

# Differential Expression of the *Chlamydomonas* [FeFe]-Hydrogenase-Encoding *HYDA1* Gene Is Regulated by the COPPER RESPONSE REGULATOR1<sup>1[C][W]</sup>

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The unicellular green alga *Chlamydomonas reinhardtii* adapts to anaerobic or hypoxic conditions by developing a complex fermentative metabolism including the production of molecular hydrogen by [FeFe]-hydrogenase isoform1 (HYDA1). *HYDA1* transcript and hydrogenase protein accumulate in the absence of oxygen or copper (Cu). Factors regulating this differential gene expression have been unknown so far. In this study, we report on the isolation of a *Chlamydomonas* mutant strain impaired in *HYDA1* gene expression by screening an insertional mutagenesis library for *HYDA1* promoter activity using the arylsulfatase-encoding *ARYLSULFATASE2* gene as a selection marker. The mutant strain has a deletion of the *COPPER RESPONSE REGULATOR1* (*CRR1*) gene encoding for CRR1, indicating that this *SQUAMOSA-PROMOTER BINDING PROTEIN* (SBP) domain transcription factor is involved in the regulation of *HYDA1* transcription. Treating the *C. reinhardtii* wild type with mercuric ions, which were shown to inhibit the binding of the SBP domain to DNA, prevented or deactivated *HYDA1* gene expression. Reporter gene analyses of the *HYDA1* promoter revealed that two GTAC motifs, which are known to be the cores of CRR1 binding sites, are necessary for full promoter activity in hypoxic conditions or upon Cu starvation. However, mutations of the GTAC sites had a much stronger impact on reporter gene expression in Cu-deficient cells. Electrophoretic mobility shift assays showed that the CRR1 SBP domain binds to one of the GTAC cores in vitro. These combined results prove that CRR1 is involved in *HYDA1* promoter activation.

Molecular dioxygen (O<sub>2</sub>) serves as substrate and reagent in a number of biosynthetic pathways (Goldfine, 1965; Raymond and Segrè, 2006) and plays an important role for both metabolism and growth of aerobic organisms (Des Marais, 1998). Under anaerobic conditions, aerobes have to adapt both energy-generating and biosynthetic mechanisms to maintain cell vitality. This modulation of metabolism is accompanied by altered gene expression, often regulated on the level of transcription as indicated by massive changes of transcript abundances in various organisms transferred to anaerobic or hypoxic conditions (Mustroph et al., 2010). As a response to anaerobiosis the unicellular green alga *Chlamydomonas reinhardtii* develops a complex fermentative metabolism (Gfeller and Gibbs, 1984; Hemschemeier and Happe, 2005; Mus et al.,

2007; Hemschemeier et al., 2008b; Philipps et al., 2011) with molecular hydrogen (H<sub>2</sub>) as one key metabolite. Hydrogen is produced by an oxygen-sensitive [FeFe]-hydrogenase (Stripp and Happe, 2009; Lambertz et al., 2011) that is linked to the photosynthetic electron transport chain via ferredoxin PETF (Winkler et al., 2009). *Chlamydomonas* expresses two hydrogenase-encoding genes, [FeFe]-hydrogenase isoform1 (*HYDA1*) and *HYDA2* (Forestier et al., 2003), but the *HYDA1* protein seems to be the primarily active hydrogenase isoform in the green alga (Godman et al., 2010; Meuser et al., 2012).

One experimental approach to induce anaerobic or hypoxic conditions in *C. reinhardtii* cultures is the incubation of the cells in sulfur (S)-free medium in the light (Melis et al., 2000; Hemschemeier et al., 2009). S-deprived algae produce significant amounts of H<sub>2</sub>. This can be attributed to the fact that PSII (Wykoff et al., 1998) and carbon dioxide fixation (Zhang et al., 2002; Hemschemeier et al., 2008a) activities decrease strongly, resulting in anaerobic (or microaerobic) conditions and a severe diminution of both respiratory and photosynthetic electron sinks (Melis, 2007; Ghysels and Franck, 2010; Hemschemeier and Happe, 2011). Under these conditions, *HYDA1* acts as an alternative electron acceptor of photosynthetic electron flow. However, small but significant amounts of H<sub>2</sub> are also produced by anaerobically adapted *C. reinhardtii* cultures in the dark (Gfeller and Gibbs, 1984; Philipps et al., 2011).

<sup>1</sup> This work was supported by the Federal Ministry of Education and Research, Germany (H<sub>2</sub>-Designzelle), the European Union (Seventh Framework Program, SolarH2 Consortium), and the Volkswagen foundation (LigH2t).

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[www.plantphysiol.org/cgi/doi/10.1104/pp.112.200162](http://www.plantphysiol.org/cgi/doi/10.1104/pp.112.200162)

A major part of the regulation of hydrogenase activity in *Chlamydomonas* occurs via differential *HYDA1* gene transcription. *HYDA1* transcript levels increase in algae transferred to anaerobic conditions in the dark (Happe and Kaminski, 2002) or in illuminated, S-deprived algae (Hemschemeier et al., 2008b), paralleled by the appearance of *HYDA1* protein (Happe and Kaminski, 2002; Zhang et al., 2002; Jacobs et al., 2009). A region from position –128 to –21 relative to the transcription start site of the *HYDA1* gene was shown by reporter gene studies to be sufficient for measurable *HYDA1* promoter activity (Stirnberg and Happe, 2004). However, oxygen sensors, downstream signaling cascades, transcriptional regulators, or essential promoter motifs regulating *HYDA1* expression are currently unknown. While in general, no specific oxygen-responsive transcription factor is known in *C. reinhardtii*, it was noted some years ago that the copper (Cu) deficiency responsive genes encoding cytochrome *c<sub>6</sub>* (*CYC6*), oxidative coproporphyrinogen oxidase1 (*CPX1*), and Copper Response Defect1 (*CRD1*), one isoform of magnesium-protoporphyrin IX monomethyl ester aerobic oxidative cyclase, were also transcriptionally induced upon hypoxic conditions (Quinn et al., 2002; Eriksson et al., 2004; Kropat et al., 2005). Additionally, the amounts of the transcript encoding *Chlamydomonas* ferredoxin isoform *FDX5*, which were first noted to increase strongly in anaerobic algal cells (Mus et al., 2007; Jacobs et al., 2009), were also shown to rise under conditions of Cu depletion (Terauchi et al., 2009; Lambertz et al., 2010; Castruita et al., 2011). In all cases mentioned above, the transcription factor COPPER RESPONSE REGULATOR1 (CRR1) was shown to be responsible for differential transcription (Quinn et al., 2002; Eriksson et al., 2004; Kropat et al., 2005; Lambertz et al., 2010). This multidomain transcription factor belongs to the *SQUAMOSA*-PROMOTER BINDING PROTEIN (SBP) family and has a characteristic zinc-finger domain that binds to the cis-acting DNA element GTAC (Birkenbihl et al., 2005; Kropat et al., 2005). A recent study proposed that the coregulation of genes during the two environmental conditions of Cu and O<sub>2</sub> deficiency might be due to a coordinated activation of the CRR1 protein through the SBP domain and a C-terminal Cys-rich region (Sommer et al., 2010). Lately, it was shown by deep sequencing of the transcriptome of Cu-deficient *C. reinhardtii* cells that the group of genes regulated in response to both Cu and O<sub>2</sub> limitation is larger than expected (Castruita et al., 2011). Transcript levels of several genes known to be induced in anaerobiosis increased during Cu deficiency (Castruita et al., 2011). Among those were *HYDA1*, *HYDG*, and *HYDEF*, the two latter encoding maturases of the [FeFe]-hydrogenases in *C. reinhardtii* (Posewitz et al., 2004), or *PYRUVATE FERREDOXIN OXIDOREDUCTASE1* (Mus et al., 2007). However, a direct influence of CRR1 on *HYDA1* expression was not postulated.

This study aimed at identifying regulative elements of *HYDA1* gene expression. For this purpose, a mutant library was generated using a *C. reinhardtii* strain carrying

the arylsulfatase reporter gene (Davies et al., 1992) fused to the *HYDA1* promoter (Stirnberg and Happe, 2004). Screening of the insertional mutant library resulted in the identification of a transformant impaired in *HYDA1* expression that had a partial deletion of the *CRR1* gene. Complementation of this strain with the full-length *CRR1* genomic sequence restored the phenotype, indicating that the CRR1 transcriptional regulator is involved in *HYDA1* promoter regulation. In silico analysis of the *HYDA1* promoter sequence showed the presence of two GTAC motifs as putative CRR1 binding sites. Reporter gene analyses employing the codon-optimized *Renilla reniformis* luciferase encoding gene (termed *CRLUC*; Fuhrmann et al., 2004) revealed that the presence of intact GTAC motifs is necessary for complete *HYDA1* promoter activity. The combined results of this study indicate that CRR1 is involved in regulating *HYDA1* gene transcription, probably in concert with (a) yet-unknown transcription factor(s).

