a glucogen (starch) synthase, a peptide methionine sulfoxide reductase (involved in repair of oxidatively damaged proteins) and a dTDP-4-dehydroharmannose reductase (involved in cell envelope or wall synthesis), respectively. We then amplified and cloned each of these four individual ORF with flanking sequences from pfs1 and used clones in complementation analysis to define the complementing gene. An 830-bp fragment covering the entire 609-bp sll1392, found to be responsible for the complementation, was sequenced. The mutations were found to be a G to A transition that resulted in changing of the Glu-111 (codon GAA) of the deduced Sll1392 protein into a Lys (codon AAA).
O2 evolution was most prominent in 4Xhli, which showed a more than 2-fold increase with a 6-h delay in peaking time. The transient increase in O2 evolution in all four strains diminished following longer period of incubation. In wild type and the pfsR− mutants, O2 evolution reached a steady-state level in about 24 h. In contrast, in the 4Xhli mutant, O2 evolution continued to decline (although slowly) until it reached a lower steady-state level several days later (Fig. 4A, inset). When PS-II-mediated activity was evaluated (in the presence of electron acceptors) as described under “Experimental Procedures,” a similar transient increase of O2 evolution was also observed, and again the dramatic transient increase in O2 evolution was most pronounced in the 4Xhli mutant. The O2 evolution reached a similar steady-state level in about 48 h in all four strains, although it was repeatedly found to be somewhat higher in the 4Xhli strain than in other strains (in great contrast to the whole chain situation). Under LL conditions, there is no significant difference between these strains, both in whole chain and PS-II-mediated electron transport activities.

To determine whether the transient increase in O2 evolution is associated with a major change in photosystem proteins, we used Western blotting to determine the abundance of the PS-II reaction center protein D1, the PS-I protein PsaC, and an ATP synthase subunit AtpB in cells that were exposed to intermediate HL for 6 or 12 h (Fig. 5). The level of D1 protein, presumably representing the PS-II level, was similar in all four strains in LL (50 μmol photon m−2 s−1). The level was reduced by about 30% for all four strains after cells were exposed to intermediate HL (300 μmol photon m−2 s−1) for 6 h. The D1 protein in each strain was then recovered to about 90% of the original level by 12 h. The PsaC protein, a representative chosen for estimation of PS-I level, was slightly lower (less than 10%) in the 4Xhli strain than in the wild type either in LL or in intermediate HL for 6 or 12 h. The PsaC level in all four strains was reduced to about 60% of their original level upon 6-h incubation in intermediate HL, and the level continued to decline to about 50% of the original level in all strains except the pfsR− single mutant. The pfsR− strain, which contained similar levels of PsaC as other strains in LL or in intermediate HL for 6 h, retained a fraction more (about 10–15%) of PsaC than in other strains when cells were exposed to intermediate HL for 12 h. The level of AtpB (presumably the level of ATP synthase) was

125% of the initial level, in 3 days. In contrast, the carotenoid level in the 4Xhli declined continuously after an initial increase to a very high level, and it did not reach steady state in 4 days. The wild type and the pfsR−/4Xhli mutant showed similar acclimation trends; the carotenoid level reached a steady-state level (about 85–95% of initial level) in 2 days. The two pfsR− strains retained a much higher concentration of carotenoids as compared with their corresponding parental strains.

Under LL conditions (50 μmol photon m−2 s−1), wild-type cells contained somewhat more chlorophyll as compared with the other strains, but this was not highly significant. The chlorophyll content of all four strains decreased upon exposure of cells to HL. The loss of chlorophyll content under HL conditions was most prominent in the 4Xhli mutant; its chlorophyll content was reduced to 14% of the initial level upon a 96-h exposure to intermediate HL at 300 μmol photon m−2 s−1. The chlorophyll content in pfsR− cells reached a steady-state level that is about 45% of the initial chlorophyll level in 24–36 h, whereas the chlorophyll contents of the wild type and the pfsR−/4Xhli mutant declined to a steady-state level that is 30% of the initial level in about 2 days. These data further verified that pfsR deletion have alleviated light stress experienced by the 4Xhli mutant in higher intensity light.

