

# Anaerobic Expression of the Ferredoxin-Encoding *FDX5* Gene of *Chlamydomonas reinhardtii* Is Regulated by the Crr1 Transcription Factor<sup>∇</sup>

Camilla Lambertz, Anja Hemschemeier, and Thomas Happe\*

Ruhr Universität Bochum, Fakultät für Biologie und Biotechnologie, Lehrstuhl für Biochemie der Pflanzen, AG Photobiotechnologie, 44780 Bochum, Germany

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**The unicellular green alga *Chlamydomonas reinhardtii* has a complex anaerobic metabolism and reacts to hypoxic or anaerobic conditions with the induced expression of many genes. One gene which is upregulated particularly strongly is the *FDX5* gene, encoding one of at least six ferredoxin isoforms in *C. reinhardtii*. Fdx5 is a typical plant-type 2Fe2S protein that is located in the chloroplast. The *FDX5* promoter region contains three GTAC motifs, which are known to be the binding sites for copper response regulator 1 (Crr1) and other *SQUAMOSA* promoter binding proteins (SBPs). This study shows that two of these GTAC sites are essential to confer oxygen and also copper responsiveness to a reporter gene. The SBP domain of Crr1 is able to bind to both of these GTAC sites in *in vitro* binding assays. Moreover, in a Crr1-deficient *C. reinhardtii* strain, *FDX5* is not expressed. These results clearly indicate that Crr1 is involved in the transcriptional regulation of the *FDX5* gene in the absence of oxygen or copper.**

Aerobic organisms depend on molecular oxygen (O<sub>2</sub>) for efficient energy production as well as for several biosynthetic pathways such as heme, ubiquinone, or cholesterol synthesis. However, O<sub>2</sub> availability can be limited depending on the environment or developmental stage. Organisms react to hypoxic or anaerobic conditions by differential gene expression in order to adjust metabolic and biosynthetic pathways (26). The unicellular eukaryotic green alga *Chlamydomonas reinhardtii* has a complex anaerobic metabolism (10, 19), which is accompanied by the differential expression of many genes (25, 29). Among these genes are transcripts encoding fermentative enzymes like HydA1 (FeFe-hydrogenase) (8) and Pfl1 (pyruvate formate lyase) (10) or enzymes that need O<sub>2</sub> as a substrate for certain steps in biosynthetic pathways such as Cpx1 (oxidative coproporphyrinogen oxidase 1) (33) or Crd1 (magnesium-protoporphyrin IX monomethyl ester aerobic oxidative cyclase) (34).

While the complex anaerobic metabolism of *C. reinhardtii* and the concomitant differential expression of genes, respectively, have been analyzed for years, there is little knowledge about the messengers and factors that are involved in low-O<sub>2</sub> sensing and adjusting the transcription of certain genes.

Only one transcriptional factor is known to be responsible for the transcriptional activation of several genes under hypoxic conditions (33, 34). This factor is the Crr1 (copper response regulator 1) protein (16), which has been identified to be essential for the adaptation of *C. reinhardtii* to copper (Cu) deficiency (4).

Crr1 is a member of the *SQUAMOSA* promoter binding protein (SBP) family carrying a characteristic zinc finger domain, which is responsible for the interaction of Crr1 with the

*cis*-acting DNA sequence GTAC (2, 16). SBP box transcription factors have been identified in many plant species, including green algae, moss, lycophyta, gymnosperms, and angiosperms (7), and have been shown to have functions in different plant organs and diverse stages of development (e.g., see references 17, 22, 38, and 40).

In *C. reinhardtii*, Crr1 is an essential transcriptional activator of several genes that are transcriptionally upregulated under conditions of Cu deficiency, such as *CYC6* (encoding cytochrome *c*<sub>6</sub>), *CPX1*, and *CRD1* (1, 33, 34), as well as the Cu transporter-encoding genes *CTR1*, *CTR2*, and *CTR3* (30). The 5' upstream regions of these genes contain at least one critical GTAC core within so-called CuREs (copper response elements), which are essential for the interaction with Crr1 (30, 34). Notably, the transcripts of the Cu-regulated genes mentioned above also accumulate under hypoxic or anaerobic conditions (30, 34). It was shown previously that this transcriptional response is also Crr1 regulated and dependent on GTAC motifs (16, 34).

We reported previously that the *FDX5* gene, one of at least six ferredoxin-encoding genes in *C. reinhardtii* (21), is strongly upregulated at both the transcript and the protein levels in anaerobic algae (13). The Fdx5 protein is a chloroplast-localized plant-type 2Fe2S-ferredoxin with a redox potential similar to that of the photosynthetic ferredoxin PetF (Fdx) (13), but the metabolic function of Fdx5 is still unknown. Very recently, it was shown that the *FDX5* transcript also accumulates in Cu-deficient *C. reinhardtii* cultures (39).

In order to gain deeper insights into the transcriptional regulation of the *FDX5* gene, we made use of a luciferase-encoding gene (*RLUC*) from the soft coral *Renilla reniformis*, which has been codon adapted (*CRLUC*) and established as a reporter gene for *C. reinhardtii* (6). By analyzing algal transformants carrying the *CRLUC* gene under the control of the putative *FDX5* promoter, we could identify two critical GTAC

\* Corresponding author. Mailing address: Ruhr Universität Bochum, Lehrstuhl für Biochemie der Pflanzen, AG Photobiotechnologie, ND2/170, 44780 Bochum, Germany. Phone: 49 234 32 27026. Fax: 49 234 32 14322. E-mail: thomas.happe@rub.de.

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motifs that are essential for the transcriptional activation of the *FDX5* gene. The SBP domain of *C. reinhardtii* Crr1 is able to bind these GTAC cores, as shown by electrophoretic mobility shift assays (EMSA). Eventually, we confirmed that *FDX5* is a strict Crr1 target by showing that a Crr1-deficient *C. reinhardtii* strain is not able to accumulate *FDX5* transcripts or the Fdx5 protein.

## MATERIALS AND METHODS

**Organisms and growth conditions.** All *C. reinhardtii* strains (wild type CC-125 [mt + 137C], cell-wall-less strain CC-425, and *crr1* mutant strain CC-3960 *crr1-2arg7* [16]) were obtained from the *Chlamydomonas* Culture Collection at Duke University. If not indicated otherwise, stocks and precultures were grown in Tris-acetate-phosphate (TAP) medium (9) at a light intensity of 80  $\mu\text{mol of photons} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$  and at a temperature of 20°C.