## RESULTS

### Isolation of *C. reinhardtii* Transformants with Impaired *HYDA1* Expression

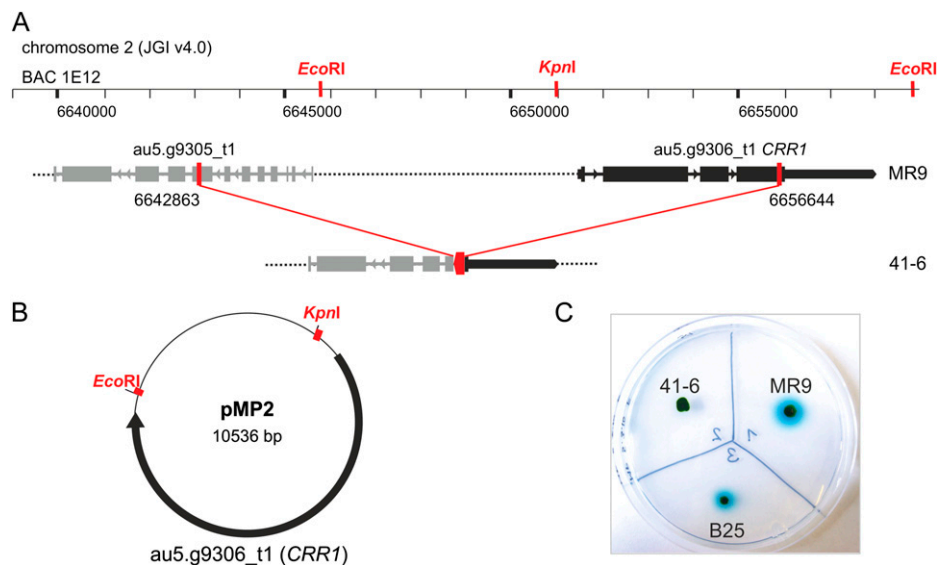
*C. reinhardtii* strain MR9 carries a *pHYDA1-ARYLSULFATASE2 (ARS2)* reporter gene construct (Supplemental Fig. S1A). The region of the *HYDA1* promoter that was fused to the promoterless *ARS2* gene (–1,020 to +158 relative to the transcription start site) was chosen to be sufficiently long to include possible regulating elements. It carried about 500 bp at its 5'- and 114 bp at its 3'-end, respectively, in addition to the longest promoter fragment (–474 to +44) already shown to be able to confer hypoxia responsiveness to the *ARS2* reporter gene (Stirnberg and Happe, 2004). To identify genes whose products are involved in *HYDA1* gene expression, a mutant library was generated by DNA insertional mutagenesis of strain MR9 using the paromomycin resistance cassette derived from plasmid pSL18 (Sizova et al., 2001). About 10,000 transformants were exposed to anaerobiosis to induce *HYDA1* promoter activity and thus *ARS2* reporter gene expression. *ARS* activity was assayed using the artificial *ARS* substrate 5-bromo-4-chloro-3-indolyl sulfate (X-SO<sub>4</sub>). Most of the transformants exhibited anaerobically inducible arylsulfatase activity, showing both that the chosen *HYDA1* upstream region was able to activate *ARS2* gene expression and that the construct was stable in the individual colonies (Supplemental Fig. S1B). The absence of a blue staining thus served as an indicator that an individual transformant was impaired in *HYDA1* promoter activity. We identified three transformants that stayed colorless during the screening. One of these, named strain 41-6 in the following, contained an intact *pHYDA1-ARS2* construct as indicated by the presence of the *HYDA1*-promoter/*ARS2* gene junction analyzed by PCR (data not shown). The transformant was also able to produce active arylsulfatase upon S deficiency (data not shown).

### The *CRR1* Gene Is Partially Deleted in *C. reinhardtii* Strain 41-6

According to Southern-blot analyses using an *APHVIII*-specific probe, transformant 41-6 has a single insertion of the paromomycin resistance cassette (Supplemental Fig. S2). Both 5'- and 3'-regions flanking the cassette were identified by inverse PCR (Fig. 1A). The 5'-end of the cassette is located at position 6,656,644 on chromosome 2 (JGI4.0, <http://genome.jgi-psf.org/Chlre4/Chlre4.home.html>), while the 3'-end is at position 6,642,863 on the same chromosome. According to the most recent genome annotations on JGI4.0 and Phytozome (<http://www.phytozome.net/chlmy.php>; Merchant et al., 2007), the integration of the selection marker resulted in the deletion of 13,781 bp and the partial disruption of two genes. The functional annotation of the 5'-flanking gene (JGI3.0 protein ID 148820, Augustus5 model au5.g9305\_t1, Augustus10 model on Phytozome: Cre02.g125200) on Phytozome is Panther: 19418 (homeobox protein). Motif and domain searches conducted in this work using various publicly available search engines resulted in only one hit on InterProScan (Zdobnov and Apweiler, 2001), indicating that the putative gene product might belong to an unnamed Panther family PTHR15377. The family includes proteins with putative DNA-binding and transcription factor activity.

The gene flanking the 3'-end of the paromomycin cassette in strain 41-6 is the *CRR1* gene (JGI3.0 protein ID 195928, Augustus5 model au5.g9306\_t1, Augustus10 model Cre02.g125250; Kropat et al., 2005), whose first four exons have been deleted (Fig. 1A).

In silico analyses of the *HYDA1* promoter region identified two GTAC motifs (see below). GTAC cores are known to be essential for the DNA-binding activity of *CRR1* (Quinn et al., 2000; Kropat et al., 2005) and other SBP-box transcription factors (Birkenbihl et al., 2005). Therefore, the *CRR1* gene was chosen as a first candidate for complementation of *C. reinhardtii* strain 41-6. The *CRR1* gene including 171 bp up- and downstream from the annotated transcriptional start and stop sites (as annotated on JGI4.0, model au5.g9306\_t1) was obtained by subcloning fragments of the bacterial artificial chromosome BAC1E12 as described in details in the "Materials and Methods" section (Fig. 1, A and B). Strain 41-6 was then cotransformed with the *CRR1* construct and a hygromycin B resistance cassette (Berthold et al., 2002). Reconstruction of the phenotype was tested applying the same arylsulfatase screening that had been used to identify strain 41-6. Out of 114 hygromycin-resistant transformants, 17 showed a restoration of the blue staining after addition of X-SO<sub>4</sub> solution to anaerobically adapted colonies. Transformant B25 was chosen for further experiments (Fig. 1C).



**Figure 1.** Genetic characterization and complementation of *C. reinhardtii* strain 41-6. A, Scheme of the genomic region that is altered in strain 41-6. The wild-type region on chromosome 2 as annotated on JGI4.0 contains a putative gene encoding a protein with unknown function (Augustus 5 model au5.g9305\_t1) and the *CRR1* gene (au5.g9306\_t1). In strain 41-6, the integration of the paromomycin resistance cassette resulted in a deletion of 13.78 kb from position 6,642,863 to 6,656,644 of chromosome 2. The sequence present in BAC1E12 including restriction sites used in this study is shown above the gene graph. B, Map of plasmid pMP2 used for complementation of strain 41-6 with the *CRR1* gene derived from BAC1E12. C, Photograph of a TAP agar plate with colonies of *C. reinhardtii* strains MR9, 41-6, and complemented strain B25. Strains were subjected to the same conditions that were applied during the screening of the MR9 progenies.

### In Vitro Hydrogenase Activity and *HYDA1* Gene Expression Are Impaired in *C. reinhardtii* *crr1* Mutant Strains

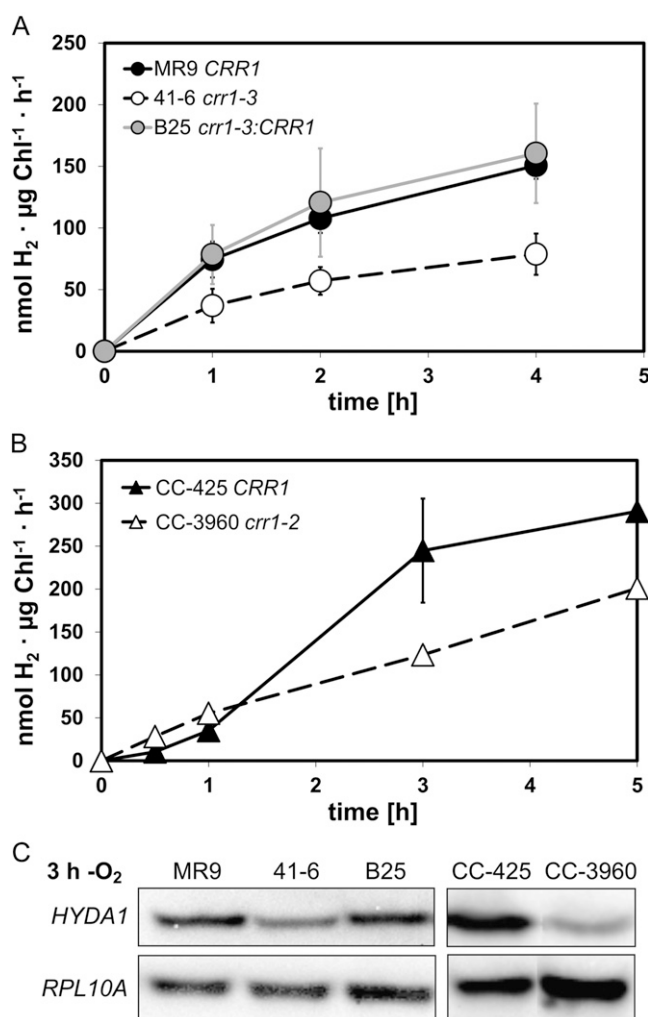
We analyzed *C. reinhardtii* strains MR9 (*pHYDA1-ARS2*), 41-6 (*pHYDA1-ARS2 crr1-3*), and B25 (*pHYDA1-ARS2 crr1-3:CRR1*) regarding hydrogenase activity and *HYDA1* transcript amounts. *C. reinhardtii crr1-2* mutant CC-3960 was analyzed to confirm that the results were due to the absence of CRR1, and CC-425 (*CRR1*) was used as a wild-type control for this strain (Kropat et al., 2005). During anaerobiosis established by nitrogen flushing, both *crr1* mutant strains showed a lower in vitro hydrogenase activity when compared with the respective wild types. In strain 41-6, hydrogenase activity reached about 50% of wild-type activity, while the same rates as determined in MR9 were detected in complemented strain B25 (Fig. 2A). Strain CC-3960 showed 65% of the activity determined in *C. reinhardtii* CC-425 (Fig. 2B).

