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A unique ferredoxin acts as a player in the low-iron response of photosynthetic organisms

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Iron chronically limits aquatic photosynthesis, especially in marine environments, and the correct perception and maintenance of iron homeostasis in photosynthetic bacteria, including cyanobacteria, is therefore of global significance. Multiple adaptive mechanisms, responsive promoters, and posttranscriptional regulators have been identified, which allow cyanobacteria to respond to changing iron concentrations. However, many factors remain unclear, in particular, how iron status is perceived within the cell. Here we describe a cyanobacterial ferredoxin (Fed2), with a unique C-terminal extension, that acts as a player in iron perception. Fed2 homologs are highly conserved in photosynthetic organisms from cyanobacteria to higher plants, and, although they belong to the plant type ferredoxin family of [2Fe-2S] photosynthetic electron carriers, they are not involved in photosynthetic electron transport. As deletion of fed2 appears lethal, we developed a C-terminal truncation system to attenuate protein function. Disturbed Fed2 function resulted in decreased chlorophyll accumulation, and this was exaggerated in iron-depleted medium, where different truncations led to either exaggerated or weaker responses to low iron. Despite this, iron concentrations remained the same, or were elevated in all truncation mutants. Further analysis established that, when Fed2 function was perturbed, the classical iron limitation marker IsiA failed to accumulate at transcript and protein levels. By contrast, abundance of IsiB, which shares an operon with isiA, was unaffected by loss of Fed2 function, pointing the site of Fed2 action in iron perception to the level of posttranscriptional regulation.

Iron limits the growth of photosynthetic organisms, especially in marine environments. Understanding the response of photosynthetic organisms to changing iron concentrations is therefore important for agriculture and biotechnology. We have identified a protein that is essential for the correct response to changing iron concentrations in photosynthetic bacteria (cyanobacteria). This protein was previously annotated as an electron transfer component of photosynthesis, called Fed2, and contains an iron–sulfur cluster. We tested Fed2, and found that it cannot act in photosynthetic electron transport. The corresponding gene is essential, and is highly conserved between cyanobacteria, algae, and higher plants. By specifically perturbing its function, we could show that it is essential for the low-iron response at the posttranscriptional level.

Significance

Iron limits the growth of photosynthetic organisms, especially in marine environments. Understanding the response of photosynthetic organisms to changing iron concentrations is therefore important for agriculture and biotechnology. We have identified a protein that is essential for the correct response to changing iron concentrations in photosynthetic bacteria (cyanobacteria). This protein was previously annotated as an electron transfer component of photosynthesis, called Fed2, and contains an iron–sulfur cluster. We tested Fed2, and found that it cannot act in photosynthetic electron transport. The corresponding gene is essential, and is highly conserved between cyanobacteria, algae, and higher plants. By specifically perturbing its function, we could show that it is essential for the low-iron response at the posttranscriptional level.

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While the photosynthetic Fd (petF) gene is repressed in response to iron limitation, most cyanobacteria also contain at least three other genes encoding [2Fe-2S] Fds (22). These include a specific heterotrophic FdxH involved in heterocyst or dark metabolism, and one to two Fds with extended C-termini. Homologs of these Fd proteins with C-terminal extensions have been named FdC1 (Fd with C-terminal extension) and FdC2 in higher plants (Fig. 1A). In the model cyanobacterium Synechocystis sp. PCC 6803 (hereafter called Synechocystis), the closest homolog of higher plant FdC2 has been named Fed2 (sll1382), and has a 22-amino acid C-terminal extension relative to PetF (22) (Fig. 1B). Due to the presence of up to 10 different genes encoding [2Fe-2S] Fds in higher plant and algae genomes (23), the nomenclature of Fed2/FdC2 homologs is highly variable, and they have variously been named Fd6 or FdC2 (AT1G32550.1) in Arabidopsis thaliana (24), FdC2 (Os03g0685000) in rice (Orzya sativa) (25, 26), and Fdx6 (ABC88605.1) in the alga Chlamydomonas reinhardtii (27).

A Fed2/FdC2 homolog seems to be present in all photosynthetic organisms (23), and, intriguingly, it was found to be essential in cyanobacteria (22). While no transposable element insertion (T-DNA) knockout has been identified, to date, in higher plants, a single amino acid substitution results in slower growth and a pale green leaf phenotype in rice (25, 26). Although Synechocystis, expression of fed2 is up-regulated in response to oxidative and heavy metal stress (22), and expression of the homologous Fdx6 in Chlamydomonas is increased in low iron (27), its biological function remains unknown. In a previous study on recombinant, His-tagged Arabidopsis Fdfs/FdC2, Kolton et al. (28) report that the protein is capable of electron transport between PSI and NADPH, and can be reduced by Fd:NADP(H) oxidoreductase (FNR), although the affinity is very low between FdC2 and FNR in both assays. Moreover, although the protein was detected bound to thylakoid membranes and mRNA, no specific functional role for the protein was identified. In this study, we aimed to understand the physiological role of Fed2 by disrupting its function in Synechocystis. Our data indicate that Fed2 is a critical component in cyanobacterial iron perception and/or homeostasis.