Photochemical Activity and Photosystem Protein Levels—Photosynthetic electron transport activity under intermediate HL was determined by measuring oxygen evolution in the absence (whole chain activity) or presence (PS-II mediated activity) of electron acceptors (Fig. 4). The whole chain O2 evolution in cells of the wild type and the two pfsR− strains (pfsR− and pfsR−/4Xhli) showed a transient peak at 6 h after the cells were transferred to intermediate HL. The transient increase in

PfsR Deletion Suppresses HLIP Defect

TABLE 1
Properties of Synechocystis PCC 6803 strains

| Strain     | O2 evolution | Relative PS-II content | Doubling time |
|------------|--------------|------------------------|--------------|
|            | Whole chain  | PS-II mediated         |              |
|            | LL | HL | LL | HL | LL | HL | LL | HL | LL | HL | LL | HL |
| WT         | 258±39 | 379±59 | 325±84 | 415±80 | 100±8 | 45±4 | 10±1 | 8±1 | 12±2 |
| pfsR       | 266±30 | 360±55 | 335±86 | 398±42 | 92±6 | 49±6 | 11±1 | 7±1 | 13±2 |
| 4Xhli      | 274±42 | 95±27 | 338±67 | 112±35 | 89±10 | 24±3 | 11±2 | 13±2 | ND* |
| pfsR−/4Xhli| 275±48 | 408±71 | 317±60 | 474±52 | 90±10 | 52±5 | 11±1 | 7±1 | 14±2 |

ND*, not determined (the strain dies in HL after incubation for longer than 2 days).

| Strain     | Oxidative stress | MDA | MDA + HAE | MDA | MDA + HAE |
|------------|------------------|-----|-----------|-----|-----------|
|            | LL               |     |           |     |           |
| WT         | 0.58±0.11 | 1.16±0.23 | 1.50±0.28 | 1.71±0.30 |
| pfsR       | 0.64±0.18 | 1.08±0.27 | 1.43±0.30 | 1.58±0.26 |
| 4Xhli      | 0.61±0.15 | 1.19±0.23 | 1.57±0.42 | 3.91±0.38 |
| pfsR−/4Xhli| 0.66±0.17 | 1.02±0.20 | 1.26±0.34 | 1.43±0.34 |

* O2 evolution was most prominent in 4Xhli, which showed a more than 2-fold increase with a 6-h delay in peaking time. The transient increase in O2 evolution in all four strains diminished following longer period of incubation. In wild type and the pfsR− mutants, O2 evolution reached a steady-state level in about 24 h. In contrast, in the 4Xhli mutant, O2 evolution continued to decline (although slowly) until it reached a lower steady-state level several days later (Fig. 4A, inset). When PS-II-mediated activity was evaluated (in the presence of electron acceptors) as described under “Experimental Procedures,” a similar transient increase of O2 evolution was also observed, and again the dramatic transient increase in O2 evolution was most pronounced in the 4Xhli mutant. The O2 evolution reached a similar steady-state level in about 48 h in all four strains, although it was repeatedly found to be somewhat higher in the 4Xhli strain than in other strains (in great contrast to the whole chain situation). Under LL conditions, there is no significant difference between these strains, both in whole chain and PS-II-mediated electron transport activities.

To determine whether the transient increase in O2 evolution is associated with a major change in photosystem proteins, we used Western blotting to determine the abundance of the PS-II reaction center protein D1, the PS-I protein PsaC, and an ATP synthase subunit AtpB in cells that were exposed to intermediate HL for 6 or 12 h (Fig. 5). The level of D1 protein, presumably representing the PS-II level, was similar in all four strains in LL (50 μmol photon m−2 s−1). The level was reduced by about 30% for all four strains after cells were exposed to intermediate HL (300 μmol photon m−2 s−1) for 6 h. The D1 protein in each strain was then recovered to about 90% of the original level by 12 h. The PsaC protein, a representative chosen for estimation of PS-I level, was slightly lower (less than 10%) in the 4Xhli strain than in the wild type either in LL or in intermediate HL for 6 or 12 h. The PsaC level in all four strains was reduced to about 60% of their original level upon 6-h incubation in intermediate HL, and the level continued to decline to about 50% of the original level in all strains except the pfsR− single mutant. The pfsR− strain, which contained similar levels of PsaC as other strains in LL or in intermediate HL for 6 h, retained a fraction more (about 10–15%) of PsaC than in other strains when cells were exposed to intermediate HL for 12 h. The level of AtpB (presumably the level of ATP synthase) was
rather similar among all four strains in either LL or intermediate HL conditions. Therefore, the exacerbated, dramatic, transient increase in O2 evolution seen in 4Xhli is unlikely caused by a major alteration in photosystem levels. Instead, it is reminiscent of the sun-type chloroplasts of plants, which have a much higher photosynthetic capacity and/or oxygen evolution on a per chlorophyll basis than the shade type of chloroplasts of LL plants (47).
PfsR Deletion Suppresses HLIP Defect