Under these conditions, *HYDA1* transcript levels of both *crr1* allelic mutants were lower than in their respective parental strains (Fig. 2C). *HYDA1* signals of *crr1-3* mutant 41-6 were 30% of the amounts detected in the parental strain MR9. In the complemented strain *crr1-3:CRR1* (B25), the *HYDA1* signal strength reached 80% of the signal in strain MR9. In strain CC-3960 (*crr1-2*), *HYDA1* mRNA abundance reached 20% of the amount detected in *C. reinhardtii* strain CC-425 (Fig. 2C). These values were similar to those obtained in Cu-deficient cells. As determined by quantitative real-time PCR (qPCR), relative *HYDA1* transcript abundance in Cu-deficient cultures of strain 41-6 was about 33% of *HYDA1* mRNA amounts detected in the parental strain MR9 (Supplemental Fig. S3). Castruita et al. (2011) showed that strain CC-3960 (*crr1-2*) had about 20% of the *HYDA1* transcript amounts of its complemented strain when subjected to Cu deficiency.

S deprivation is another well-known condition to induce hypoxia and the concomitant changes of gene expression in *C. reinhardtii* (Melis et al., 2000; Nguyen et al., 2008). Because strain MR9 did not establish hypoxic conditions and became chlorotic (data not shown), S-deprivation experiments were only performed with strains CC-3960 and CC-425. Upon S deficiency, in vitro hydrogenase activity of *crr1-2* mutant strain CC-3960 reached about 20% of the rate determined in strain CC-425 (Fig. 3A). *HYDA1* transcript and *HYDA1* protein amounts of S-deficient *C. reinhardtii* CC-3960 were significantly lower than in strain CC-425 and reached 20% on average each (Fig. 3, B and C).

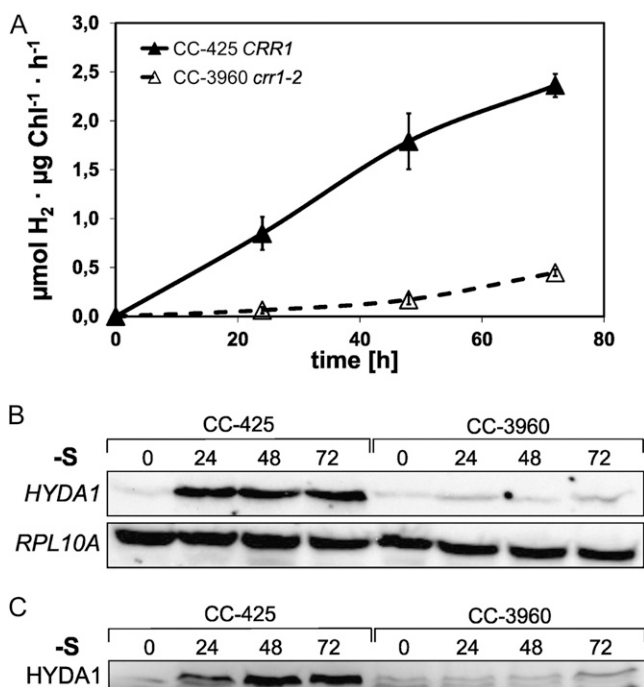
### *HYDA1* Expression Is Inhibited by Mercuric Ions

Hg(II) can deactivate the expression of CRR1-dependent genes both in Cu and O<sub>2</sub> deficiency (Hill et al., 1991; Quinn et al., 2000, 2002). We investigated the effect of subtoxic concentrations of HgCl<sub>2</sub> (10 μM; Quinn et al., 2002) on hydrogenase activity as well as



**Figure 2.** Analysis of in vitro hydrogenase activity and *HYDA1* transcript levels in *crr1* mutant strains compared with the respective parental strains upon artificially induced anaerobiosis. A and B, Concentrated cell suspensions were subjected to anaerobic conditions by nitrogen flushing. At the indicated time points, cell samples were withdrawn and analyzed in an in vitro hydrogenase activity assay containing Triton X-100 as a detergent to lyse the cells and reduced methyl viologen as an artificial electron donor of the [FeFe]-hydrogenase. Hydrogen generated in these assays was quantified by gas chromatography. Average values  $\pm$  SD (error bars) of three independent experiments are shown. A, *C. reinhardtii* strains MR9 (*pHYDA1-ARS2 CRR1*; black circles), 41-6 (*pHYDA1-ARS2 crr1-3*; white circles), and B25 (*pHYDA1-ARS2 crr1-3:CRR1*; gray circles). B, Strains CC-425 (*CRR1*; black triangles) and CC-3960 (*crr1-2*; Kropat et al., 2005; white triangles). C, RNA-hybridization analyses using DIG-labeled *HYDA1*- and *RPL10a*-specific probes. Total RNA was isolated from *Chlamydomonas* cells after 3 h of anaerobic incubation. *HYDA1* transcript amounts in all strains were analyzed in three independent experiments. Each one representative result is shown. The quantitative analysis of the signal strength is described in the text.

on transcript levels of several marker genes known to be induced upon Cu deficiency and/or anaerobiosis. For this purpose, *C. reinhardtii* CC-124 cultures were



**Figure 3.** Analysis of in vitro hydrogenase activity (A), *HYDA1* transcript (B), and *HYDA1* protein (C) levels in *crr1* mutant CC-3960 and strain CC-425 upon S deficiency. A, Cells were subjected to S deprivation in sealed flasks in the light. At the indicated time points, cell samples were withdrawn and analyzed by in vitro hydrogenase activity assays. Black triangles: CC-425 (*CRR1*), white triangles: CC-3960 (*crr1-2*; Kropat et al., 2005). Average values  $\pm$  SD of two independent experiments are shown. B, RNA-hybridization analyses using DIG-labeled *HYDA1*- and *RPL10a*-specific probes on RNA samples isolated from S-deprived cells after the indicated time points. C, *HYDA1* immunoblot analyses using polyclonal anti-*C. reinhardtii*-*HYDA1* antibody on crude protein extracts prepared from S-starved cultures. *HYDA1* transcript and *HYDA1* protein amounts were analyzed from both strains examined in two independent experiments. One representative result is shown.

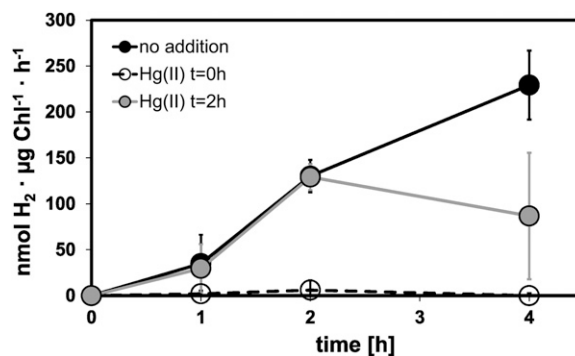
shaded and sealed to let them consume  $\text{O}_2$  by respiration and establish anaerobic conditions. One culture remained untreated and served as the control. Hg(II) was added to a second culture at the beginning of the incubation ( $t = 0$  h), and to a third culture after 2 h of anaerobic induction, when the cells had already developed hydrogenase activity and accumulated *HYDA1* transcript ( $t = 2$  h; Fig. 4; Table I).

Cells exposed to Hg(II) from the beginning of the experiment did not show any in vitro hydrogenase activity. Addition of Hg(II) after 2 h of anaerobic induction resulted in a decrease of the in vitro hydrogenase activity developed by then (Fig. 4). Relative abundances of transcript amounts of the indicated genes (Table I) were analyzed by qPCR. *HYDA1* transcript amounts in cells treated with Hg(II) at  $t = 0$  h remained almost as low as in the aerobic control cells. When mercuric ions were added after 2 h, they returned to the control value (Table I). Abundances of

*FDX5*, *HYDEF*, and *HYDG* transcripts showed the same patterns as described for the *HYDA1* gene in Hg(II)-treated and control cells (Table I). *CRR1* transcripts did also respond to Hg(II) in this assay and decreased 4- to 7-fold upon its addition (Table I). The amounts of the PYRUVATE FORMATE LYASE1 (*PFL1*) encoding *PFL1* transcript were not significantly influenced by Hg(II) (Table I).