**Results**

Fed2 Shows Unique Structural Features and Lacks Activity with FNR. Fed2/FdC2 proteins are highly conserved (Fig. 1A) and distant from the photosynthetic PetF proteins, but present through the “green cut” of cyanobacterial genes with homologs conserved through algae to higher plants (29). The characteristically extended C terminus is well conserved, usually beginning with a Gly–Phe–Gly–Arg/Lys–Tyr–Phe motif, and ending with several acidic residues at the C terminus (Fig. 1B). These 22 to 36 extra residues at the C terminus are significant, as it is known that the C-terminal region of PetF (which lacks them) plays a critical role in interaction with PSI during PetF reduction (30, 31). Following cloning into a protein expression vector, the [2Fe-2S] cluster of purified Synechocystis Fed2 proved to be unstable, and we therefore purified recombinantly expressed Fed2 from the thermophilic cyanobacterium Thermosynechococcus elongatus BP-1 to investigate protein function. Remarkably for an Fd, the purified T. elongatus Fed2 showed migration over a size exclusion column as an oligomer, part of which forms a stable dimer during SDS/PAGE (Fig. 2A), with purity and band identity confirmed by mass spectrometry (SI Appendix, Table S1). The protein showed a characteristic [2Fe-2S] cluster UV–visible (UV-VIS) spectrum (Fig. 2B), although it had very poor ability to transfer electrons with the classical FNR compared with cyanobacterial PetF (Fig. 2C). Our data contrast with the findings of Kolton et al. (28), who measured reasonable electron transport with FNR by the higher plant homolog, using a His-tagged higher plant (A. thaliana) FdC2. This could relate to species differences, or the noncleaved His-tag used in the study by Kolton et al., where the protein also showed an atypical UV-VIS spectrum for a [2Fe-2S] cluster. To further establish whether Fed2 could function in photosynthetic electron transport, we measured the redox midpoint potential of the recombinant protein. The spectra in SI Appendix, Fig. S2 indicate a midpoint potential of −465 ± 3 mV vs. Ag/AgCl/KCl (3 M). Corrected for normal hydrogen electrode (NHE) reference, this gives a value of −243 ± 3 mV. This is much more positive than the values of −412, −320, and −320 mV measured for Synechocystis PetF (32), cyanobacterial FNR (33), and NADP(H), respectively, making electron donation from cyanobacterial Fed2 to FNR and then NADP⁺ energetically unfavorable. These factors, in combination with the very low protein content reported for the algal homolog (27), indicate that it is highly unlikely that Fed2/FdC2 proteins function directly in photosynthetic electron transport or are reduced by FNR. Moreover, this redox potential also indicates that electron donation from Fed2/FdC2 proteins to many other classical Fd-dependent enzymes, such as nitrite reductase (34) or glutamate synthase (35), is also energetically less favorable.

Fed2 Is Essential in Synechocystis. To identify the physiological role of Fed2, we aimed to knock out the gene in Synechocystis. fed2 (SII1382) forms a transcriptional unit with suhB (inositol 1,2,3-triphosphatase), sll111382 (34), and sll131381 (35), indicating that it is highly unlikely that Fed2/FdC2 proteins are involved in the classical Fd/NADP(H) oxidoreductase. Remarkably for an Fd, the Synechocystis homolog of higher plant FdC2 has been named Fed2 (sll1382), and is part of the same operon in various other cyanobacteria (22). This study, we chose to insert all selectable markers downstream of fed2, as only 71 bp separate the first codon of the fed2 gene and the first nucleotide of a unique RNA-Ser gene on the opposite strand, whose expression we considered it critical not to disturb. Moreover, we hypothesize that any impact on suhB expression will make a small contribution to the phenotype, because its knockout in Synechococcus only results in a mild phenotype related to osmotic stress (37). In contrast, disruption of the fed2 gene by insertion of a kanamycin resistance cassette in Synechocystis failed to yield a fully segregating strain (SI Appendix, Fig. S3A) even after restreaking cells multiple times in the presence of glucose, suggesting that Fed2 is essential.

We next tried to knock down fed2 using two independent inducible expression systems (Fig. 3B): firstly, with an inducible 3′ promoter running antisense to fed2 (fed2i′) and, secondly, by replacing an inducible promoter for the fed2 gene (fed2i). As fed2i′ transcript abundance responds to some metals (22), we avoided the classical Cu-inducible system (38) and used the nitrate-inducible nra promoter from Synechococcus (39). These strains readily segregated (SI Appendix, Fig. S3B) in nitrogen regimes that facilitate fed2 expression (ammonia for antisense, nitrate for sense). On transfer to the alternative N source (nitrate for fed2a, ammonia for fed2i), to repress transcript abundance, no changes in Fed2 protein were observed in fed2a lines, but fed2i lines showed a large decrease in Fed2 abundance (Fig. 3C). Surprisingly, the fed2i strain showed no significant changes in cell growth or chlorophyll a content in these conditions (Fig. 3 D and E), indicating that even very low abundance of Fed2 is sufficient for regular cyanobacterial growth.

Attenuated Fed2 Function Results in a Growth Penalty. Rather than decreasing protein abundance, we then sought to disrupt, but not eliminate, Fed2 function by mutation. The most obvious conserved feature of Fed2 proteins is the C-terminus region (Fig. 1A), so we performed a sequential truncation, introducing stop codons to remove amino acids from the C terminus in groups of three (Fig. 4). Truncations of up to 18 amino acids (fed2T18) readily segregated to full mutation (SI Appendix, Fig. S3C), while deletions of more than 21 amino acids (fed2T21 and fed2T24) only segregated after extensive rounds of screening were performed (SI Appendix, Fig. S3 C and D). To compare phenotypes to the fed2i line, we also performed experiments on the truncation lines in
Iron Import Is Not Disturbed in Fed2 Truncation Lines. A major pathway of iron acquisition by *Synechocystis* relies on reduction of both ammonium and nitrate growth media. Western blot analysis detected much less Fed2 protein in both the *fed2T18* and *fed2T24* lines, similar to that seen in the *fed2i* knockdown line (Fig. 4B). Because expression of *fed2T18* and *fed2T24* is driven by the native promoter in these lines, the decreased Fed2 protein content is probably due to structural destabilization caused by truncation, leading to faster protein turnover. In contrast to *fed2i*, however, the truncation mutants both showed decreased growth on agar (Fig. 4C). This phenotype is almost certainly due to perturbed Fed2 function, rather than disruption of other genomic elements or compensatory mutation, as it is seen in the two independent truncation lines (one of which segregated rapidly), and not observed in *fed2a*, where the kanamycin resistance gene is inserted in an identical position in the genome. For *fed2T24*, this translated into significantly slower growth in liquid media (Fig. 4D). Interestingly, we also observed significantly decreased chlorophyll $a$ content relative to cell density for the truncation lines in both nitrate and ammonium liquid growth conditions (Fig. 4D), while the *fed2i* line did not differ significantly from the WT in ammonium growth conditions (Fig. 4E).