To analyze *CRLUC* expression in recombinant *C. reinhardtii* strains carrying chimeric constructs of *CRLUC* under the control of various fragments of the putative *FDX5* promoter, individual colonies from each transformation were grown in 12 ml TAP in 15 ml Sarstedt centrifuge tubes (Sarstedt, Nümbrecht, Germany) in light (80  $\mu\text{mol of photons} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ ). After the cultures had reached a chlorophyll concentration of about 20  $\mu\text{g} \cdot \text{ml}^{-1}$ , conditions known to induce *FDX5* gene expression were applied, as described below.

Sulfur (S) deprivation of strains CC-425 and CC-3960 was achieved as described previously (11). For analyzing a large number of individual *C. reinhardtii* transformants, experiments were conducted with 12-ml-headspace bottles (Fisher Scientific, Schwerte, Germany) with an 11-ml culture volume, sealed with 20-mm butyl rubber stoppers (Alltech, Rottenburg-Hailfingen, Germany). A 5-mm glass bead was added to each vessel to improve mixing of the cells. The flasks were incubated on a reciprocal shaker and illuminated from the bottom with 80  $\mu\text{mol of photons} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ . The algal cells were used for luciferase activity assays after 72 h of S deficiency. At this time point, the cultures were hypoxic and producing hydrogen, as confirmed by gas chromatography (using a GC-2010 gas chromatograph [Shimadzu, Kyoto, Japan] equipped with a Plot fused silica-coated Molsieve column [5Å, 10 m by 0.32 mm; Varian, Palo Alto, CA]) (data not shown).

Anaerobic adaptation by  $\text{N}_2$  flushing was conducted as described previously (11). To anaerobically adapt many individual *C. reinhardtii* transformants, the protocol was modified: *C. reinhardtii* cultures were grown in 15-ml Sarstedt tubes, concentrated to 80  $\mu\text{g}$  chlorophyll  $\cdot \text{ml}^{-1}$ , filled in 8-ml headspace bottles, and sealed with red rubber Suba-Seals 25 (Sigma-Aldrich, St. Louis, MO). The flasks were wrapped in aluminum foil and bubbled with  $\text{N}_2$  via syringes piercing through the septa for 30 min. The vessels were then incubated on a shaker for 3 h in the dark without gassing, before samples for determining luciferase activity were taken. When indicated, *in vitro* hydrogenase activity in the cell cultures was determined as described in detail previously by Hemschemeier et al. (11).

To induce Cu deficiency, *C. reinhardtii* cultures were grown once in TAP ENEA2 medium as reported previously by Ferrante et al. (5), before a main culture was inoculated with 1 ml of the preculture and incubated at a light intensity of 80  $\mu\text{mol of photons} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$ . As soon as the cells had reached a chlorophyll concentration of 3  $\mu\text{g} \cdot \text{ml}^{-1}$ , the Cu chelator triethylenetetramine dihydrochloride (TETA) was added to a final concentration of 50  $\mu\text{M}$ . For analyzing *C. reinhardtii* transformants, Cu depletion was carried out in a culture volume of 40 ml in 50-ml Sarstedt tubes for 96 h before luciferase activity was determined. For RNA isolation, 200 ml of Cu-deficient culture was prepared in 500-ml Erlenmeyer flasks.

**Transformation of *C. reinhardtii*.** The transformation of *C. reinhardtii* strain CC-125 was done according to a glass bead method (15) after treatment with autolysin. Cells were transformed by using 1.5  $\mu\text{g}$  of ScaI-linearized plasmids (described below) and plated onto TAP agar plates. After incubation in low light for 24 h, the agar blocks were transferred onto fresh petri dishes containing a filter paper soaked with paromomycin solution to obtain a final concentration of 5  $\mu\text{g} \cdot \text{ml}^{-1}$ .

**Luciferase activity assay.** For determining luciferase activity, 200  $\mu\text{l}$  of sulfur-deprived or anaerobically adapted cultures was removed with a syringe from the incubation flask by piercing through the rubber seals and transferred into the individual wells of a 96-well plate (96-well white microplate; Berthold, Bad Wildbad, Germany). Background light emission was detected for 10 s on a photon-counting microplate luminometer (Orion microplate luminometer connected to a personal computer [PC] with Simplicity software 2.1; Berthold Detection Systems, Pforzheim, Germany). Next, 0.5  $\mu\text{l}$  of 2 mM coelenterazine