#### Two GTAC Motifs Have an Influence of *HYDA1* Promoter Activity

An in silico analysis of the *HYDA1* promoter revealed two GTAC sites in position  $-173$  and  $-84$  relative to the transcription start site. Fragments of the putative *HYDA1* promoter region in which the GTAC sequences were individually or simultaneously mutagenized were examined regarding their capacity to activate the expression of the luciferase-encoding reporter gene *CRLUC* (Fig. 5). Furthermore, two additional stretches of bp were mutated. These were tentatively named motif 1 (M1) and motif 2 (M2). Their analysis appeared interesting because M1 (AAGCTCGC,  $-23$  relative to the transcription start site) appeared twice in the putative *HYDA1* promoter and the same sequence of bp was identified in the upstream region of *HYDA2* of *Chlamydomonas moewusii* (Kamp et al., 2008; data not shown). M2 (CGCAGGCAC,  $+26$  relative to the transcription start point) is present in the *C. reinhardtii* and *C. moewusii* *HYDA2* upstream regions. These observations and the fact that both M1 and M2 are located in a region that is able to allow reporter gene expression (Stirnberg and Happe, 2004; and this study, see below) prompted us to introduce mutations in each of these sequences, too (Fig. 5). Furthermore, the



**Figure 4.** Analysis of the effect of mercuric ions on in vitro hydrogenase activity in *C. reinhardtii* strain CC-124. Cell suspensions were transferred to sealed flasks in the dark to allow respiratory  $\text{O}_2$  consumption. One culture was treated with  $10 \mu\text{M}$   $\text{HgCl}_2$  from the beginning on ( $t = 0$  h; white circles), and another after 2 h of dark incubation ( $t = 2$  h; gray circles). Untreated cells served as a control (black circles). In vitro hydrogenase activity was determined at the indicated time points. Values shown are means of three independent experiments. Error bars indicate the SD.

**Table 1.** Analysis of the effect of mercuric ions on relative transcript abundances of the genes indicated in the first row

RNA was isolated from the same *C. reinhardtii* CC-124 cultures described in the legend of Figure 4, treated with DNase, and used as a template for cDNA synthesis. Relative transcript abundances were calculated according to Pfaffl (2001), using the constitutively expressed *CBLP/RCK1* gene (Cre13.g599400 on Phytozome) as reference gene. Each value represents the average  $\pm$  SD of technical triplicates from two independent biological experiments.

Treatment	Sampling	<i>HYDA1</i>	<i>HYDEF</i>	<i>HYDG</i>	<i>PFL1</i>	<i>FDX5</i>	<i>CRR1</i>
	0 h	2.7 $\pm$ 0.1	0.1 $\pm$ 0.01	0.2 $\pm$ 0.05	6.9 $\pm$ 1.9	0.03 $\pm$ 0.02	7.7 $\pm$ 2.4
No addition	2 h	227.1 $\pm$ 74.8	9.4 $\pm$ 1.3	19.3 $\pm$ 2.2	488.0 $\pm$ 60.8	69.7 $\pm$ 17.3	13.7 $\pm$ 0.5
Hg(II) at <i>t</i> = 0 h	2 h	10.4 $\pm$ 2.8	0.5 $\pm$ 0.02	1.7 $\pm$ 0.3	196.5 $\pm$ 51.5	0.1 $\pm$ 0.01	2.3 $\pm$ 7.8
Hg(II) at <i>t</i> = 2 h	2 h <sup>a</sup>	82.1 $\pm$ 3.5	5.3 $\pm$ 0.1	15.5 $\pm$ 1.7	217.3 $\pm$ 13.7	22.8 $\pm$ 15.3	7.8 $\pm$ 1.8
Hg(II) at <i>t</i> = 2 h	4 h	11.4 $\pm$ 0.7	0.9 $\pm$ 0.3	2.1 $\pm$ 0.3	194.2 $\pm$ 33.9	2.4 $\pm$ 2.5	0.9 $\pm$ 0.7

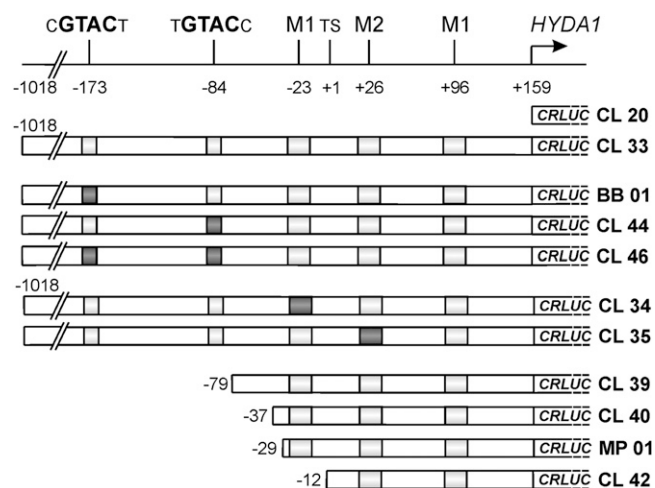
<sup>a</sup>Culture samples for RNA isolation were withdrawn before the addition of Hg(II).

*HYDA1* upstream region was sequentially truncated to refine the absolute promoter length requirements. Figure 5 shows a schematic overview of the constructs analyzed in this study. *C. reinhardtii* wild-type strain CC-124 served as the recipient of the respective plasmids. Paromomycin-resistant progenies were subjected to S deprivation in sealed flasks to induce hypoxic conditions (Melis et al., 2000) or to Cu deficiency. Luciferase activity of several randomly chosen colonies of each transformant library was determined under both conditions.

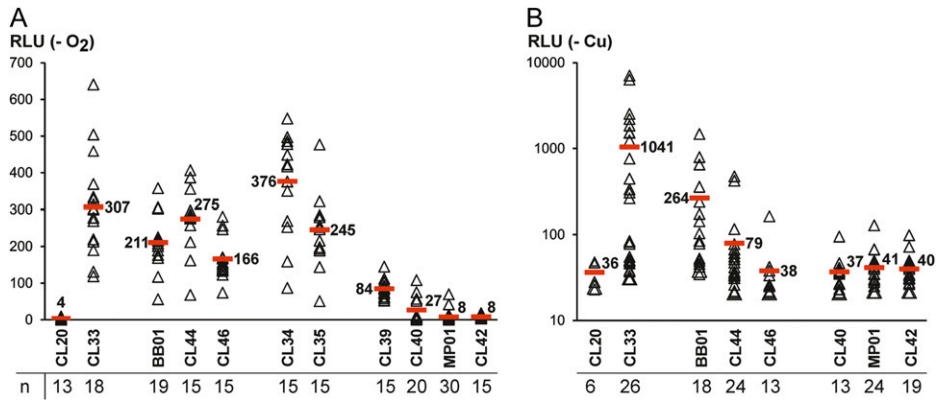
Upon S deficiency, transformants carrying the promoterless *CRLUC* gene (CL20) exhibited an average luciferase activity of 4 relative light units (RLUs). This was as low as the average luciferase activity detectable in transformants in which *CRLUC* was under the control of the longest *HYDA1* upstream region of 1,177 bp (construct CL33) incubated aerobically in replete medium (3.2  $\pm$  2.1 RLU, *n* = 15). In contrast, cells carrying construct CL33 reached 307 RLU under S deprivation. Mutation of either GTAC motif caused a moderate decrease of luciferase activity, which accounted to 211 (minus 31%) when the distal GTAC (construct BB01) and 275 (minus 10%) when the proximal GTAC (CL44) was changed. S-starved transformants carrying a construct in which both GTAC sites were mutated exhibited a still-lower average luciferase activity of 166 RLU (minus 46%; Fig. 6A). Nucleotide exchanges within motif M1 (CL34) resulted in a mean of 376 RLU (plus 22%) and an altered motif M2 (CL35) led to moderately lower luciferase activities of the transformants (245 RLU, minus 20%; Fig. 6A). *C. reinhardtii* strains carrying constructs in which the *HYDA1* promoter was truncated showed a strongly reduced luciferase activity. When a *HYDA1* upstream region from -79 to +159 (relative to the transcription start point) was fused to the *CRLUC* gene (CL39), S-deprived algae exhibited 84 RLU (minus 73%) on average, and a further truncation by 40 nucleotides (CL40) led to a mean luciferase activity of 27 RLU (minus 91%). Fragments from -29 (MP01) and -12 (CL42), respectively, to +158 resulted in only 8 RLU in the respective *Chlamydomonas* strains (Fig. 6A).