Response to Low Iron Is Disrupted in *fed2* Truncation Lines. To investigate how decreased chlorophyll $a$ content impacted the photosynthetic apparatus, we monitored the 77 K emission spectra (Fig. 4E), where emission peaks at 685 and 695 nm correspond to PSI reaction centers, and the peak at 720 nm corresponds to PSII reaction centers. *Fed2T24* cells grown in nitrate medium demonstrated that, after growth in liquid medium lacking iron, *O. sativa* displayed a much smaller increase in 685-nm fluorescence, and the *fed2i* only deviated very slightly from the WT spectrum. A relative increase at 685 nm, such as that seen in the *fed2T24* line in nitrate growth conditions, has previously been ascribed to the IsA antenna and is typically associated with iron depletion in cyanobacteria (12, 16).

Since previous studies detected increased *fed2* expression in response to high cadmium and zinc (22) and expression of *Fd6*, the Fed2/Fd2C homolog in *Chlamydomonas*, is up-regulated in response to low iron (27), this result prompted us to examine the sensitivity of the truncation mutants to altered metal contents (*SI Appendix*, Fig. S4), with a particular focus on iron (Fig. 5). Although increased growth of the truncation mutants at increased iron concentrations is still perturbed relative to the WT, use of media with depleted iron results in similar growth of all genotypes (Fig. 5A). Following transfer of cells to depleted iron liquid media, cell proliferation is the same for WT and the *fed2T24* line, but total chlorophyll concentrations are severely affected in the truncation line (Fig. 5B). The 77 K fluorescence spectra shown in Fig. 5C demonstrate that, after growth in liquid medium lacking iron, the fluorescence peaks of PSIII increase relative to PSI in both genotypes. However, this is much more dramatic in the *fed2T24* line than in WT, irrespective of the N source. This contrasts to the 77 K phenotype of *fed2T24* under iron-replete conditions, which is only seen in nitrate media. Although growth on solid media with elevated copper, cobalt, and zinc also eliminated the difference in growth between the WT and truncation mutants (*SI Appendix*, Fig. S4), only low iron induced the dramatic increase in PSI relative to PSII fluorescence. After identifying this phenotype in *fed2T24*, we subjected *fed2T18* to iron-depleted media and measured 77 K spectra (Fig. 5D). Unexpectedly, *fed2T18* showed a trend opposite to that of *fed2T24*. Instead of an exaggerated increase in PSI/PSII fluorescence, this genotype is less responsive than the WT. Thus, a difference of just six amino acids at the C terminus of Fed2 results in opposite responses at the level of PSI to PSII ratio.
of ferric iron (Fe$^{3+}$) to ferrous iron (Fe$^{2+}$) during import (40). Although some of the components of this machinery are identified (40–43), the original source of these electrons remains

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**Fig. 2.** Unusual properties of the recombinant purified Fed2 protein. (A) Coomassie-stained SDS/PAGE gel showing two bands in eluting fractions from size exclusion chromatography in the final step of recombinant T. elongatus Fed2 (Tfed2) purification. (B) Comparison of UV-Vis spectra of recombinant purified PetF (green), cloned from Synechocystis sp.PCC 6803, and TeFed2 (blue). Spectra measured at 0.1 mM concentration. (C) Electron transport between FNR and Fds. Activity was measured in cytochrome c coupled assay with 20 nM FNR over a range of concentrations of Synechocystis PetF (green circles) and TeFed2 (blue squares).

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**Fig. 3.** Genomic environment and disruption of fed2 in Synechocystis. (A) Genomic context of fed2 in Synechocystis. The sll1382 (fed2) and sll1383 (suhB) form a single transcriptional unit (TU1). In the region 5′ from fed2 there are two more transcriptional units. One encodes a unique t-RNA-Ser (t20) and the other a predicted signal recognition particle (SRP) in combination with slr1471, a protein of unknown function. (B) Strategies for fed2 knockdown and knockout. Arrows show direction of promoter action; HR, homologous region taken for cloning; KmR, kanamycin resistance; NP, fed2 native promoter; NirA, nitrate responsive promoter. From top to bottom: WT genomic arrangement, knockout insertion of KmR, inducible knockdown by replacing the native promoter with NirA, and inducible antisense by introduction of the NirA promoter 3′ of fed2 in the anticoding direction. (C) Protein abundance of Fed2 following transfer of single colony derived cultures of inducible antisense and inducible expression lines between inducing and noninducing media, detected by Western blotting for Fed2 (see SI Appendix, Fig. S3 for segregation of strains). For nitrate to ammonium transfer, Top is Fed2 and Bottom is a loading control of a nonspecific band on the same blot. (D) Growth and (E) chlorophyll content of Synechocystis WT (black) and fed2i (orange) cells following transfer to ammonium media (suppression of fed2 expression in fed2i line). Chlorophyll a content is expressed as a function of the cell density. Values are mean ± SD of at least three independent measurements. Data from ref. 36.
unclear (44). We initially hypothesized that Fed2 might be involved in providing this reducing power to the plasma membrane, and we therefore measured iron concentrations in WT
and truncation mutants of Fed2 by inductively coupled plasma mass spectrometry (ICP-MS). Cellular iron contents were compared for iron-replete and iron-deprived cells (Fig. 6). Significant amounts of unimported iron may be stored in the Synechocystis periplasm (41), so we also measured iron contents of EDTA-washed cells (pale bars, Fig. 6). Interestingly, despite the apparent iron stress of fed2T24 under normal growth conditions indicated by the 77 K spectrum (Fig. 4E) and the disturbed PSI/PSII emission ratios in 77 K spectra of both mutants following iron depletion (Fig. 5), neither line showed decreased cellular iron relative to WT. In fact, contents of both total cellular iron and intracellular iron (EDTA-washed cells) increased relative to the WT (up to 1.8 times higher, depending on the growth conditions).