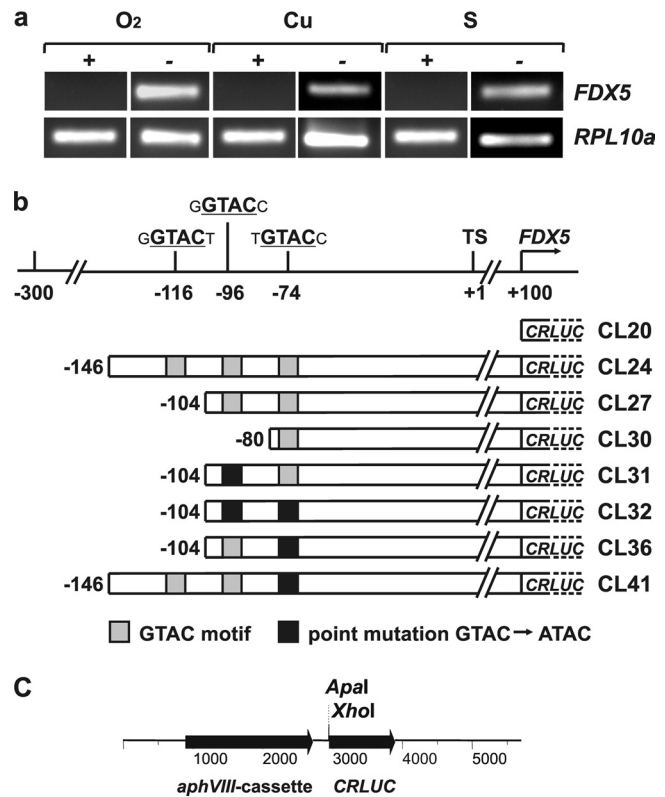


FIG. 1. RT-PCR analyses of *FDX5* transcript abundance and schematic diagrams of reporter gene constructs generated in this study. (a) RT-PCR analyses using mRNA isolated from *C. reinhardtii* strain CC-125 cultures flushed with  $\text{N}_2$  in the dark ( $\text{O}_2$ , -), S-deprived cells (S, -), and Cu-deficient algae (Cu, -). mRNA from cultures grown in TAP medium in the light served as controls (each +). Data for the *RPL10a* transcript served as controls. (b, top) Scheme of a fragment of the putative *FDX5* promoter from positions -300 to +100 relative to the transcription start site (TS) (position +1) showing the localization of three GTAC motifs, which are highlighted and underlined within a 6-nucleotide sequence showing the 3'- and 5'-adjacent nucleotides. The numbers below the motifs indicate their positions relative to the TS. (Bottom) Scheme of the reporter gene constructs generated in this study, in which the *CRLUC* gene was fused to various fragments of the putative *FDX5* promoter. Numbers below the respective constructs indicate the positions of the 5' ends relative to the TS. Squares indicate GTAC motifs, either in their wild-type sequence (gray) or in mutated forms (GTAC → ATAC) (black). (c) Schematic drawing of ScaI-linearized plasmid pCL20 used for generating the reporter gene constructs carrying both the paromomycin resistance cassette (*aphVIII* cassette) and the codon-adapted *CRLUC* gene. *FDX5* promoter constructs were inserted via *ApaI* and *XhoI*.

(PJK GmbH, Kleinblittersdorf, Germany) in methanol was added to the cell samples, and light emission was detected for 10 s again.

In the case of Cu-depleted cells, 2 ml of each culture was disrupted by freezing in liquid nitrogen, thawing, and subsequent sonication three times for 30 s (Transsonic T 460; Elma, Singen, Germany). The cell lysates were stored in liquid nitrogen until the analysis occurred. For measurements of luciferase activity, 200  $\mu\text{l}$  of thawed lysates was transferred into multiwell plates as described above.

**Construction of plasmids.** In order to circumvent the necessity of cotransformation, plasmids containing both the codon-optimized luciferase gene from *R. reniformis* (*CRLUC*) (6) and the paromomycin resistance cassette *aphVIII*, which was under the control of the *HSP70* promoter (Fig. 1c), were constructed.

*CRLUC* was obtained by digesting plasmid pCrLuc (6) with *Bam*HI and *Xho*I and ligating the fragment into the *Bam*HI- and *Xho*I-cut vector pBS(-) (Stratagene, La Jolla, CA) to obtain plasmid pBSLuc.

TABLE 1. Oligonucleotides used for the amplification of various fragments of the putative *FDX5* promoter and the corresponding constructs used for reporter gene analyses

Oligonucleotide	Sequence <sup>a</sup>
FDX5_rev.....	CACTCGAGTCTTTCGGAAGAGTCGCGACGGAC
FDX5_CL24.....	CGGGCCCTACACCTTGGAGCCGAGATT
FDX5_CL27.....	TGGGCCCTCACTGGAGGTACCGCAGGTGG
FDX5_CL30.....	AGGGCCCGCGAGTGTACCTGAGGTC
FDX5_CL31.....	TGGGCCCTCACTGGAGATACCGCAGGTGG
FDX5_CL32.....	TGGGCCCTCACTGGAGATACCGCAGGTGGCGC GCGAGTACTCTGAGGTC
FDX5_CL36.....	TGGGCCCTCACTGGAGGTACCGCAGGTGGCG CGCGAGTACTCTGAGGTC
FDX5_CL41.....	CGGGCCCTACACCTTGGAGCCGAGATTGGCTT GGCGGTACTGGGTGTCACTGGAGGTACCG CAGGTGGCGCGAGTACTCTGAGGTC

<sup>a</sup> ApaI and XhoI restriction sites are underlined. Inserted mutations are indicated in boldface type.

Vector pSL18 (37) was used as a template to amplify the *aphVIII* gene by PCR, including the 5' and 3' untranslated regions (UTRs) of the *C. reinhardtii* *RBCS2* gene and the 5' region of *HSP70A*, and to add flanking PdiI restriction sites by using oligonucleotides TGCGGGCGGTACCCGCTCAAATACGC and CGCCGGCGAGCTCGCTGAGGCTTGACA (PdiI sites are underlined). The amplicase was inserted into pGemT-Easy (Promega, Madison, WI) to obtain plasmid pGemParo.

The *aphVIII* cassette was cut from pGemParo by PdiI digestion and inserted into PdiI-cut pBSLuc, which resulted in plasmid pBSLucParo. Finally, the KpnI restriction site in plasmid pBSLucParo was removed, since the KpnI site contains a GTAC motif, which might have falsified the reporter gene analysis. To eliminate the KpnI site, pBSLucParo was digested with Acc65I, treated with S1 nuclease (Gibco BRL/Invitrogen, Carlsbad, CA), and finally religated, resulting in plasmid pCL20.

pCL20 was used to produce chimeric constructs in which the *CRLUC* gene was under the control of various fragments of the putative *FDX5* promoter. Different fragments of the *FDX5* upstream region were amplified by PCR using *Pfu* polymerase (Stratagene) and the oligonucleotides listed in Table 1 on genomic DNA as a template. Genomic DNA was isolated from *C. reinhardtii* strain CC-125 by using a slightly modified version of a method described previously by Newman et al. (28).

The individual PCR products were digested with XhoI and ApaI and inserted into pCL20 digested by the same endonucleases. Mutations within some fragments of the putative *FDX5* promoter were inserted by site-directed mutagenesis using oligonucleotides carrying the desired mutation (Table 1). All constructs were sequenced at the sequencing facility at the Ruhr University of Bochum, Germany (Department of Biochemistry I, Receptor Biochemistry).