When the algal transformants were subjected to Cu deficiency, the average activity of strains carrying the longest *HYDA1* promoter construct (CL33, -1,018 to +158) was 1,041 RLU (Fig. 6B). Mutation of the GTAC

motif at position -173 (BB01) led to a decrease of measurable light emission by 75% down to 264 RLU, and when the GTAC motif at position -84 was altered (CL44), it decreased by 92% down to 79 RLU (Fig. 6B). In transformants carrying constructs in which both GTAC motifs were mutated, the average luciferase activity was as low as 38 RLU, which was very close to the activity



**Figure 5.** Scheme of the *HYDA1* upstream region (top) and of chimeric constructs of various fragments of the *HYDA1* promoter fused to the luciferase-encoding *CRLUC* reporter gene analyzed in this study (bottom). A schematic of the *HYDA1* upstream region from position -1,018 to +159 is shown at the top. Positions and 6-nucleotide sequences of GTAC sites including the 3' and 5' adjacent nucleotides are indicated. M1 and M2 refer to tentatively assigned motifs which appeared in the upstream regions of the *C. reinhardtii* and *C. moewusii* *HYDA2* genes in the exact same sequence (see text for details). An arrow labeled with *HYDA1* indicates the translation start codon ATG. Chimeric constructs are depicted below. Names of the constructs are indicated on the right. Length of the *HYDA1* promoter fragments are indicated by numbers on the left, which refer to the position relative to the transcription start site (TS). Gray boxes indicate the presence of the wild-type sequences of the GTAC sites and the sequences termed M1 and M2. Black boxes show that the respective sequence has been mutated. GTAC sites were exchanged by GGCC (distal) and TGAC (proximal), respectively. M1 (AAGCTCGC) was changed to CTCATCGC, and M2 (CGCAGGCAC) was altered to ACAAGGCAC.



**Figure 6.** A and B, Luciferase activity determined in *C. reinhardtii* transformants equipped with the *CRLUC* gene under the control of various wild-type and mutated *HYDA1*-promoter fragments. Names of constructs which were used to transform strain CC-124 are written below the x axes and represent the sequences schematically shown in Figure 5. Cells were either subjected to S deficiency in sealed flasks in the light (A) or to Cu deficiency (B). After 72 h of incubation in either condition, 200- $\mu$ L cell samples were either directly used for the assay (A) or frozen in liquid nitrogen until luciferase activity was measured after addition of coelenterazine. Several transformants of each construct library were analyzed (indicated as n below the graphs). Each triangle represents the RLU value obtained with a single transformant. Red bars indicate the mean values, which are also shown as numbers next to the bars. The average luciferase activity detectable in CL33 transformants incubated aerated in replete medium reached  $3.2 \pm 2.1$  RLU ( $n = 15$ ; not shown). [See online article for color version of this figure.]

measured in case of the promoterless construct CL20 (36 RLU; Fig. 6B). The effect of mutations in either or both GTAC sites on *HYDA1* promoter activity was therefore much stronger in Cu-deficient than in hypoxic cultures. Strains that were transformed with constructs in which truncated *HYDA1* promoter fragments were fused to the *CRLUC* gene (CL40, MP01, and CL42) exhibited luciferase activity close to the control strain in Cu-deficient medium (Fig. 6B).

### The SBP Domain of CRR1 Binds to *HYDA1* Promoter Fragments in Vitro

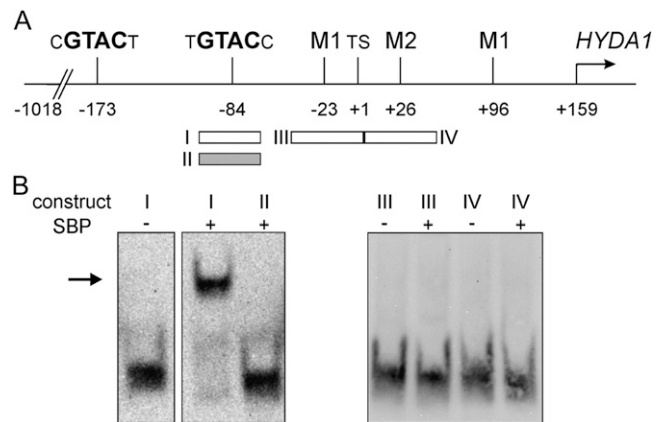
The heterologously produced SBP domain of CRR1 (Sommer et al., 2010) was examined by electrophoretic mobility shift assays (EMSA) regarding its ability to bind to a GTAC motif of the *HYDA1* promoter. A labeled fragment containing the wild-type sequence of the proximal GTAC (position -84; Fig. 7A) showed a shift when incubated with the CRR1-SBP domain, while the same fragment containing a TAGA mutation did not shift (Fig. 7B). As a control it was tested if the SBP domain would be able to cause a shift of fragments carrying motifs M1 or M2, but no shift was observed (Fig. 7B).

## DISCUSSION

### The Absence of an Active CRR1 Transcriptional Regulator Impairs *HYDA1* Gene Expression

Expression of the *C. reinhardtii* *HYDA1* gene encoding the [FeFe]-hydrogenase *HYDA1* is up-regulated in anaerobic cultures in the dark as well as in illuminated S-, nitrogen-, or Cu-deprived cells (Happe and Kaminski,

2002; Zhang et al., 2002; Hemschemeier et al., 2008b; Castruita et al., 2011; Philipps et al., 2012). So far, regulatory signals or transcription factors affecting *HYDA1* expression were not known. By applying an *ARS2* reporter-gene-based screening procedure we isolated *Chlamydomonas* transformant 41-6 (termed *crr1-3*). This strain has a truncation of the *CRR1* gene and is impaired



**Figure 7.** EMSAs analyzing the capacity of the heterologously produced CRR1 SBP domain to bind to *HYDA1* promoter fragments. A, Schematic drawing representing the genomic upstream region of the *HYDA1* gene as shown in Figure 5. Boxes below this scheme indicate the fragments used for EMSAs in their wild-type form (white boxes) or in a mutated form (gray box; GTAC was exchanged by TAGA). The individual fragments are labeled with Roman numerals, which assign them to the respective EMSA shown in B. B, DIG-labeled fragments I to IV were incubated without (-) or with (+) 200 ng of the recombinant CRR1 SBP domain, separated by native PAGE, and visualized using antidigoxigenin antibody.

in *HYDA1* promoter activity and *HYDA1* transcript accumulation. The well-studied *crr1-2* mutant strain CC-3960 (Eriksson et al., 2004; Kropat et al., 2005) also showed lower *HYDA1* mRNA amounts and *HYDA1* in vitro activity in anaerobic cultures, corroborating the results obtained with strain 41-6.

The transcriptional response of several CRR1 target genes has been shown to be inactivated in the presence of mercuric ions (Hill et al., 1991; Quinn et al., 2002). A recent study showed that Hg(II) interferes with DNA binding of the SBP domain and it was discussed that mercury, which is able to replace Cu in various Cu-binding proteins, acts as a Cu mimic (Sommer et al., 2010). The fact that the addition of HgCl<sub>2</sub> to anaerobic *C. reinhardtii* wild-type cultures prevented *HYDA1* transcript accumulation in this study is a further indication that *HYDA1* gene expression is affected by a Cu signaling pathway. Both the repressing influence of mercuric ions (this study) and the activating effect of Cu deficiency (Castruita et al., 2011; this study) could also be observed in cases of *HYDEF* and *HYDG* transcripts, encoding *HYDA1* maturases (Posewitz et al., 2004) and coaccumulating with *HYDA1* in all conditions tested so far (Mus et al., 2007; Nguyen et al., 2008). These observations support the shared O<sub>2</sub> and Cu responsiveness of genes essential for H<sub>2</sub> production in *C. reinhardtii*.

The effect of mercuric ions on *HYDA1* transcript levels observed in this study differed from previous results in which Hg(II) was shown to have no effect on *HYDA1* mRNA amounts (Quinn et al., 2002). However, both the experimental setup and the final Hg(II) concentrations varied in the two studies. Since the inactivating influence of mercury on *CPX1* and *CYC6* transcription was shown to be concentration dependent (Hill et al., 1991; Quinn et al., 2002), our observation of impaired *HYDA1* gene expression was probably due to the higher amounts of Hg(II) (10 μM). This mercuric ion concentration has been discussed not to be toxic for *Chlamydomonas* cells (Hill et al., 1991; Quinn et al., 2002), and the observation that *PFL1* transcript levels were not strongly affected by mercury in this study is in line with this view.

#### GTAC Sites Are Important for Maximum *HYDA1* Promoter Activity

CRR1 binds to a GTAC core sequence of Cu or hypoxia response elements (Quinn et al., 2000, 2002). The two GTAC motifs found at positions -173 and -84 relative to the transcription start site of the *HYDA1* gene are in a similar proximity to the transcription start site as has been shown for several essential GTAC sites of other CRR1 target genes (Quinn et al., 2000, 2002; Allen et al., 2008; Page et al., 2009; Lambertz et al., 2010). We analyzed the influence of these GTAC motifs on *HYDA1* promoter activity in S- and Cu-depleted algal transformants.