Iron Is Not Correctly Perceived on Fed2 Truncation. The contradiction between the photosystem response to decreased iron (Fig. 5) and actual cellular iron content (Fig. 6) prompted us to investigate whether other cellular responses to low iron were activated in the Fed2 truncation lines. We therefore checked the abundance of classical iron depletion marker proteins in cells grown with either iron-replete or iron-limited media (Fig. 7A). As this phenotype is seen in both N conditions, we limited the experiment to nitrate growth only. While iron limitation leads to a decrease in the PSI protein PsaD, this is less pronounced in the fed2T18 line, consistent with its greater PSI fluorescence than the WT, under these conditions (Fig. 5D). The change in PSI acceptors, with decreased Fd (PetF) and increased Fld (IsiB), occurs in all genotypes, although the increase in Fld levels is more pronounced in fed2T24 than in WT or fed2T18. Dramatically, abundance of IsiA, the classical marker for iron depletion, does not increase in either Fed2 truncation line after transfer to low-iron conditions. This is especially striking, as isiA and isiB are thought to form a single transcriptional unit, with the promoter region upstream of isiA (45). Our data indicate that processing and translation of this cotranscript is more complex than previously believed. Although it was reported that Synechocystis fed2 transcripts do not respond to iron, we examined Fed2 protein contents over time in the WT following iron depletion (Fig. 7B). Increased abundance of Fed2 is visible 10 h after iron depletion, and, by 72 h, a new band at the same predicted migration size as a Fed2 dimer (~30 kDa) appears. This is of interest in the context of the Fed2 dimer obtained on purification of the recombinant protein (Fig. 24).

As Fed2 appears critical for IsiA accumulation, but does not negatively impact IsiB, we investigated whether misregulation occurs at the protein or transcript level, by detecting isiA transcript abundance following the same treatment (Fig. 7C). In the WT, 24 h of iron limitation is sufficient for a large induction of isiA transcript expression. However, neither truncation mutant shows a significant response, although a small amount of transcript is visible, especially in fed2T24.

As the iron depletion response is disrupted on truncation of Fed2, we checked whether this was also true when Fed2 contents are decreased in the fed2T line (SI Appendix, Fig. S5). While growth rate was not affected, the chlorophyll content was slightly decreased in low iron (SI Appendix, Fig. S5A), and the 685-nm peak in 77 K responded more than in the WT (SI Appendix, Fig. S5B). This mirrors the response in the fed2T24 line but is less severe. These observations were confirmed by Western blotting, where fed2T18 showed an intermediate increase in IsiA contents between WT and fed2T18 (SI Appendix, Fig. S5E). These effects were most severe in ammonium media, but also seen in nitrate media. In the fed2T line, fed2 expression is driven by the nirA promoter, which can be additionally down-regulated in iron starvation (46, 47), explaining the decreased Fed2 content in the fed2T18 line (SI Appendix, Fig. S5C), and the corresponding phenotypic changes in chlorophyll concentration (SI Appendix, Fig. S5A), 77 K fluorescence (SI Appendix, Fig. S5B), and protein response (SI Appendix, Fig. S5C). It is also clear from the corresponding Northern blots that, although isiA induction is perturbed in the fed2T24 line (SI Appendix, Fig. S5D), this is to a lesser extent than in the truncation lines (Fig. 7C).

Disrupted Thylakoid Structure in Fed2 Truncation Lines. It was previously reported that thylakoid structure was disrupted in mutants of isiA (16), and we therefore checked whether this feature was consistent with the Fed2 truncation lines, in which IsiA does not accumulate. Transmission electron microscopy (TEM) was performed on Synechocystis WT, fed2T18, and fed2T24 lines that had been grown in either iron-replete or iron-deprived conditions (Fig. 8). Greater numbers of cells at lower magnification are shown in SI Appendix, Fig. S6. Under iron-deprived conditions, all genotypes show large numbers of electron-dense particles in the cytosol, principally between the thylakoids. Interestingly, the fed2T24 line also shows some accumulation of these structures under iron-replete conditions. Even in iron-replete conditions, cells of the fed2T24 line appear stressed, with large inclusion bodies present in the cytosol of most cells. On iron depletion, a significant proportion of fed2T24 cells appear to have decreased abundance of high-density cytosolic particles relative to the other genotypes. Some of these cells contain very little internal membrane, with the remainder highly disorganized. This is not a uniform phenotype but is present in a large percentage of the cells (~21% for fed2T24), some of which are clearly dividing (SI Appendix, Fig. S6). If these cells were dead, a decreased growth rate of fed2T24 on low iron would be expected. However, cellular growth is very similar to the WT (Fig. 5), indicating that these cells are alive but lack significant thylakoid structures. Nonviable cells are easily identified by their
green fluorescence following chlorophyll excitation (48), so we compared the genotypes following iron depletion using confocal microscopy (SI Appendix, Fig. S7). As seen in SI Appendix, Fig. S7B, the T18 and T24 lines have a similar, or even a lower, frequency of nonviable cells compared with the WT, indicating that the low membrane density T24 cells are viable. The fed2T18 line shows the same phenotype under iron deprivation, but, in this case, a smaller proportion of the cells (around 8%) are affected (SI Appendix, Fig. S6B). Both lines are completely segregated (SI Appendix, Fig. S3), so the binary nature of this phenotype (either WT-like or lacking organized thylakoids) must reflect either induction or absence of a specific developmental response, to which cells with perturbed Fed2 function are more prone.