**RNA analysis, RT-PCR, and 5'-RACE PCR.** Total RNA was isolated according to a method described previously by Johanningmeier and Howell (14). Most parts of genomic DNA contamination were removed by LiCl precipitation. Ten micrograms of total RNA was treated with Turbo DNase (Ambion, Austin, TX), and 0.5 µg of the DNase-treated RNA was used as a template for reverse transcription-PCR (RT-PCR) analyses using the Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany) to detect the transcripts of *CRLUC* (using oligonucleotides CGATGGCCTTGATCTTGTCTGGT and GCAGATGAACGTGCTGGACAGCTT), *FDX5* (CGGCTTCATCCTCATGTGCT and ACGCTGACACGAATGGTACG), and *RPL10a* (CCAAGTGCAGCATCAAGTTC and CACGTTCTGCCAGTTCTTCT).

For identifying the transcription start point (TS) of the *FDX5* gene, 5' rapid amplification of cDNA ends (5'-RACE) PCR was applied by using a SMART RACE cDNA amplification kit from Clontech (Mountain View, CA) according to the manufacturer's recommendations.

RNA hybridization analyses were conducted with 20 µg of each sample of total RNA according to standard techniques (35), using digoxigenin-labeled probes complementary to *RPL10a* (encoding ribosomal protein L10a; JGI v4.0 protein identification 195585) and *FDX5* (JGI v4.0 protein identification 156833). Probe fragments were produced by PCR using oligonucleotides FDX5for (CGGCTTCATCC TCATGTGCT), FDX5rev (ACGCTGACACGAATGGTACG), L10afor (CCAA GTGCAGCATCAAGTTC), and L10arev (CACGTTCTGCCAGTTCTTCT).

**Heterologous expression of the Crr1 SBP domain, electrophoretic mobility shift assay, and other biochemical methods.** The SBP domain of *C. reinhardtii* Crr1 was heterologously produced in *Escherichia coli* and purified by His tag

chromatography as described previously (2), except that *Escherichia coli* strain BL21(DE3)pLysS (Novagen/Merck, Darmstadt, Germany) was used. The *CRR1* cDNA fragment encoding a truncated polypeptide from amino acids 428 to 524 (numbers correspond to the Crr1 sequence reported previously [16]) was present in plasmid pet15SBP, which was kindly donated by Frederik Sommer, Potsdam, Germany. After purification of the polypeptide, the success of the purification was proven by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining. The protein concentration was determined by a Bradford assay (2a) (data not shown).

An electrophoretic mobility shift assay (EMSA) was done by using a second-generation digoxigenin (DIG) gel shift kit (Roche, Basel, Switzerland), according to the manual provided by the manufacturer, except that 1 mM ZnCl<sub>2</sub> was added to the DNA-protein samples. Thirty-six-base-pair-long oligonucleotides corresponding to the wild type or mutated parts of the putative *FDX5* promoter (details are shown in Fig. 3a) were obtained from Sigma-Aldrich. Two hundred nanograms of the heterologously produced Crr1 SBP domain and 60 fmol of the individual double-stranded fragments were used for each sample. After incubation, samples were separated via 0.5× TBE (Tris-borate-EDTA buffer) native PAGE and blotted onto a nylon membrane by electroblotting (400 mA for 30 min). Fragments were visualized via chemiluminescence after immunodetection with anti-digoxigenin antibodies coupled with horseradish peroxidase.

Western blot analyses with polyclonal anti-*C. reinhardtii* Fdx5 antibody were done as described previously (13).

## RESULTS

**FDX5 transcription depends on two GTAC motifs in both anaerobiosis and copper depletion.** *FDX5* transcripts accumulate in the absence of both O<sub>2</sub> and Cu (Fig. 1a), as was shown previously (13, 39). By applying RT-PCR analyses using mRNA samples isolated from *C. reinhardtii* cells under different conditions of O<sub>2</sub> or Cu availability, no *FDX5* transcript could be detected in aerobic *C. reinhardtii* cultures in the presence of Cu, whereas strong signals appeared in samples from algal cells that had become anaerobic due to sulfur depletion in the light (18) or N<sub>2</sub> flushing in the dark or from Cu-depleted, aerobic cells (Fig. 1a).

To gain insights into the regulatory elements within the putative *FDX5* promoter, we first carried out a 5'-RACE analysis. The *FDX5* transcription start (position +1) was found 99 nucleotides upstream of the translation start codon (position +100). This results in a 5' untranslated region (UTR) that is 14 bp longer than that indicated by gene model au5.g7064\_t1 in JGI 4.0.

*In silico* analyses of the putative *FDX5* promoter region 1 to 300 bp upstream of the transcription start point (TS) revealed the presence of three GTAC motifs at positions -116, -96, and -74 relative to the TS (Fig. 1b). GTAC cores were shown previously to be essential for the activation of transcription by the Crr1 transcription factor under both Cu-deficient and hypoxic conditions (30, 33, 34). To analyze the role of these three GTAC motifs in the regulation of the *FDX5* gene, chimeric constructs of the *CRLUC* gene (6) under the control of various fragments of the putative *FDX5* promoter were generated. The *FDX5* promoter fragments were chosen to contain three, two, or only one of the GTAC motifs as well as GTAC motifs in which single point mutations (GTAC→ATAC) were introduced (Fig. 1b). These constructs were used to transform *C. reinhardtii* wild-type strain CC-125, and the resulting transformants were exposed to S deprivation in the light, N<sub>2</sub> bubbling in the dark, or Cu deficiency to analyze the dimensions of luciferase activity under these conditions.

Several (19 to 55) randomly chosen transformants of each library were analyzed. Smaller numbers of strains were exam-