In Cu deficiency, mutations in either one or both of the GTAC sites resulted in a pronounced effect. As

indicated by luciferase activity, Cu-responsive *HYDA1* promoter activity reached 25% of the full promoter activity when the distal and 8% when the proximal GTAC site was mutated. The *HYDA1* promoter in which both GTAC motifs were mutated had no significant activity at all. Since the absence of GTAC sites should mimic the effect of CRR1 deficiency, the latter result is not consistent with the moderate, but significant increase of *HYDA1* transcript levels in the Cu-deficient *crr1-2* mutant CC-3960 (Castruita et al., 2011) or strain 41-6 (*crr1-3*, this study). A simple explanation for the discrepancy might be an insufficient resolution or sensitivity of the *CRLUC*-based reporter gene assay (Shao and Bock, 2008).

Upon S deficiency, *HYDA1* promoter activity was less impaired by mutations of the GTAC sites. Only when both GTAC motifs were altered, a decrease of the luciferase activity by 50% was observed. Mutations in individual GTAC motifs resulted in only a 31% (distal) or 10% (proximal) decrease of RLU values. These results indicate that the factors and/or signaling pathways influencing *HYDA1* promoter activity differ in hypoxia versus Cu deficiency.

#### Activation of *HYDA1* Expression Requires Additional Regulatory Components

By comparing transcript amounts under Cu and O<sub>2</sub> deficiency (Quinn et al., 2002; Kropat et al., 2005) as well as number and importance of GTAC motifs in the promoter regions of CRR1 targets (Quinn et al., 2000, 2002; Allen et al., 2008; Page et al., 2009; Lambertz et al., 2010), it has been noted before that the regulation of CRR1-activated genes does not follow a simple pattern. For example, *CPX1* transcription seems to be more responsive to anaerobiosis than to the absence of Cu, while it is the opposite in case of the *CYC6* gene (Quinn et al., 2002). For Cu-dependent expression of the *CPX1* gene, a single Cu Response Element is sufficient, but an additional GTAC motif termed Hypoxia Response Element is necessary for hypoxia responsiveness of *CPX1* (Quinn et al., 2002). A previous elaborate study examining the function of individual domains of CRR1 showed that the accumulation of *CYC6* transcript under anaerobic conditions depended on the presence of the Cys-rich C terminus, and, to a lower extent, an ankyrin-repeat region of CRR1, while this was not the case upon Cu depletion (Sommer et al., 2010). A conclusion that can be drawn is that CRR1-dependent gene expression is multifaceted and that triggering signals are integrated in different ways.

It has been shown recently that the effect of CRR1 on transcription of *CYC6*, *CPX1*, and *CRD1* is mediated by an opening of the chromatin structure of the respective promoter regions (Strenkert et al., 2011). Due to the constitutively open chromatin state of the *CRD1* promoter under noninducing conditions, the authors suggested that an additional unknown factor is probably involved in *CRD1* gene expression (Strenkert et al., 2011).



**Table II.** List of oligonucleotides used for amplification and mutation of *HYDA1* promoter fragments for generation of reporter gene constructs as well as oligonucleotides used in EMSAs

*Apal* and *XhoI* restriction sites are underlined. Inserted mutations are indicated in boldface type.

Name	Template	Oligonucleotide
Constructs created by PCR		
CL33	gDNA	HYDA1_rev: GCCTCGAGCTTGTCCGCTCTACGATATTAG CL33_for: GGGGCCCCGATGATATGGATCGTCTGGTG
CL39	CL33	CL39_for: GGGGCCCCGCCGAGTGTCCGCTGCATTC
CL40	CL33	CL40_for: ATGGGCCCCGTCGCGCATAGGCAAGCTC
MP01	CL33	MP01_for: TGGGGCCCCATAGGCGTACTCGCAAATGCTGT
CL42	CL33	CL42_for: CTGGGCCCTGCTGTACGCTTATCTTACAT
Constructs created by site-directed mutagenesis		
CL34	CL33	1: SDM_UP: GGGGCCCCGATGATATGGATCGTCTGGTG 2: SDM_RP: GCCTCGAGCTTGTCCGCTCTACGATATTAG A: SDM_pCL34_A: ACAGCATTTGCGAT <b>GAGGC</b> CTAT B: SDM_pCL34_B: CATAGG <b>CCTCAT</b> CGCAAATGCTGT
CL35	CL33	A: SDM_pCL35_A: TTGAGGCTAGTGCCT <b>TTG</b> TCGAGTGTT B: SDM_pCL35_B: AACACTCG <b>ACA</b> AGGCACTAGCCTCAA
BB01	CL33	A: SDM_pBB01_A: CTCCTATACT <b>AGGCCG</b> CATTGTTC B: SDM_pBB01_B: GGAACAAT <b>GCGGC</b> CTAGTATAGGA
Constructs created by QC-PCR		
CL44	CL33	QC_for: <b>GTTGAC</b> CGCCGAGTGTCCGCTGCATTC QC_rev: CACTCGGCG <b>GTCA</b> ACCCCGTCAGCAC
CL46	BB01	QC_for: <b>GTTGAC</b> CGCCGAGTGTCCGCTGCATTC QC_rev: CACTCGGCG <b>GTCA</b> ACCCCGTCAGCAC
Fragments used in EMSAs		
I		I-pHYDA1for: GACGGGGTGTACCGCCGAGTGTC I-pHYDA1rev: GACACTCGGCGGTACACCCCGTC
II		II-pHYDA1for: GACGGGG <b>TTAGAC</b> CGCCGAGTGTC II-pHYDA1rev: GACACTCGGCG <b>TCTA</b> ACCCCGTC
III		III-pHYDA1for: GGTCGCGCATAGGCAAGCTCGCAAATGCTGTACGCT III-pHYDA1rev: AGCTGACAGCATTTGCGAGCTTGCTATGCGCGACC
IV		IV-pHYDA1for: TGAACACACAACACTCTCGCAGGCACTAGCCTCAA IV-pHYDA1rev: TTGAGGCTAGTGCCTGCGAGAGTGTGGTGTGTTCA

The results reported here show that CRR1 is involved in *HYDA1* promoter activation and *HYDA1* transcript accumulation, but they also indicate that additional factors specific for each particular environmental condition influence its transcription. In luciferase assays, only the truncation of the *HYDA1* promoter down to  $-79$  or  $-37$  resulted in a strong (minus 70% and minus 90%, respectively) decrease of promoter activity in hypoxic *C. reinhardtii* transformants. Therefore, this region might be vital for the binding of an additional factor specific for the condition of  $O_2$  limitation.

The regulation of one gene by distinct cis-acting elements and environmental stimuli is a common

phenomenon. The Arabidopsis (*Arabidopsis thaliana*) *ADH1* gene encoding alcohol dehydrogenase is induced upon cold and dehydration stress and by hypoxia, and some of the critical *ADH1* promoter elements were shown to be specific for individual stress conditions (Dolferus et al., 1994). In *C. reinhardtii*, the *HIGH-CO<sub>2</sub>-INDUCIBLE 43-KD PROTEIN/FE ASSIMILATION1* gene is regulated both under high- $CO_2$ - and iron-deficiency conditions, which act independently via two different response elements (Baba et al., 2011). Multiple stimuli acting on the *HYDA1* promoter could help adjusting *HYDA1* protein levels to metabolic needs. The identification and biochemical characterization of the

**Table III.** Oligonucleotides used for qPCR analyses

Gene and locus names refer to the annotation on Phytozome.

Gene (locus) Name	Forward Oligonucleotide	Reverse Oligonucleotide
<i>RCK1</i> (Cre13.g599400)	GCCACACCGAGTGGGTGTCGTGCG	CCTTGCCGCCCGAGGCGCACAGCG
<i>RPL10a</i> (Cre02.g101350)	GCTTCGGACTCCGTCATCAA	CCATGCACAGCACCTTCTTC
<i>HYDA1</i> (Cre03.g199800)	GCGATTGACGTTGGTGTAGG	GTGCTTACAAGCGGCTGATG
<i>HYDEF</i> (Cre06.g296750)	CTGCATGATTGACGCCGAGA	AGGCGGCCCAAGAGAAGAAC
<i>HYDG</i> (Cre06.g296700)	CCGGTCAATGACGCTGACTT	ATCTGGCTCATGCCGCACTT
<i>PFL1</i> (Cre01.g044800)	CAAGTACGGCAACGACGATG	ACGTTGGAGGTGATGGTACG
<i>FDX5</i> (Cre17.g700950)	CGGCTTCATCCTCATGTGCT	ACGCTGACACGAATGGTACG
<i>CRR1</i> (Cre02.g125250)	TGCGTGTGGTGTGTTTCAGG	GCCAGGTGTGATGGAGAGAC

putative additional factor(s) binding to the *HYDA1* promoter might throw light on the orchestrated Cu- and O<sub>2</sub>-responsive signaling in *C. reinhardtii*.