Discussion
In this article, we show that Fed2, an Fd with a uniquely extended C terminal, which is conserved throughout photosynthetic organisms (Fig. 1), is involved in the response to low iron in cyanobacteria. Following iron depletion, the Synechocystis lines with perturbed Fed2 function, fed2T18 and fed2T24, failed to induce isiA transcript (Fig. 7C) and protein accumulation (Fig. 7A), demonstrating that a functional Fed2 is critical for the cyanobacterial response to iron depletion. IsiA forms an operon with isiB (36), but, under the same conditions, the isiB protein accumulated to similar or even higher levels in fed2i, fed2T18, and fed2T24 than in WT (Fig. 7A and SI Appendix, Fig. S5C), and was therefore independent of Fed2 function. When we additionally probed for the isiB transcript by Northern blotting (SI Appendix, Fig. S5D), we confirmed the original finding that it is predominantly present as a cotranscript with isiA (49). Our findings indicate that isiB can be translated before other regulatory processes prevent translation of IsiA. This indicates that Fed2 might impact at the level of RNA stability rather than transcriptional processes. IsiA transcript abundance is known to be regulated by the small antisense RNA IsrR, forming a double-stranded RNA that is targeted for destruction (20). The latest model proposes that IsrR expression is constant and prevents short-term iron fluctuations from causing expensive induction of isiA, driven by the iron-sensing transcriptional regulator FurA (50). Our data show that a functional Fed2 also plays a critical role in accumulating isiA transcript. A previous study demonstrated that recombinant higher plant FdC2 protein is capable of binding chloroplast transcripts (28), although it is still unclear whether this interaction is part of any significant regulatory process.

In Synechocystis, Fed2 is critical for the up-regulation of IsiA, and the phenotype of the truncation mutants resembles that of isiA mutants under low-iron conditions (16), with a similar growth rate to WT, but low chlorophyll content. However, in iron-replete conditions, the isiA mutant is not distinguishable from the WT, whereas the failure of fed2 insertion mutants to
iron response, such as an enhanced accumulation of IsiB relative to the WT (Fig. 7A), the difference between the two truncation lines likely reflects changes in the speed or amplitude of the low-iron response at the photosystem level. This corresponds to a faster response than WT in the fed2T24 and fed2i lines, but a slower response than WT in fed2T18.

We initially conducted our growth experiments on two separate N sources, due to the use of the nitrate-inducible promoter in fed2i line (Fig. 3), and noted that, for fed2T24, the 685-nm emission phenotype is more severe when cyanobacteria are grown on nitrate than on ammonia media. It may be that perception of iron deprivation is more sensitive under nitrate growth conditions, as iron deprivation regulates expression of several genes involved specifically in nitrate assimilation (46, 47, 52). The typical induction of IsiA is also attenuated on low N (53), although differences between separate nitrogen sources were not investigated until now. In addition, two more enzymes are required for assimilation of nitrate than for assimilation of ammonium, nitrate reductase and nitrite reductase, both of which possess essential iron-containing cofactors.

There is a highly complex network of factors involved in regulating the response of cyanobacteria to low iron. These include multiple players at the level of posttranscriptional regulation, with 10 small RNAs and 62 antisense RNAs identified so far (52, 54). Our data show that Fed2 is a critical player in specific parts of this response. It will be interesting to see whether the regulatory role of Fed2 is conserved in higher plants, and, although Fed2/FdC2 appears to be the only C-terminal type Fd that is almost uniformly conserved (Fig. 1), some species contain multiple Fds with extended C termini (27) that could potentially also act as posttranslational regulators. Future studies should aim to identify the interaction partners and functional mechanism of Fed2/FdC2 proteins, which may be related to dimerization (Figs. 2A and 7B), as has been demonstrated for other iron- and redox-sensing regulators (55–57).

Materials and Methods

Strains and Culture Conditions. A glucose-tolerant Synechocystis strain sp. PCC 6803 (58) obtained from N. Murata, National Institute for Basic Biology, Okazaki, Japan, was used as WT in this work. Cells were grown photoautotrophically on BG11, NH4+ as nitrogen source) or BG11, NO3− as nitrogen source) medium (59) at 30 °C under continuous illumination of 50 μEin−2 sec−1. For plate cultures, media was supplemented with 1% (wt/vol) agar. If not otherwise indicated, kanamycin was added to a final concentration of 50 μg·mL−1. Experiments were performed using cultures from the midlogarithmic phase (2–3 μg chlorophyll per milliliter) in BG11_NH or BG11_NO medium supplemented with the indicated amounts of CuSO4·5H2O, CoCl2, ZnCl2, and ferric ammonium citrate. Escherichia coli D18 cells were grown in LB medium and supplemented with 200 μg·mL−1 of ampicillin or 12.5 μg·mL−1 of kanamycin when required.