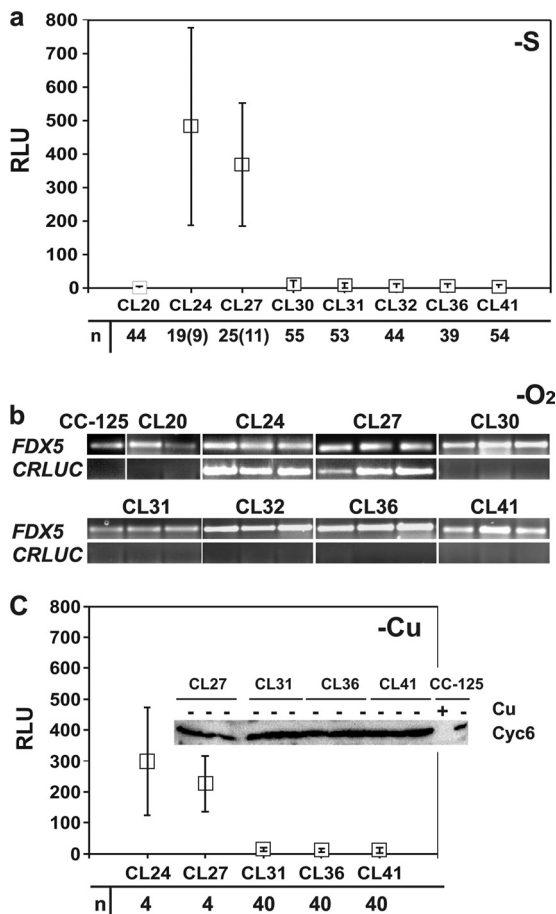


FIG. 2. Expression of the *CRLUC* reporter gene controlled by various fragments of the putative *FDX5* promoter in *C. reinhardtii* transformants under different physiological conditions. (a and c) Average luciferase activities in RLU (relative luminescence units) for S- and Cu-depleted *C. reinhardtii* transformants carrying the respective chimeric constructs. Bars indicate standard deviations, and n indicates the number of individual transformants analyzed for each library. (a) Luciferase activity in S-depleted cells. In the cases of CL24 and CL27, only the luminescent transformants (numbers in parentheses) were included in the calculation. (b) Transcript analysis of *CRLUC* via RT-PCR using mRNA of *C. reinhardtii* transformants that had been subjected to anaerobic conditions by N<sub>2</sub> flushing in the dark. The results shown are representative of three individual strains of each construct library. mRNA isolated from strain CC-125 served as an untransformed control, and mRNA from CL20 (in which *CRLUC* is promoterless) served as the background control. Each RNA sample was also tested for *FDX5* transcript accumulation to verify the physiological status of the cells and RNA quality. (c) Average luciferase activity in Cu-deficient *C. reinhardtii* transformants. In the case of strains carrying CL24 and CL27, the activities of four individual luminescent strains were measured. Forty individual strains of each library, CL31, CL36, and CL41, were analyzed. (Inset) Immunoblot analysis with anti-*C. reinhardtii* Cyc6 antibody using crude protein extracts of randomly chosen Cu-depleted transformants in order to verify the Cu-deficient status of the cells. +, sample isolated from Cu-replete CC-125 cells.

ined when significant luciferase activity was detected. In cases where no activity was measured, we increased the amount of sample to clearly confirm the result. The luciferase activities in S-depleted *C. reinhardtii* transformants carrying the various chimeric constructs are shown in Fig. 2a. Only for strains car-

rying the constructs CL24 and CL27, including all three or the two proximal (positions  $-96$  and  $-74$ ) GTAC motifs, respectively, could a significant luciferase activity of 400 to 500 relative luminescence units (RLU) be detected. In *C. reinhardtii*, transformants carrying the *CRLUC* gene under the control of larger parts of the *FDX5* promoter than those present in CL24 (up to 1,549 bp, including an additional GTAC motif at position  $-359$ ) did not exhibit significantly higher RLU values (data not shown). However, the deletion of the two distal GTAC motifs at positions  $-116$  and  $-96$  (CL30) or single point mutations in both or one of each of the two proximal GTAC sites (positions  $-96$  and  $-74$ ) (CL31, CL32, and CL36) were sufficient to prevent the expression of *CRLUC*, as indicated by a very low luciferase activity of about 20 RLU. This value is comparable to that of the usually observed background signal (about 12 RLU) also detected in transformants carrying the construct CL20 with a promoterless *CRLUC* gene (Fig. 2a). It is noteworthy that a construct containing all three GTAC motifs but a single point mutation in the most proximal GTAC motif at position  $-74$  (CL41) did not allow *CRLUC* expression, either.

The same *C. reinhardtii* transformants were analyzed for luciferase activity after being flushed with N<sub>2</sub> in the dark to confirm that the luciferase activity detected in sulfur-depleted, anaerobic cells was indeed due to the absence of O<sub>2</sub> rather than to the absence of sulfur. However, no luciferase activity could be detected by using the luminometer. Therefore, mRNA was isolated from 10 to 15 individual transformants of each library and used to analyze *CRLUC* transcription by RT-PCR (Fig. 2b). These analyses showed the presence of *CRLUC* transcripts in anaerobic strains carrying the constructs CL24 and CL27 but not in the other transformants (Fig. 2b) and therefore revealed the same pattern of *CRLUC* expression as what had been observed as luciferase activity in sulfur-depleted *C. reinhardtii* transformants.

Finally, the transformants were subjected to Cu deficiency. It is known that conditions of trace element deficiency are difficult to establish, since the diminutive amounts that are essential for an organism can be supplied even by diffusion out of glassware or by the entry of dust (31). We have applied a chelator-based method for inducing Cu deficiency in *C. reinhardtii* cells grown in low but sufficient Cu concentrations (0.3  $\mu$ M). This approach was shown to allow *CYC6* gene expression (5). The *C. reinhardtii* transformants that had been cultivated under these conditions indeed experienced a Cu deficiency, as shown by the accumulation of the Cyc6 protein in several selected strains (Fig. 2c, inset). After the copper-depleted state of the *C. reinhardtii* transformants had been proven, they were analyzed by the luciferase activity assay. Again, only transformants carrying the constructs CL24 and CL27 exhibited luciferase activity (Fig. 2c), while *C. reinhardtii* strains transformed with the constructs CL31, CL36, and CL41 did not.

To confirm that no *CRLUC* transcript accumulated in Cu-depleted *C. reinhardtii* transformants of the CL31, CL36, and CL41 libraries, RT-PCR analyses were conducted. RNA isolated from 11 individual strains of each construct library, which had been Cu deprived for 4 days, was tested for the presence of *CRLUC* and *FDX5* transcripts. While strong signals appeared using oligonucleotides specific for *FDX5* mRNA, no signals were obtained by using *CRLUC*-specific primers in

strains carrying the constructs CL31, CL36, and CL41 (data not shown).