The Transcript Levels of Photosynthetic and Stress-associated Genes—
To investigate the molecular mechanisms of the suppression, we examined the impacts of the pfsR deletion on the expression of a number of genes involved in pigment synthesis, photosynthesis, and stress response. Total RNA isolated from cells grown in LL or treated by HL was hybridized to gene-specific probes. The results are shown in Fig. 6. In the wild type, transcripts of bfrA and bfrB, encoding subunits of bacterioferritin, were accumulated to high level (about four times induction), while the mRNA level of a heme oxygenase (ho1) decreased by ~50% following exposure to HL. The transcript of gpx2, encoding a stress inducible glutathione peroxidase (40), was detected in HL treated cells but not in LL grown cells. The level of psbA transcripts was enhanced by 2.5-fold by HL treatment, while the psaAB transcript level dropped by ~80% upon HL exposure, consistent with previous reports (48–50).

In the pfsR− mutant, bfrA and bfrB genes become constitutively expressed at a level that is about 2-fold of that in HL-treated wild type cells, regardless of the light conditions. The ho1 gene expression was almost completely depressed in HL. As in the wild type, the gpx2 gene was induced by exposure to high light, but the degree of induction was somewhat less. The increased accumulation of psbA transcripts in HL in the pfsR− mutant was comparable to that in wild type; and the down-regulation of psaAB transcripts in HL was also observed in the pfsR− mutant as in the wild type.

Growth of pfsR− Strains under Different Iron and Light Conditions—Because bacterioferritin chelates free iron and heme oxygenase oxidizes heme (releasing ferrous iron), we hypothesized that PfsR controls the availability of iron through regulation of bacterioferritin and heme oxygenase. For this reason, we tested the growth of the pfsR− strains under conditions with different iron availability under different light conditions. Fig. 7 shows the growth of the Synechocystis PCC 6803 strains in BG-11 medium with three different iron concentrations. In LL, all four strains grew similarly well under normal or 10 times surplus iron conditions, suggesting that normal BG-11 medium contains sufficient amounts of iron to support Synechocystis PCC 6803 growth. More iron (up to 10 times surplus) in the growth medium is neither toxic nor beneficial to the cell growth, as consistent with previous reports (51). In LL under low iron condition, the wild type and 4Xhlil grew poorly especially in the later hours, presumable after iron depletion has taken place in vivo. In contrast, the pfsR− and pfsR−/4Xhlil mutants apparently were less sensitive to the iron limitation in LL; both pfsR− mutants grew well under iron limitation condi-
tions as compared with iron-replete conditions in the time frame tested. The growth of 4Xhli was highly sensitive to iron conditions. It stopped growing under very high iron conditions (about 50 times of iron in normal BG-11) whereas other strains grew well under the same conditions (data not shown). These results suggest that the pfsR/H11002 mutants have higher iron buffering capacity.

Under intermediate HL, 4Xhli grew poorly (see Table 1 for doubling time) whereas the pfsR/H11002/4Xhli mutant grew as well as the wild type. This further demonstrated that inactivation of PfsR reduced the stress experienced by the 4Xhli mutant. However, the cultures of the pfsR/H11002/4Xhli appeared to be more chlorotic, possibly reflecting an enhanced accumulation of the carotenoid myxoxanthophyll (about 10% more myxoxanthophyll was found in the quintuple mutant than in the wild type, data not shown), which is mainly associated with cytoplasmic membrane and the outer membrane (52). Interestingly, all four strains appeared to grow faster under 10 times higher iron conditions as compared with the normal iron condition in intermediate HL for up to 3 days, suggesting that iron uptake/usage may be limited or restricted by the cells under intermediate HL conditions. Longer incubation caused the 4Xhli strain, but not other strains, to lose cell viability (not shown). Under low iron conditions with intermediate HL, all four strains died, even though the iron concentration was sufficient to maintain the cell growth in LL. This might be because of a combination of low iron availability augmented by intermediate HL and oxidative stress imposed by relatively high intensity light.