Mutagenesis of Synechocystis Genes. Primers used in this work are listed in SI Appendix, Table S2. If not otherwise mentioned, all restriction enzymes were purchased from Thermo Scientific. Gene disruption of fed2 was achieved by PCR amplification of a 1,293-bp fragment from the genomic DNA of Synechocystis using primers P1 and P2 (SI Appendix, Table S2). The fragment containing the fed2 gene (369 bp) and additional homologous regions (492 bp at the 5′ end and 432 bp at the 3′ end) was gel-purified and ligated into the multiple cloning site of a pET12.blunt vector using the CloneJET PCR Cloning Kit (Thermo Scientific), resulting in the formation of pfed2KO (Fig. 3), which was finally transformed into WT Synechocystis. For the ntrA responsive promoter, a 166-bp fragment upstream of the nirA operon from Synechococcus sp. strain PCC 7942 was synthesized (Eurofins Genomics) with addition of Sacl and AgeI restriction sites flanking the 5′ and 3′ ends, respectively. The pxe-A2 vector harboring the nirA promoter was digested with KpnI and treated with blunt end enzyme, followed by ligation with a kanamycin resistance cassette derived from a pUK-4K vector cut with HincII, resulting in the formation of pxe-A2, NK.
In a parallel experiment, multiple cloning sites containing Stul and AgeI restriction sites were added at the 3' (in front of the start codon) or the 5' end (behind the translation stop codon) of the fragment sequence via site-directed mutagenesis of pET, FDC2 using primers P3 and P4 or P5 and P6 (SI Appendix, Table S2), respectively (QS Site-Directed Mutagenesis Kit; New England Biolabs). The resulting vectors pfdC2-StAge and pfdC2-StAge2 were digested with AgeI and Stul and ligated with the expression cassette containing the nira promoter and the kanamycin selection marker derived from pex-A2. N2K2 cut with the same enzymes, generating pfd21 and pfd2a (Fig. 3B), which were finally transformed into WT Synechocystis. Stepwise truncation of Fed2 was obtained by sequential site-directed mutagenesis introducing stop codons to shorten the C terminus at specific positions on the vector pfd2a with primer P7 and one of the primers P8 to P15 (SI Appendix, Table S2), resulting in the formation of plasmids pfd2trunc1 to pfd2trunc24 (Fig. 4A).

**Protein Analysis.** T. elongatus fed2 was cloned from genomic DNA (kind gift of Yuichi Fujita, Nagoya University, Nagoya, Japan) into the 6XHis-tag expression vector pT3871 by in-fusion cloning, followed by purification on Talon resin (all from Clontech Laboratories). This was followed by precipitation in 25% saturation (NH4)2SO4. The His-tag was cleaved with enterokinase and removed by passing through Talon resin, followed by size-exclusion chromatography (Superose 12 10/300 preparative). Anti-cyanobacterial Fed2 was generated by injection of this protein into a rabbit (Pineda Antikörper Service). Synechocystis petF was cloned into pTRC99A, then expressed and purified basically as described previously (24).

Proteins were detected by SDS/PAGE followed by Western blot analysis with detection by alkaline phosphatase reaction. The cells were broken in 10 consecutive cycles of vortexing in the presence of 50 mM Tris Buffer (pH 7.7) and 250 μL of glass beads (0.25- to 0.3-mm diameter) for 1 min at 4 °C, each cycle followed by 30 s resting at 4 °C. Cell debris was removed by centrifugation, and the supernatant was recovered. Primary antibodies were 1:40,000 dilutions of antiserum against Fed2, maize Fd1 (PetF), PsbA, and PsbD (both raised against synthetic peptides; Agrisera), Synechocystis CP43′ (IsiA), and Synechocystis Fid (IsiB, kind gift of Néstor Carrillo, Rosario University, Rosario, Argentina). Specificity of the anti-Fed2 antiserum was confirmed by comparing detection of Fed2 with 100 times more PetF, the most significant band. Redox Potentiometry. The spectroelectrochemical measurements were carried out with a “honeycomb” gold working electrode, gold counter electrode integrated on the same card (AKSTCKIT3; PINE Instruments), using a Ag/AgCl/KCl 3 M reference electrode. All solutions were in 100 mM Tris-buffered with 0.1 M phosphate. Sample staining of cells embedded in low-melting agarose was performed for 3.5 h with 1% OsO4, followed by consecutive dehydration of the sample in serial ethanol dilutions: 5 min in 30%, 5 min in 50%, 10 min in 70%, 10 min in 80%, and 10 min in 95% ethanol. Final dehydration was obtained by sample treatment in 95% and 100% ethanol, each for 15 min. For infiltration, the sample was transferred to a 1.3-mixture of propylene oxide and ethanol for 10 min, followed by two incubation steps in propylene oxide for 5 min and one final infiltration step with a 1.3-mixture of propylene oxide and Epon [1:1 mixture A/B plus 1.5% 2,2-dimethoxypropane (DMP)] 30. For embedding, the sample was washed three times with Epon (1:1 mixture A/B plus 1.5% DMP) followed by polymerization at 65 °C for 72 h. The sample was sectioned at the Ultracrome (Ultratome UCT, Leica) and stained for 30 min with 2% uranyl acetate and then for 20 min with lead citrate. The TEM images were recorded with the EM 902 from Zeiss.