**The SBP domain of Crr1 binds to the GTAC motifs of the *FDX5* promoter.** Crr1 is a member of the SBP box family (2, 16). SBP domains harbor a zinc finger domain that is known to interact with the GTAC motif of DNA (2). It was shown previously that the SBP domain of Crr1 binds to the functionally defined (33) CuREs of *CPX1* and *CYC6* (16). We analyzed whether the SBP domain of Crr1 can also bind to the two proximal GTAC motifs within the *FDX5* upstream region described above to be essential for *FDX5* promoter activity. By using the heterologously produced Crr1 SBP domain, electrophoretic mobility shift assays were conducted with digoxigenin-labeled 36-bp fragments of the *FDX5* promoter, including the two proximal GTAC motifs either intact or mutated to ACTG or TAGA (Fig. 3a). The labeled *FDX5* promoter fragments were incubated in the presence or in the absence of 200 ng of a heterologously produced Crr1 SBP domain. No retardation of the fragments could be observed in the absence of the SBP domain (Fig. 3b, lanes 1 to 4). However, the incubation of a fragment of the putative *FDX5* promoter including the two proximal (positions -96 and -74) GTAC motifs (construct I) (Fig. 3a) with the Crr1 SBP domain resulted in two shifted bands of the digoxigenin-labeled oligonucleotides (Fig. 3b, lane 5). The fragments carrying only one intact GTAC site (constructs II and III) (Fig. 3a) showed a single band shift, independently of which of the two GTAC motifs was present (Fig. 3b, lanes 6 and 7). The mutation of both GTAC motifs (construct IV) (Fig. 3a) prevented the retardation of the fragment (Fig. 3b, lane 8).

The specificity of the binding of the Crr1 SBP domain to the *FDX5* promoter fragments was verified by incubating the SBP domain with constant amounts (60 fmol) of labeled *FDX5* construct II (Fig. 3a) and increasing amounts (0 fmol to 6,000 fmol) of unlabeled fragment (construct II or IV) (Fig. 3a). The strength of the shifted band decreased with increasing amounts of unlabeled construct II but not in the presence of unlabeled construct IV, which carries both mutations (Fig. 3c).

***FDX5* gene expression is absent in a Crr1-deficient *C. reinhardtii* strain.** The fact that the expression of the *FDX5* gene is induced by anaerobiosis, Cu deficiency, and the addition of Ni (13, 39; this study) and the requirement for intact GTAC sites for the activity of the *FDX5* promoter, as shown above, strongly indicate that the *FDX5* gene is a target of the Crr1 transcriptional activator. To confirm this hypothesis, *FDX5* expression was analyzed in O<sub>2</sub>- or Cu-deficient cultures of a *crr1* mutant strain (CC-3960) (16). *C. reinhardtii* strain CC-425 served as the control strain. Both strains were incubated in sulfur-free medium in the light, flushed with nitrogen in the dark, or transferred into TAP ENEA2 medium including TETA to establish a Cu deficiency (5). To ensure the establishment of anaerobic conditions in the first two cases, *in vitro* hydrogenase activity as a sensitive indicator of the absence of O<sub>2</sub> (8) was analyzed (data not shown). The Cu deficiency of the cells was verified by Western blot analysis using a polyclonal anti-Cyc6 antibody (kindly donated by S. Merchant, UCLA, Los Angeles, CA) (data not shown).

Northern blot analyses using a probe specific for *FDX5* mRNA showed a typical pattern of *FDX5* transcript accumulation in *C. reinhardtii* strain CC-425. No *FDX5* mRNA was

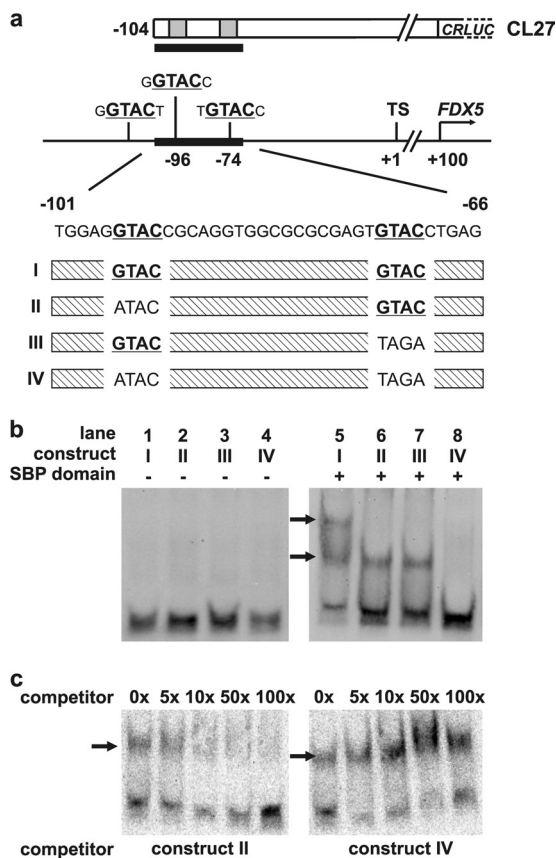


FIG. 3. EMSAs for analyzing the binding of the Crr1 SBP domain to the two proximal GTAC motifs of the putative *FDX5* promoter. (a) Schematic drawing of the 36-bp fragments used in the assays. Fragments I to IV are shown in the context of the reporter gene construct CL27 (Fig. 1b) and the putative *FDX5* promoter. The fragments were derived from positions -101 to -66 relative to the transcription start site (TS) of the *FDX5* gene as the wild-type sequence (fragment I), with individually mutated GTAC motifs (fragments II and III) or with mutations in both GTAC sites (fragment IV). (b) Digoxigenin-labeled fragments I to IV of the putative *FDX5* promoter were incubated without (-) (lanes 1 to 4) or with (+) (lanes 5 to 8) 200 ng of the recombinant Crr1 SBP domain, separated by native polyacrylamide gel electrophoresis in 0.5× TBE, and visualized after immunohybridization with anti-digoxigenin antibody coupled with horseradish peroxidase. The arrows indicate shifted *FDX5* fragments. (c) Digoxigenin-labeled construct II was incubated with 200 ng of the recombinant Crr1 SBP domain and increasing amounts (0×, 5×, 10×, 50×, and 100×) of unlabeled constructs II and IV, respectively. The arrows indicate the positions of shifted fragments.

detectable in RNA samples isolated from untreated cells (Fig. 4a), while the *FDX5* transcript levels increased significantly in RNA samples isolated from CC-425 cultures subjected to sulfur deprivation for 24, 48, and 72 h; N<sub>2</sub> flushing for 0.5, 1, and 3 h; or Cu deficiency for 45 h after the second transfer to copper-free medium (Fig. 4a). However, no *FDX5* transcript could be detected in RNA samples isolated from equally treated *C. reinhardtii* strain CC-3960 (Fig. 4a).