## MATERIALS AND METHODS

### Organisms and Growth Conditions

*Chlamydomonas reinhardtii* strains CC-124, CC-425, and *crr1-2* mutant strain CC-3960 (Eriksson et al., 2004; Kropat et al., 2005) were obtained from the *Chlamydomonas* Resource Center, University of Minnesota. Strain MR9 was generated as described before (Stimberg and Happe, 2004). It resulted from cotransforming *C. reinhardtii* 388 (*cw15 arg7 nia1 mt<sup>-</sup>*) with plasmid pARG7.8cos (Debuchy et al., 1989) and plasmid pMR9. In the latter, a *HYDA1* promoter region from position -1,020 to +158 relative to the transcription start site as indicated in Stimberg and Happe (2004), is fused to the promoterless arylsulfatase-encoding *ARS2* gene in plasmid pJD54 (Davies et al., 1992). *C. reinhardtii* cells were grown photoheterotrophically in Tris-acetate-phosphate (TAP) medium, pH 7.2 (Harris, 1989, 2009) at a light intensity of 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and a temperature of 20°C.

Cu deficiency of *C. reinhardtii* pHYDA1:CRLUC transformants generated as described below was achieved by growing the cells once in TAP ENEA2 medium as reported previously (Ferrante et al., 2008). Then, a main culture of 20 mL in 50-mL Starstedt tubes was inoculated with up to 1 mL of this pre-culture and the Cu-chelator triethylenetetramine was added to the medium to reach a final concentration of 50  $\mu\text{M}$ . The cells were incubated at a light intensity of 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  until they reached a chlorophyll content of 15  $\mu\text{g mL}^{-1}$ . S deprivation of strains CC-425 and CC-3960 was achieved as described previously (Hemschemeier et al., 2009) using a modified TAP medium, in which sulfate salts were replaced by chloride compounds. S starvation of *C. reinhardtii* pHYDA1:CRLUC transformants was done using the same medium and incubating the strains in 12-mL headspace bottles (Fisher Scientific) as described in detail in Lambertz et al. (2010).

### Anaerobic Induction and Treatment of Cells with Mercuric Chloride

Anaerobic induction by flushing concentrated *C. reinhardtii* cell suspensions with nitrogen gas and in vitro hydrogenase activity assays in lysed cell samples using methyl viologen as an artificial electron donor were conducted as described previously (Hemschemeier et al., 2009). In case of the experiments analyzing the effect of Hg(II), *C. reinhardtii* strain CC-124 was grown until the culture had reached a chlorophyll content of 20  $\mu\text{g mL}^{-1}$ . Cells were harvested by centrifugation, resuspended in the same volume of fresh TAP medium, and transferred to squared glass bottles sealed by Suba seals (Sigma-Aldrich). The culture flasks were then incubated on a magnetic stirrer in the dark to allow respiratory O<sub>2</sub> consumption by the cells. When indicated, mercuric chloride (HgCl<sub>2</sub>) was added to the cells to a final concentration of 10  $\mu\text{M}$  either from the beginning on or after the cells had been incubated for 2 h.

### Transformation of *C. reinhardtii*

*C. reinhardtii* cells were transformed using the glass bead method (Kindle, 1990). Strain MR9 was transformed using 1  $\mu\text{g}$  of the *APHVIII*-encoding paromomycin resistance cassette derived from plasmid pSL18 (Sizova et al., 2001) by *KpnI* and *StuI* digestion. After transformation, cells were streaked on TAP agar plates without paromomycin and incubated in low light (15  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for 24 h. Then, the agar blocks were transferred to petri dishes containing filter paper soaked with a paromomycin solution that resulted in a final concentration of 5  $\mu\text{g mL}^{-1}$  paromomycin in the agar. For generation of *C. reinhardtii* transformants carrying various *HYDA1*:CRLUC reporter gene fusion constructs (see below), wild-type CC-124 was treated with autolysin (Harris, 1989, 2009) prior to transformation with *ScaI*-linearized plasmids.

### Screening for Transformants with Impaired *HYDA1* Promoter Activity

Ten-thousand transformants resulting from transforming *C. reinhardtii* strain MR9 with the paromomycin resistance cassette were tested for impaired *HYDA1* promoter activity by the following screening protocol: The algal colonies were grown on TAP agar plates containing 5  $\mu\text{g mL}^{-1}$  paromomycin for

7 d. Each plate included one colony of *C. reinhardtii* CC-124 transformed with the paromomycin cassette to ensure that the endogenous arylsulfatase, which is produced in S deficiency (de Hostos et al., 1988), was not produced. After growth of the colonies, the plates were transferred to an anaerobic tent to activate the *HYDA1* promoter and 25  $\mu\text{L}$  of a 3-mM solution of the artificial arylsulfatase substrate X-SO<sub>4</sub> were dropped on each colony. Twenty-four hours later, colonies were checked for a blue staining, which would result from cleavage of X-SO<sub>4</sub> by arylsulfatase (Davies et al., 1992). Colonies that did not show a blue staining were candidates for a loss of *HYDA1* promoter activity and were picked for further analyses. To ensure that their phenotype was not due to a general inability to generate active arylsulfatase, the candidate strains were first subjected to S starvation and tested for native arylsulfatase activity. Additionally, the presence of an intact junction between the *HYDA1* upstream region and the *ARS2* gene was analyzed by PCR using the oligonucleotides *Ars02* (5'-GTCGACGGTACCGGTCGCGCATAGG-CAAGCTC-3') and *HydA01* (5'-GTCGACGGTACCGGTCGCGCATAGG-CAAGCTC-3').

### Genetic Characterization and Complementation of Mutant Strain 41-6

Genomic DNA was isolated by a method described previously (Newman et al., 1990). Southern-blot analyses according to standard techniques (Sambrook et al., 1989) were performed to determine the integration frequency of the paromomycin resistance cassette. A probe specific for the *APHVIII* gene was generated by PCR amplification including PCR DIG labeling mix (Roche) using pSL18 as a template and applying the oligonucleotides *aphVIII* (5'-ACGATGCGTTGCCTGC-3') and *aphVIIIrev* (5'-GAGACTGCGATCGAACG-3'). The integration site of the *APHVIII* cassette was determined by inverse PCR: 500 ng of genomic DNA were digested with *PstI*. After inactivation of the enzyme, religation was performed at 16°C overnight using T4 DNA-Ligase (Fermentas) according to the manufacturer's recommendations. Inverse PCR was conducted using 1  $\mu\text{L}$  of 20-fold-diluted ligation mixture in a 25- $\mu\text{L}$  PCR reaction mixture using Biotools *Taq* DNA polymerase (Biotools) as indicated in the manual, except that 2% (v/v) dimethyl sulfoxide was included. The PCR program was as follows: 5 min 96°C, followed by 35 cycles of 1 min 96°C, 1 min 60°C, and 4 min 72°C and a final extension step of 10 min at 72°C. Oligonucleotides used for the first PCR were *Paro07* (5'-GAGTGTCC-GCGGCTTCC-3') and 5'*GSP1* (5'-CATACGCACCAATCATGTCAAGCC-CAG-3'), and a nested PCR was performed using *Paro06* (5'-TACCGGCGTGTGGACGAGTTC-3') and 5'*GSP2* (5'-GTCTGCCTGACAGGAAGTGAA-CGCATGT-3'). For complementation of *C. reinhardtii* strain 41-6, the bacterial artificial chromosome clone number 1E12 (PTQ139 at JGI v4.0) was digested with *EcoRI* to generate a 9,201-bp fragment that included the *CRR1* gene and a fragment of an unknown gene (JGI v4.0 ID519527, au5.g9305\_t1). The fragment was cloned into *EcoRI* linearized pBluescript II SK+ (Stratagene) resulting in plasmid pMP1. pMP1 was then digested with *KpnI* and *EcoRI* to generate a 7,619-bp fragment carrying only the *CRR1* gene flanked by 171 bp of genomic DNA at the 5' and the 3' end. This fragment was cloned into *KpnI* and *EcoRI* digested pBluescript II SK+ resulting in plasmid pMP2. Plasmid pMP2 was then used for cotransforming *C. reinhardtii* strain 41-6 with *ScaI*-linearized plasmid pHyg, which contains a hygromycin resistance cassette (Berthold et al., 2002). Transformants were selected on agar plates containing 5  $\mu\text{g mL}^{-1}$  hygromycin B (Fluka). Grown colonies were tested for their regained ability to produce arylsulfatase under anaerobic conditions as described above.

### Construction of Plasmids for *HYDA1* Promoter Analyses

The *HYDA1* promoter region used to generate construct CL33 (from position -1,018 to +158 relative to the transcription start site) was amplified from genomic DNA by PCR using *Pfu* polymerase (Stratagene) and oligonucleotides *HYDA1\_rev* and *CL33\_for* listed in Table II. Truncated fragments were amplified using CL33 as a template and using oligonucleotide *HYDA1\_rev* and construct-specific oligonucleotides (Table II). Mutations within some fragments of the *HYDA1* promoter were inserted by site-directed mutagenesis or by quick change (QC) site-directed mutagenesis (Zheng et al., 2004; Liu and Naismith, 2008) using oligonucleotides carrying the desired mutation (Table II). For each construct designed by site-directed mutagenesis (CL34, CL35, and BB01), two first-step PCR reactions were conducted using oligonucleotides 1 and A, and 2 and B, respectively (Table II). Oligonucleotides 1 and 2 were used for amplifying all constructs. Oligonucleotides A and B contained the

respective mutation and were specific for each construct. In each case, the two amplicates overlapped at the site of the mutagenized sequence and were used as templates in the presence of the terminal oligonucleotides 1 and 2 in a final PCR reaction to synthesize the whole *HYDA1* promoter fragment. *HYDA1* promoter fragments were digested with *XhoI* and *ApaI* and inserted into vector pCL20 cut by the same endonucleases. The generation of pCL20, which contains both the paromomycin resistance cassette amplified by PCR from pSL18 (Sizova et al., 2001) and the promoterless *CRLUC* gene encoding the luciferase of *Renilla reniformis* (RLUC; Fuhrmann et al., 2004), was described in detail before (Lambertz et al., 2010). Constructs CL44 and CL46 were generated by applying QC-PCR. In both cases, oligonucleotides QC\_for and QC\_rev (Table II) were applied, which introduced a mutation in the proximal GTAC motif of the *HYDA1* promoter. Using CL33 as a template resulted in the generation of construct CL44, and template BB01 led to construct CL46 having mutations in both GTAC motifs. All constructs were sequenced at the sequencing facility at the Ruhr University of Bochum, Germany (Department of Biochemistry I, Receptor Biochemistry).