Electron Microscopy. Synechocystis cells were fixed with 2% glutaraldehyde buffered with 0.1 M phosphate. Sample staining of cells embedded in low-melting agarose was performed for 3.5 h with 1% OsO4, followed by consecutive dehydration of the sample in serial ethanol dilutions: 5 min in 30%, 5 min in 50%, 10 min in 70%, 10 min in 80%, and 10 min in 95% ethanol. Final dehydration was obtained by sample treatment in 95% and 100% ethanol, each for 15 min. For infiltration, the sample was transferred to a 1.3-mixture of propylene oxide and ethanol for 10 min, followed by two incubation steps in propylene oxide for 5 min and one final infiltration step with a 1.3-mixture of propylene oxide and Epon [1:1 mixture A/B plus 1.5% 2,2-dimethoxypropane (DMP)] 30. For embedding, the sample was washed three times with Epon (1:1 mixture A/B plus 1.5% DMP) followed by polymerization at 65 °C for 72 h. The sample was sectioned at the Ultracrome (Ultratome UCT, Leica) and stained for 30 min with 2% uranyl acetate and then for 20 min with lead citrate. The TEM images were recorded with the EM 902 from Zeiss.

ICP-MS. Cells were collected at midlogarithmic phase before rapidly washing two times in either chelex-treated water or 5 mM EDTA. Cells were then suspended in 200 μL of chelex-treated H2O and mixed with 800 μL of 65% Suprapur HNO3 (Merck Millipore) to digest, before dilution (1 in 5) with 2.5% Suprapur HNO3 for aqueous analysis of metal content was determined using an XSERIES-2 ICP mass spectrometer (Thermo Fisher Scientific) following calibration with elemental standards that were matrix-matched to the sample. Internal standards (beryllium, silver, and indium) were used to correct for any variations in analytical performance. Mean and SD values determined from three biological replicates.

**Northern Blot Analysis.** Cells grown under iron-replete conditions (time point 0) were washed two times with iron-free medium and further grown under iron depletion for 24 and 48 h (time points 24 and 48). RNA extraction, blotting, and hybridization were performed as described previously (61), and RNA was hybridized to a radioactively labeled isiA DNA probe (including the 5′ UTR region) using the Rediprime II DNA labeling system (GE Healthcare Life Sciences). As a control, the same blot was hybridized with a riboprobe against the mpfRNA using the 17 polymerase Maxscript kit (Ambion). Primers used to generate the isiA (P16 and P17), mpfRNA (P20 and P21), and isiB (P20 and P21) probes are given in SI Appendix, Table S2.

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1. Lyons TW, Reinhard CT, Planavsky NJ (2014) The rise of oxygen in Earth’s early ocean and atmosphere. Nature 506:307-315.
2. Norman L, Cabanesa DJ, Blanco-Ameijeiras S, Moisset SA, Hassler CS (2014) Iron bioavailability in the Mediterranean Sea: An ecotoxicological perspective. Front Ecol Evol 2:193.
3. Fraser JM, et al. (2013) Photophysiological and photosynthetic complex changes during iron starvation in Synechocystis sp. PCC 6803 and Synechococcus elongatus PCC 7942. PLoS One 8:e59861.
4. Kehrer JP (2000) The Haber-Weiss reaction and mechanisms of toxicity. Toxicology 149:43-50.
5. Bibby TS, Nield J, Barber J (2001) Iron deficiency induces the formation of an antenna complex in a cyanobacterium. Nature 412:743-745.
13. van der Weij-de Wit CD, et al. (2007) Fluorescence quenching of isiA in early stage of iron deficiency and at cryogenic temperatures. Biochim Biophys Acta 1767:1393–1400.

14. Sun J, Golbeck JH (2015) The presence of the isiA-PSI supercomplex leads to enhanced photosystem I electron throughput in iron-starved cells of Synechocystis sp. PCC 7942. J Biol Chem 290:14080–14088.

15. Kranzler C, Lis H, Shaked Y, Keren N (2011) The role of reduction in iron uptake processes in a unicellular, planktonic cyanobacterium. Environ Microbiol 13: 2990–2999.

16. Badarau A, et al. (2008) FutA2 is a ferric binding protein from Synechocystis PCC 6803. J Biol Chem 283:12520–12527.

17. Bradley RW, Bombelli P, Lea-Smith DJ, Hove CJ (2013) Terminal oxidase mutants of the cyanobacterium Synechocystis sp. PCC 6803 show increased electronic activity in biological photo-voltaic systems. Phys Chem Chem Phys 15:13611–13618.

18. Katoh H, Hagino N, Ogawa T (2001) Iron-binding activity of FutA1 subunit of an ABC-type iron transporter in the cyanobacterium Synechocystis sp. Strain PCC 6803. Plant Cell Physiol 42:823–827.

19. Thorne RJ, Schneider K, Hu H, Cameron PJ (2015) Iron reduction by the cyanobacteria Synechocystis sp. PCC 6803. Bioelectrochemistry 105:103–109.

20. Dühring U, Axmann IM, Hess WR, Wilde A (2006) An internal antisense RNA regulates iron deficiency in Synechocystis sp. PCC 6803. Mol Plant 10:143–154.

21. Burnap RL, Troyan T, Sherman LA (1993) The highly abundant chlorophyll-protein complex of iron-deficient Synechocystis sp. PCC7942 (CP43 proteins) is encoded by the isiA gene. Plant Physiol 103:895–902.

22. Andrews SC, Robinson AK, Rodriguez-Quirones F (2003) Bacterial iron homeostasis. FEMS Microbiol Rev 27:215–237.

23. González A, Bes MT, Valladares A, Peleato ML, Fillat MF (2012) FurA is the main regulator of iron homeostasis and modulates the expression of tetratypic binary bpsynthesis genes in Anabaena sp. PCC 7120. Environ Microbiol 14:3175–3187.

24. Kuniert A, Vinnemeyer J, Ehrmann D, Hagemann M (2003) Repression by Fur is not the main mechanism controlling the iron-inducible isA4B operon in the cyanobacterium Synechocystis sp. PCC 6803. FEMS Microbiol Lett 227:255–262.