In the case of sulfur-deprived algal cultures, the results obtained by RNA hybridization were confirmed at the protein level (Fig. 4b). By using polyclonal anti-Fdx5-antibodies (13), the Fdx5 protein was detected in crude protein extracts isolated from strain CC-425 subjected to sulfur deficiency for 24,

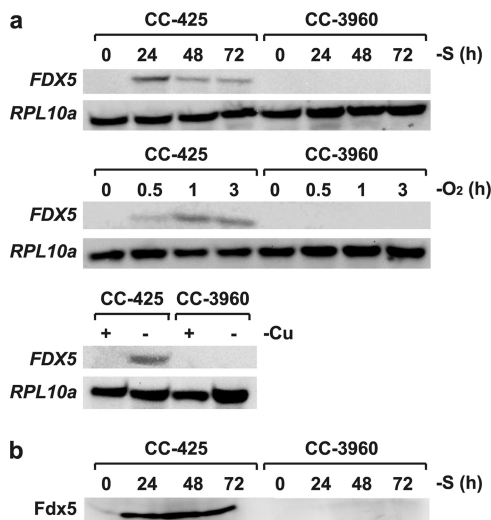


FIG. 4. Expression of the *FDX5* gene in *C. reinhardtii* strain CC-425 and Crr1-deficient strain CC-3960 at the transcript level (a) and the protein level (b). (a) RNA hybridization analyses of RNA isolated from strains CC-425 and CC-3960 subjected to S deprivation (–S), N<sub>2</sub> bubbling in the dark (–O<sub>2</sub>), or Cu deficiency (–Cu) using probes specific for *FDX5* or *RPL10a*. RNA was isolated from the cells after the indicated time points of S depletion and N<sub>2</sub> flushing and after 48 h of Cu deficiency (–Cu), respectively. Control samples (0 h in the case of –S and –O<sub>2</sub> and + in the case of Cu) were isolated from the strains grown in TAP medium in the light. (b) Immunoblot analysis using anti-Fdx5 antibody of crude protein extracts isolated from S-deficient strains CC-425 and CC-3960 after the indicated time points of S deprivation. A protein amount equivalent to 2 μg of chlorophyll was loaded into each lane.

48, and 72 h, while no signals were visible using protein extracts from sulfur-deprived Crr1-deficient strain CC-3960 (Fig. 4b).

## DISCUSSION

The expression of the *FDX5* gene of *C. reinhardtii* has been shown to be strongly upregulated under anaerobic conditions, either in dark-adapted and N<sub>2</sub>-flushed cells (13, 25, 39) or in illuminated, sulfur-deprived cultures (13). *FDX5* transcripts also accumulate significantly in Cu-limited cultures (39; this study). The transcription of several genes is activated by both O<sub>2</sub> and Cu deprivation in *C. reinhardtii* (1, 23, 30, 34), and this dual response is mediated by one transcriptional factor, called Crr1 (16, 30, 34). Crr1 has been identified to be a SBP domain protein (16), and the recombinant Crr1 SBP domain is able to bind GTAC motifs (2, 16).

In the upstream regions of *CYC6*, *CPX1*, *CRD1*, and *CTR1*, one or more GTAC motifs have been detected, and their influence on gene expression was analyzed by using reporter gene assays. The GTAC motif was shown to be the core of copper response elements (CuREs), and in the case of the *CPX1* gene, it was also proven that GTAC is a hypoxia response element (HyRE) (33, 34).

In view of the accumulation of the *FDX5* transcript under both hypoxic and Cu-deficient conditions, a regulation of the *FDX5* gene similar to that of the reported genes mentioned above was compelling. *In silico* analyses of the region at positions –300 to +1 relative to the transcription start site of the

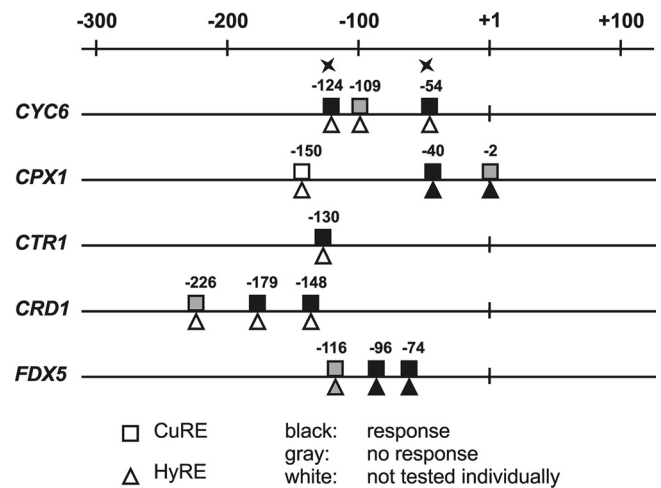


FIG. 5. Schematic comparison of the promoter regions of several Crr1 target genes. Sequences were derived from the original research articles (*CYC6* [33], *CPX1* [32], *CTR1* [30], and *CRD1* [1]) and from the Augustus 5 gene model annotated in the *C. reinhardtii* genome sequence in JGI 4.0 for *FDX5*: au5.g7064\_t1 (transcription start site 14 bp upstream at position chromosome\_17:702568 in JGI 4.0 [identified in this work]). Positions of GTAC sites are indicated as squares and triangles and numbered relative to the transcription start site at position +1. In the case of *CPX1*, the transcription start point of the longest transcript described previously by Quinn et al. (32) was chosen. Black symbols indicate that a function of the respective GTAC motif as a CuRE (black squares) or HyRE (black triangles) was proven, gray symbols represent GTAC sites which were shown to have no function, and white symbols stand for GTAC sequences that have not been analyzed regarding their function. The asterisks above the CuRE symbols in the *CYC6* scheme indicate that both sites are able to confer Cu responsiveness individually.

putative *FDX5* promoter indeed showed the presence of three GTAC sites at positions –116, –96, and –74 relative to the transcriptional start point of the *FDX5* gene.