**DISCUSSION**

Extragenic suppressor analysis offers one of the best ways to analyze the functions of a gene and to ascertain which gene products interact with each other in performing their functions (35). Several mechanisms explain the phenomenon of genetic suppression of lethality. These include the most common way of restoring the viability of the cell by preventing the accumulation of a toxic intermediate or by altering regulatory mechanisms. Indeed, our genetic screen resulted in the discovery of the regulatory protein PfsR. Upon deletion of PfsR from 4Xhli by targeted mutagenesis, we found that the resulting quintuple mutant pfsR/H11002/4Xhli was capable of growth under HL conditions with a long term growth rate comparable to the wild type or the pfsR/H11002 single mutant. In addition, the loss of photosynthesis seen in the 4Xhli mutant in HL was brought back to the wild-type level, as evaluated by the whole chain or PS-II mediated oxygen evolution (Table 1). These results confirmed that the inactivation of PfsR rescued the 4Xhli from HL sensitivity, demonstrating the effectiveness of the suppressor screen in genetic analysis of HL acclimation.

The PfsR protein resembles a transcriptional regulator in protein sequence and secondary structure. It contains a HTH-TETR motif, a signature for the HTH DNA-binding domain of the tetR family of transcription factors. Many of these proteins function as repressors that control various cellular processes,
PfsR Deletion Suppresses HLIP Defect

ranging from drug resistance (by TetR) to transcription of cytochrome P450 genes (by Bm3R). However, these repressors often can also activate sets of genes. To decipher potential target genes of PfsR, we examined the impacts of the PfsR deletion on the expression of a number of genes involved in pigment synthesis, photosynthesis, and stress response. The most striking result obtained was that bfrA and bfrB genes became constitutively expressed upon deletion of PfsR. This suggests that PfsR, functioning as a repressor as the secondary protein structure predicts, represses the expression of bfr genes under low light conditions. Like ferritins or bacterioferritin in other systems, the Synechocystis PCC 6803 bacterioferritin plays a critical role in iron homeostasis. It stores iron and ensures that very little free iron is present while maintaining availability when it is needed (26, 53). Therefore, the up-regulation of bfr genes upon deletion of pfsR in Synechocystis PCC 6803 is likely to have an impact on iron metabolism that affects photosynthesis and stress response as well. Indeed, deletion of pfsR from 4Xhli suppressed the lethality of HL to the quadruple mutant. In addition, the pfsR− and pfsR−/4Xhli mutants were much less sensitive to the iron limitation in LL; both pfsR− mutants grew well for several generations under iron limitation conditions as compared with iron-replete conditions, at least during the time period tested. Therefore, deletion of PfsR resulted in an enhanced iron storage (before the onset of iron limitation), which is subsequently used to enhance cell growth under iron-deplete conditions.

The down-regulation of ho1 in HL would also have profound impact on the survival of Synechocystis PCC 6803. The Heme oxygenases catalyze the oxygen-dependent cleavage of heme to produce biliverdin IX, releasing ferrous iron (54). In Synechocystis PCC 6803, two paralogs of ho genes (ho1 and ho2) have been identified, both of which have been shown to encode active enzymes in vitro (55–57). However, only the ho1 gene is expressed to a level that is readily detectable by Northern blot analysis (Fig. 6; also see Ref. 54). In this study, we have shown that the ho1 gene is repressed under HL conditions. This down-regulation, in concert with up-regulation of bfr genes, is likely to be important for HL acclimation and/or for alleviation of oxidative damage as it would reduce the synthesis of phycobilin (synthesized from biliverdin IX) and the release of ferrous iron. This is supported by the fact that the pfsR−/4Xhli mutant suffered less lipid peroxidation (normally triggered by hydroxyl radicals) than the 4Xhli strain in HL (Table 2).

The significance of further down-regulation of ho1 in the pfsR− mutant (as compared with the wild type) is unclear. At a first glance, this might seem to contribute to an even tighter control of phycobilin synthesis and/or iron release. However, we did not see differences in the phycobilisome amount between the pfsR− strain and wild type in any light conditions tested (data not shown). Therefore, the functions of ho1 might be as complex as in animal systems; in the HO1 knock-out mice, tissue stores of iron are elevated, while serum iron levels are low (58, 59). In addition, the mechanism by which HO1 activity is linked to cellular iron mobilization remains obscure, even though it has been demonstrated that expression of HO1 is linked to cellular iron efflux (60, 61). Nonetheless, it might be beneficial that an enhanced accumulation of bfr mRNA occurs concurrently with down-regulation of ho1 since bacterioferritin binds heme, the substrate of heme oxygenase. Such coordination would allow a precise adjustment of iron homeostasis to respond to light stress.