25. Dühring U, Aumann IM, Hess WR, Wilde A (2000) An internal antisense RNA regulates expression of the photosynthesis gene isiA. Proc Natl Acad Sci USA 107:7054–7058.

26. Noddo A, et al. (2008) Transcript profiling reveals new insights into the acclimation of the photosynthetic fresh-water cyanobacterium Synechocystis elongatus PCC 7942 to iron starvation. Plant Physiol 147:747–763.

27. Cassier-Chauvat C, Chauvat F (2014) Function and regulation of ferredoxins in the cyanobacterium, Synechocystis PCC6803: Recent advances. Life (Basel) 4:666–680.

28. Handke G, Mulo P (2012) Plant type ferredoxins and ferredoxin-dependent metabolismo. Plant Environ Sci 36:1071–1084.

29. Kolton M, et al. (2011) Plastidic redox switches: Ferredoxins as novel RNA-binding proteins. J Endotoxins Cell Res 21:1–18.

30. Karpovich SJ, Prochnik SE, Grossman AR,Merchant SS (2011) The GreenC2t resource, a phylogenetically derived inventory of proteins specific to the plant lineage. J Biol Chem 286:21427–21439.

31. Gou P, et al. (2014) Higher order structure contributes to specific differences in redox potential of different ferredoxin transfer efficiency of root and leaf ferredoxins. Biochemistry 53:14389–14396.

32. Sancho J, Pelato ML, Gomez-Moreno C, Edmondson DE (1998) Purification and properties of ferredoxin-NAADP oxidoreductase from the nitrogen-fixing cyanobacteria Anabaena variabilis. Arch Biochem Biophys 260:200–207.

33. Hirasawa M, Tollin G, Salamon Z, Knaff DB (1994) Transient kinetic and oxidation-reduction studies of spinach ferredoxin:nitrite oxidoreductase. Biochim Biophys Acta 1185:336–345.

34. Ravasio S, et al. (2002) Properties of the recombinant ferredoxin-dependent glutamate synthase of Synechocystis PCC6803. Comparison with the Azospirillum brasilense NADPH-dependent enzyme and its isolated alpha subunit. Biochemistry 41:1820–1833.

35. Mitschke J, et al. (2011) An experimentally anchored map of transcriptional start sites in the model cyanobacterium Synechocystis sp. PCC6803. Proc Natl Acad Sci USA 108: 2124–2129.

36. Wang FK, Latifi A, Chen WL, Zhang CC (2012) The isositol monophosphatase AII927 (IMPA1) is involved in osmotic adaptation in Anabaena sp. PCC7120. Environ Microbiol Rep 4:622–632.

37. Kuchmim E, Wallner T, Kryazhov S, Zinchenko VV, Wilde A (2012) An expression system for regulated protein production in Synechocystis sp. PCC6803 and its application for construction of a conditional knockout of the ferrocobaltelase enzyme. J Biotechnol 162:75–80.

38. Schorsch et al. 201810379115

39. Maeda S, Kawaguchi Y, Ohe TA, Omata T (1998) cis-acting sequences required for nitrite-dependent, nitrite-responsive positive regulation of the nitrate assimilation operon in the cyanobacterium Synechococcus sp. strain PCC 7942. J Bacteriol 180: 4080–4088.

40. Wilson A, Boulay C, Wilde A, Kerfeld CA, Kirilovsky D (2007) Light-induced energy dissipation in iron-starved cyanobacteria: Roles of OCP and isiA proteins. Plant Cell 19: 656–672.

41. Hernandez-Prieto MA, et al. (2012) Iron deprivation in Synechocystis: Inference of pathways, non-coding RNAs, and regulatory elements from comprehensive expression profiling. G3 (Bethesda) 2:1475–1495.

42. Vinnemeyer J, Ehrmann D, Hagemann M (1998) Transcriptional analysis of the isiAB operon in salt-stressed cells of the cyanobacterium Synechocystis sp. PCC 6803. FEMS Microbiol Lett 169:323–330.

43. Legewie S, Dienst D, Wilde A, Herzel H, Aumann IM (2008) Small RNAs establish delays and temporal thresholds in gene expression. Biophys J 95:3232–3238.

44. Schulze K, Lopez DA, Ferrer CA, Kirilovsky D (2007) Light-induced energy dissipation in iron-starved cyanobacteria: Roles of OCP and isiA proteins. Plant Cell 19: 656–672.

45. Hernández-Prieto MA, et al. (2012) Iron deprivation in Synechocystis: Inference of pathways, non-coding RNAs, and regulatory elements from comprehensive expression profiling. G3 (Bethesda) 2:1475–1495.

46. Schrader PS, Milligan AJ, Behrenfeld MJ (2011) Surplus photosynthetic antennae and regulatory elements from comprehensive expres-

47. Hernandez-Prieto MA, et al. (2012) Iron deprivation in Synechocystis: Inference of pathways, non-coding RNAs, and regulatory elements from comprehensive expression profiling. G3 (Bethesda) 2:1475–1495.

48. Reischauer D, Wolter E, Herzel H, Axmann IM (2008) Light-regulated plastidic redox switches: Ferredoxins as novel RNA-binding proteins. J Endotoxins Cell Res 21:1–18.

49. Karpovich SJ, Prochnik SE, Grossman AR, Merchant SS (2011) The GreenC2t resource, a phylogenetically derived inventory of proteins specific to the plant lineage. J Biol Chem 286:21427–21439.

50. Gou P, et al. (2014) Higher order structure contributes to specific differences in redox potential of different ferredoxin transfer efficiency of root and leaf ferredoxins. Biochemistry 53:14389–14396.