Using the *Renilla* luciferase-encoding gene *CRLUC* as a reporter gene (6), we were able to show that a fragment of the putative *FDX5* promoter involving bases –146 to +100 relative to the transcriptional start point (construct CL24) was able to activate the expression of the luciferase-encoding gene in response to anaerobiosis or to Cu deficiency. The successive truncation of this promoter region revealed the two proximal GTAC sites at positions –96 and –74 to be necessary and sufficient for conferring Cu and hypoxia responsiveness to the expression of *CRLUC*. Various mutations of one or both of these two critical GTAC sites showed that both sites have to be present in their wild-type sequence to allow the *FDX5* promoter to be active and that the distal GTAC site cannot replace the proximal motif (CL41).

Comparing these results with available literature, the Cu response of *FDX5* resembles that of the *CRD1* gene (1). Besides *FDX5*, this is the only Crr1 target reported so far to depend on two GTAC sites for transcriptional activation under conditions of Cu deficiency (1) (Fig. 5). In both the *CPX1* (33) and *CTR1* (30) promoters, one single GTAC motif has been shown to be the CuRE. The *CYC6* promoter contains two Cu-responsive GTAC sites, which, however, are both able to confer Cu responsiveness independently (33) (Fig. 5).

Regarding the response to hypoxia, only the GTAC sites of

the *CPX1* promoter have been analyzed in detail. In contrast to the Cu response, a single CuRE is not sufficient for an enhanced expression of a reporter gene under hypoxic conditions. Rather, the CuRE and a second GTAC motif, referred to as the HyRE, have to be present in the promoter region to allow hypoxia-responsive expression of the reporter gene (34) (Fig. 5).

Thus, regarding the transcriptional activation of the *FDX5* gene as a response to hypoxia, the *FDX5* promoter is similar to the promoter of the *CPX1* gene (34) (Fig. 5).

It is noteworthy that while both proximal GTAC sites of the *FDX5* promoter are necessary to induce gene transcription *in vivo*, the SBP domain of Crr1 is able to bind to both GTAC sites of the *FDX5* promoter individually, as shown by a clear band shift of all constructs of the respective region that contain at least one wild-type GTAC motif. Obviously, the fact that Crr1 is able to bind to one GTAC site via its SBP domain is not sufficient to confer transcriptional activation *in vivo*.

It has been suggested from a detailed mutational analysis of the *CYC6* promoter that additional nucleotides in the *CYC6* promoter are important for the proper expression of a reporter gene (33). Again, this indicates that the binding of the SBP domain to a GTAC core alone is not sufficient to activate transcription properly. The appearance of a double-shift band in EMSAs using the wild-type *FDX5* promoter fragment, containing both GTAC sites, might hint at a double binding of the SBP domain to sequences containing two GTAC motifs (27). Extrapolating this hypothesis to the *in vivo* background of living *C. reinhardtii* cells, this might indicate that two SBP domain proteins are necessary to activate the transcription of the *FDX5* gene. This has to be analyzed in more detail in the future, also taking into account additional domains of the responsible transcriptional activator that influence the binding and/or dimerization capacity of the factor.

The fact that the Crr1 SBP domain binds to the GTAC sites of the putative *FDX5* promoter is still no ultimate evidence for *FDX5* being regulated by the Crr1 transcriptional activator. Several SBP domains have been shown to bind to GTAC motifs, and the SBP domain of Crr1 also binds an *Arabidopsis thaliana* promoter fragment, including a GTAC site, while on the other hand, a *C. reinhardtii* *CYC6* promoter fragment can be retarded by SBP domains from *Arabidopsis* and *Physcomitrella* (2). At least seven genes that putatively encode SBP domain proteins have been detected in the *C. reinhardtii* genomic sequence (7). However, the hypothesis that the *FDX5* gene is under the transcriptional control of the Crr1 protein was confirmed by the fact that a *C. reinhardtii* *crr1* mutant strain does not show any detectable accumulation of *FDX5* transcripts or the Fdx5 protein. Thus, *FDX5* is clearly regulated by Crr1 in response to Cu and O<sub>2</sub> limitation.

It remains a matter of debate as to why the two environmental conditions of Cu and O<sub>2</sub> deficiencies are sensed and signaled by the same pathway. Cu is less soluble and bioavailable under anaerobic conditions, so the evolution of one signaling pathway to react to these two coupled conditions might make physiological sense (3). The proteins encoded by the known Cu- and hypoxia-responsive genes have specific functions in Cu or in O<sub>2</sub> deficiency but not always obviously in both. While the role of the Cyc6 protein is evident in Cu-depleted *C. reinhardtii* cells, where it replaces the Cu protein plastocyanin

(20), a role of Cyc6 under anaerobic conditions is not known. The same is true for copper transporters like Ctr1 (30). Cpx1 is involved in heme biosynthesis, which might be enhanced to provide a sufficient amount of heme as the cofactor of Cyc6 (12). On the other hand, the enhanced expression of the *CPX1* and *CRD1* genes under hypoxic conditions (23, 24, 34) might be an attempt to scavenge O<sub>2</sub> molecules, since both encoded enzymes are O<sub>2</sub> dependent.

Ferredoxins are small soluble electron carriers like cytochrome *c*<sub>6</sub>, and there are manifold pathways that involve electron transfer reactions mediated by ferredoxins. Some of these are O<sub>2</sub> dependent, such as fatty acid desaturation (36), which might provide a link to the O<sub>2</sub> responsiveness of a ferredoxin-encoding gene.

*C. reinhardtii* has at least six ferredoxin-encoding genes, which are differentially expressed under various environmental conditions (13, 39). Interestingly, the putative promoter regions of *PETF*, *FDX2*, *FDX4*, and *FDX6* each contain one GTAC motif as well. However, *FDX5* is the only gene whose transcript strongly and significantly accumulates upon treatments known to elicit Crr1-dependent responses (13, 39). Obviously, the small electron carrier ferredoxin 5 has a specific role in anaerobic and/or Cu-deficient *C. reinhardtii* cells, which, however, remains to be elucidated.

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