The heavily augmented and extended transient increase in oxygen evolution during early hours of exposure to intermediate HL is a readily detectable phenotype that distinguishes 4Xhli from other strains studied here. This exacerbated, albeit transient, increase in oxygen evolution in 4Xhli upon exposure to intermediate HL could cause over-reduction of plastoquinone pool to a greater extent, which could stimulate regulatory and/or protective stress responses until a new homeostasis has been achieved. Indeed, when the cells lacking HLIPs was exposed to intermediate HL for a longer period of time, the whole chain oxygen evolution in the 4Xhli mutant was compromised, and cells grew slower; however, it was able to reach a new, albeit lower, steady-state level (Fig. 4A, inset). A lowered redox potential might also lead to a reduction in chlorophyll biosynthesis and/or accumulation (Fig. 3), as was also observed in scp mutants with PS-I-less background (32). While the inverse statement (i.e. reduction in chlorophyll biosynthesis affects electron transport activities) could also be possible, we do not favor this explanation as there is no significant difference in chlorophyll content among all four strains in LL and as this small difference in chlorophyll content was not augmented during the early hours of exposure to intermediate HL (Fig. 3). Interestingly, when oxygen evolution was measured in the presence of electron acceptors, all four strains reached a steady-state level in about 48 h, and the 4Xhli strain retained a fraction higher level of oxygen evolution capability than in the other three strains. These data suggest that the electron transport chain downstream of PS-II was hampered in the 4Xhli cells by the deletion of the hli genes. Therefore, our data suggest that HLIPs might be responsible for the fine adjustment of the structure/assembly of photosystems and/or the photosynthetic electron transport. While the details of this hypothesis invite further investigation, it is clear that the increased photochemical activity (or electron draining) is not sufficient to compensate for the deletion of the four HLIPs, highlighting the importance of HLIPs in HL acclimation.

Based on the data presented above, we propose that suppression of the lethality of HL to 4Xhli by the inactivation of PfsR involves up-regulation of bfr genes and down-regulation of the ho1 gene, which results in a tighter control of free iron (II). This in turn reduces the formation of hydroxyl radicals, a cytotoxic agent that could initiate, for example, lipid peroxidation processes, a chain of highly damaging reactions. The iron buffering characteristics (Fig. 7) and a strong reduction in lipid peroxidation (Table 2) observed in the quintuple mutant strengthen this interpretation.

The studies reported here have revealed a critical role that PfsR plays in regulation of iron homeostasis that is important for photosynthesis and cell survival. In addition, our data suggest that HLIPs might be involved in fine adjustment of the structure/assembly of photosystems. Because iron affects the physiology of photosynthetic organisms in many ways, as an essential micronutrient for cell growth, as redox-active cofac-
PfsR Deletion Suppresses HLIP Defect