## Luciferase Activity Assay

Luciferase activity of Cu-deficient transformants carrying various p*HYDA1*:*CRLUC* constructs was determined in 1.5-mL cell suspension aliquots withdrawn from the cultures after 72 h of Cu starvation. The cell aliquots were sonicated three times for 30 s in an ultrasonic bath (Transsonic T 460, Elma) and then frozen in liquid nitrogen until analyzing luciferase activity. The latter was determined in 200- $\mu$ L lysate aliquots transferred to 96-well plates (Berthold Technologies, microplate 96 well, white). Light emission was measured using a photon-counting microplate luminometer (orion microplate luminometer, Berthold detection systems, operated by software simplicity 2.1). Before determining luciferase activity, background light emission was measured for 10 s. Afterward, 0.5  $\mu$ L of 2 mM coelenterazine (pjk-gmbH) in methanol was added to cell lysates and light emission was again recorded for 10 s. In S-deficient transformants, luciferase activity was determined as described above after 72 h of nutrient starvation, except that the cells were withdrawn from the incubation flask by a syringe and directly measured as described. To ensure that the transformants experienced S deficiency and established a hydrogen metabolism, the concentration of H<sub>2</sub> in head space of each culture vessel was determined (data not shown). In case of Cu deficiency, protein samples were removed from randomly chosen transformants and analyzed regarding CYC6 protein accumulation (Supplemental Fig. S4).

## Heterologous Expression of the CRR1 SBP Domain and EMSA

Heterologous expression and purification of the *C. reinhardtii* CRR1 SBP domain were conducted as described previously (Birkenbihl et al., 2005; Lambertz et al., 2010) using plasmid pet15SBP, which contains a complementary DNA (cDNA) fragment encoding amino acids 428 to 524 of the CRR1 polypeptide (Kropat et al., 2005). Vector pet15SBP was kindly donated by Dr. Frederik Sommer, Max-Planck-Institute for Molecular Plant Physiology.

EMSA oligonucleotides corresponding to wild-type or mutated parts of the *HYDA1* promoter were obtained from Sigma-Aldrich (Table II). EMSAs were conducted as described in Lambertz et al. (2010), using the second-generation digoxigenin (DIG) gel shift kit from Roche.

## RNA and Protein Analyses

Total RNA was isolated as described before (Schloss et al., 1984), except that RNA was precipitated with 1 volume of 8 M LiCl overnight. For cDNA synthesis, total RNA was first treated with DNase (TURBO DNase from Ambion/Applied Biosystems) according to the manual. Afterward, cDNA was synthesized from 20  $\mu$ g of DNase-treated RNA using oligo(dT)<sub>18</sub> oligonucleotides and M-MLV reverse transcriptase (Invitrogen) as recommended by the manufacturer.

qPCR reactions were performed with the peqGOLD *Taq* DNA polymerase kit (PeqLab) using buffer 5 and 5x Enhancer solution included in the kit in 20  $\mu$ L reactions. The latter contained 2  $\mu$ L 10-fold diluted cDNA, 2  $\mu$ L 10x SYBR-mix (0.1% [v/v] SYBR Green 1 nucleic acid gel stain from Sigma-Aldrich, 1% [v/v] Tween20; 1 mg per mL bovine serum albumin; 50% [v/v]

dimethyl sulfoxide), 6 pmol of each forward and reverse oligonucleotides (Table III), and 1  $\mu$ L *Taq* (1 unit/ $\mu$ L). Each sample was analyzed in technical triplicate using DNA Engine Opticon 2 from MJ Research Inc. and a PCR program applying the following steps: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 63°C for 20 s, and 72°C for 25 s. Fluorescence was measured after each cycle at 72°C. Melting curves were performed from 70°C to 95°C with plate reads every 0.5°C. Transcript levels of *CBLP/RCK1* (Cre13.g599400), which were constant in all samples, were used as a reference to calculate relative transcript abundances (Pfaffl, 2001).

RNA hybridization analyses were conducted according to standard techniques (Sambrook et al., 1989). Hybridization signals of the *RPL10a* transcript were used for normalization. *RPL10a* encodes ribosomal protein L10a, a component of the 60S large subunit of cytosolic 80S ribosomes. *HYDA1*- and *RPL10a*-specific probes were generated from cloned fragments by in vitro transcription using DIG-labeled dUTPs (Roche). The *HYDA1*-specific probe was deduced from the 3'-untranslated region of the transcript and amplified from cDNA using oligonucleotides hydA1-5-1629 (5'-GAGGAGAAGG-ACGAGAAGAA-3') and hydA1-3-1883 (5'-TTAGGCGTAGGTACTGGCA-3'). The *RPL10a* fragment was generated applying oligonucleotides L10a-1-5-445 (5'-CCAAGTGCAGCATCAAGTTC-3') and L10a-1-3-596 (5'-CACGTTTCTGCCAGTCTCTCT-3').

Crude protein extracts were prepared from 1 mL of cell culture harvested by centrifugation at maximum speed for 1 min. The pellet was resuspended in 5x SDS-PAGE sample buffer (0.25 M Tris-HCl pH 8.0, 25% [v/v] glycerol, 7.5% [w/v] SDS, 0.25 mg mL<sup>-1</sup> bromophenol blue, 12.5% [v/v]  $\beta$ -mercaptoethanol). After denaturation of proteins at 95°C for 5 min, samples were stored at 4°C. SDS-PAGE and blotting were performed as described previously (Hemscheimer et al., 2008a), except that proteins were transferred to polyvinylidene fluoride membranes. *HYDA1* and *FDX5* were detected by polyclonal anti-*C. reinhardtii*-*HYDA1* (Happe et al., 1994), and anti-*C. reinhardtii*-*FDX5* antibody, respectively (Jacobs et al., 2009). CYC6 immunoblot analyses were conducted using anti-CYC6 antibody, which was kindly donated by S. Merchant, University of California, Los Angeles.

Chemiluminescence of RNA and protein hybridization analyses from each three independent experiments was measured using the FluorChem 8800 system and quantified with AlphaEase FCTM Software version 3.1.2 ( $\alpha$  Innotech Corporation) according to the manufacturer's instructions. For each RNA sample, the Integrated Density Value, (sum of all pixel values after background correction) of the *HYDA1* signal was related to the respective *RPL10a* value.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers XP\_001691972 (*ARS2*), XP\_001701729 (*CPX1*), XP\_001692557 (*CRD1*), XP\_001695062 (*Cre02.g125200*), XP\_001694940 (*CRR1*), XP\_001698242 (*CYC6*), XP\_001691603 (*FDX5*), XP\_001693376 (*HYDA1*), XP\_001694503 (*HYDA2*), XP\_001691465 (*HYDEF*), XP\_001691319 (*HYDG*), XP\_001692808 (*PETF*), XP\_001689719 (*PFL1*), XP\_001701208 (*PFR1*), XP\_001698065 (*RCK1*), and XP\_001699807 (*RPL10a*).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Scheme of the screening which identified *Chlamydomonas* strain 41-6 (*crr1-3*).

**Supplemental Figure S2.** Determination of the integration frequency of the paromomycin resistance cassette in strain 41-6 (*crr1-3*).

**Supplemental Figure S3.** *HYDA1* transcript abundance upon Cu deficiency.

**Supplemental Figure S4.** CYC6 protein amounts in Cu-deprived *CRLUC*-expressing transformants.

## ACKNOWLEDGMENTS

We are very thankful for the generous gift of anti-*C. reinhardtii*-CYC6 antibody from Sabeeha Merchant (University of California, Los Angeles, CA). We would also like to thank Sabeeha and Janette Kropat (University of California, Los Angeles, CA) for fruitful discussions. Frederik Sommer (Max-Planck-Institute for Molecular Plant Physiology, Potsdam-Golm, Germany) kindly donated the plasmid for heterologous production of CRR1 SBP domain. Not at least, we thank Klaus Überla, Thomas Grunwald, and Bastian

Grewe (Institute for Hygiene and Microbiology, Ruhr-University of Bochum, Germany) for the opportunity to utilize the luminometer.

Received May 10, 2012; accepted June 2, 2012; published June 5, 2012.

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