References

1. Asada, K. (1994) in Causes of Photodioxidative Stress and Amelioration of Defense Systems in Plants (Foyer, C. H., and Mullineaux, P. M., eds) pp. 77–104, CRC Press, Boca Raton, FL.
2. Halliwell, B., and Gutteridge, J. M. C. (1999) Free Radicals in Biology and Medicine, 3rd Ed., pp. 1–104, Oxford University Press, Oxford, New York.
3. Mittler, R. (2002) Trends Plant Sci. 7, 405–410.
4. Niyogi, K. K. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 333–359.
5. Durnford, D. G., and Falkowski, P. G. (1997) Photosyn. Res. 53, 229–241.
6. Yabuta, Y., Motoki, T., Yoshimura, K., Takeda, T., Ishikawa, T., and Baker, N. R. (2002) Annu. Rev. Plant Physiol. Plant Mol. Biol. 53, 599–626.
7. Abdul-Tehrani, H., Hudson, A. J., Chang, Y. S., Timms, A. R., Hawkins, C., Williams, J. M., Harrison, P. M., Guest, J. R., and Andrews, S. C. (1999) J. Bacteriol. 181, 1415–1428.
8. Dolganov, N. A., Bhaya, D., and Grossman, A. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 636–640.
9. Funk, C., and Vermaas, W. (1999) Biochemistry 38, 9397–9404.
10. He, Q., Dolganov, N., Bjorkman, O., and Grossman, A. R. (2001) J. Biol. Chem. 276, 306–314.
11. Coleman, M. L., Sullivan, M. B., Martiny, A. C., Steglich, C., Barry, K., Delong, E. F., and Chisholm, S. W. (2006) Science 311, 1768–1770.
12. Xu, H., Vavilin, D., Funk, C., and Vermaas, W. (2004) J. Biol. Chem. 279, 27971–27979.
13. Xu, H., Vavilin, D., Funk, C., and Vermaas, W. (2002) Plant Mol. Biol. 49, 149–160.
14. Havaux, M., Guedeney, G., He, Q., and Grossman, A. R. (2003) Biochim. Biophys. Acta 1557, 21–33.
15. St Johnston, D. (2002) Nat. Rev. Genet. 3, 176–188.
16. Dolganov, N., and Grossman, A. R. (1999) J. Bacteriol. 181, 610–617.
17. Xie, D. X., Feys, B. F., James, S., Nieto-Rostro, M., and Turner, J. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7530–7535.
18. He, Q., and Vermaas, W. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5830–5835.
19. Haio, H. Y., He, Q., Van Waasbergen, L. G., and Grossman, A. R. (2004) J. Bacteriol. 186, 3882–3888.
20. Meetam, M., Keren, N., Ohad, I., and Pakrasi, H. B. (1999) Plant Physiol. 121, 1267–1272.
21. Peter, G. F., and Deusch, R. J. (1994) Biochemistry 33, 6137–6149.
22. Peter, G. F., and Thorner, J. P. (1991) J. Biol. Chem. 266, 16745–16754.
23. Chamovitz, D., Sandmann, G., and Hirschberg, J. (1993) J. Biol. Chem. 268, 17348–17353.
24. Moran, R. (1982) Plant Physiol. 69, 1376–1381.
25. Xie, D. X., Feya, B. F., James, S., Nieto-Rostro, M., and Turner, J. G. (1998) Science 280, 1091–1094.
26. Lichtenhaler, H. K., Buschmann, C., Doll, M., Fietz, H., Bach, T., Kozel, U., Mei, D., and Ramsdorf, U. (1981) Photosynth. Res. 2, 115–141.
27. Mohamed, A., and Janssen, C. (1989) Plant Mol. Biol. 13, 693–700.
28. Herranen, M., Aro, E. M., and Tyystjarvi, T. (2001) Physiol. Plant. 112, 531–539.
29. Muramatsu, M., and Hihara, Y. (2003) Plant Cell 15, 446–453.
30. Laulhere, J. P., Laboure, A. M., Van Wuytswinkel, O., Gagnon, J., and Briat, J. F. (1992) Biochem. J. 281, 785–793.
31. Jurgens, U. J., and Weckesser, I. (1986) J. Bacteriol. 168, 568–573.
32. Kaklkon, O., and Cahn, J. Z. (2002) Free Radic. Biol. Med. 33, 1037–1046.
33. Cornejo, J., Willows, R. D., and Beale, S. I. (1998) Plant J. 15, 99–107.
34. Sugishima, M., Hagiwara, Y., Zhang, X., Yoshida, T., Migita, C. T., and Fukuyama, K. (2005) Biochemistry 44, 4257–4266.
35. Sugishima, M., Migita, C. T., Zhang, X., Yoshida, T., and Fukuyama, K. (2004) FEBS J. 272, 1012–1022.
36. Sugishima, M., Migita, C. T., Zhang, X., Yoshida, T., and Fukuyama, K. (2003) FEBS Lett. 537, 91–95.
37. Di Mascio, P., Kaiser, S. P., Devasagayam, T. P., and Sies, H. (1991) Adv. Exp. Med. Biol. 283, 71–77.
38. Di Mascio, P., Devasagayam, T. P., Kaiser, S., and Sies, H. (1990) Biochem. Soc. Trans. 18, 1054–1056.
39. Cozzi, A., Santambrogio, P., Levi, S., and Arosio, P. (1990) FEBS Lett. 277, 119–122.
40. Wai, S. N., Nakayama, K., Umene, K., Moriya, T., and Amako, K. (1996) Mol. Microbiol. 20, 1127–1134.
41. Keren, N., Aurora, R., and Pakrasi, H. B. (2004) Plant Physiol. 135, 1666–1673.
42. Abdul-Tehrani, H., Hudson, A. J., Chang, Y. S., Timms, A. R., Hawkins, C., Williams, J. M., Harrison, P. M., Guest, J. R., and Andrews, S. C. (1999) J. Mol. Microbiol. 20, 1127–1134.
43. Keren, N., Aurora, R., and Pakrasi, H. B. (2004) Plant Physiol. 135, 1666–1673.
44. Abdul-Tehrani, H., Hudson, A. J., Chang, Y. S., Timms, A. R., Hawkins, C., Williams, J. M., Harrison, P. M., Guest, J. R., and Andrews, S. C. (1999) J.

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