Differential Expression of the *Chlamydomonas* [FeFe]-Hydrogenase-Encoding *HYDA1* Gene Is Regulated by the COPPER RESPONSE REGULATOR1

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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 (O₂) serves as substrate and reagent in a number of biosynthetic pathways (Goldfine, 1965; Raymond and Segré, 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; Philipp et al., 2011) with molecular hydrogen (H₂) 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 H₂. This can be attributed to the fact that PSI (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; Gysels 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 H₂ are also produced by anaerobically adapted *C. reinhardtii* cultures in the dark (Gfeller and Gibbs, 1984; Philipp et al., 2011).
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*₅₅ (CYC6), oxidative coproporphyrinogen oxidase1 (CPX1), and Copper Response Defect1 (CRD1), one isoform of magnesium-protoporphyrin IX monomethyl ester aerobic oxidative cyclease, 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 SQUALOSA-PROMOTER BINDING PROTEIN (SBP) family and has a characteristic zinc-finger domain that binds to the cis-acting DNA element GTAC (Birknbihl et al., 2005; Kropat et al., 2005). A recent study proposed that the coregulation of genes during the two environmental conditions of Cu and O₂ 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 O₂ 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 regulatory 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-SO₄). 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 APH VIII-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 (JGI 4.0, 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 JGI 4.0 and Phytozome (http://www.phytozome.net/chlamy.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 (JGI 3.0 protein ID 148820, Augustus 5 model au5.g9305_t1, Augustus 10 model 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 (JGI 3.0 protein ID 195928, Augustus 5 model au5.g9306_t1, Augustus 10 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 JGI 4.0, 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-SO4 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 JGI 4.0 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 O2 deficiency (Hill et al., 1991; Quinn et al., 2000, 2002). We investigated the effect of subtoxic concentrations of HgCl2 (10 μM; Quinn et al., 2002) on hydrogenase activity as well as 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
shaded and sealed to let them consume O₂ 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 mutated 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 point) 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). Moreover, the

![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 ± 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.

![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 O₂ consumption. One culture was treated with 10 \(\mu\)M HgCl₂ 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.](image-url)
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 ± 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

<table>
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<th>Treatment</th>
<th>Sampling</th>
<th>HYDA1</th>
<th>HYDEF</th>
<th>HYDG</th>
<th>PFL1</th>
<th>FDX5</th>
<th>CRR1</th>
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<tr>
<td>No addition</td>
<td>0 h</td>
<td>2.7 ± 0.1</td>
<td>0.1 ± 0.01</td>
<td>0.2 ± 0.05</td>
<td>6.9 ± 1.9</td>
<td>0.02 ± 0.02</td>
<td>7.7 ± 2.4</td>
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<tr>
<td>Hg(II) t = 0 h</td>
<td>2 h</td>
<td>227.1 ± 74.8</td>
<td>9.4 ± 1.3</td>
<td>19.3 ± 2.2</td>
<td>488.0 ± 60.8</td>
<td>69.7 ± 17.3</td>
<td>13.7 ± 0.5</td>
</tr>
<tr>
<td>Hg(II) t = 2 h</td>
<td>4 h</td>
<td>82.1 ± 3.5</td>
<td>5.3 ± 0.1</td>
<td>15.5 ± 1.7</td>
<td>217.3 ± 13.7</td>
<td>22.8 ± 15.3</td>
<td>7.8 ± 1.8</td>
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*Culture samples for RNA isolation were withdrawn before the addition of Hg(II).
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-deicient 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-deicient 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 (EMSAs) 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...
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 HgCl₂ 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 O₂ and Cu responsiveness of genes essential for H₂ 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 O₂ 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).
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 −279 or −237 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 O2 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-CO2-INDUCIBLE 43-KD PROTEIN/FE ASSIMILATION1 gene is regulated both under high-CO2- 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

<p>| Table III. Oligonucleotides used for qPCR analyses |</p>
<table>
<thead>
<tr>
<th>Gene and locus name</th>
<th>Forward Oligonucleotide</th>
<th>Reverse Oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCK1 (Cre13.g599400)</td>
<td>GCCACACCCAGTGGTCTGCTGAGC</td>
<td>CAGCTGCGCCAGCGCAGAGCC</td>
</tr>
<tr>
<td>RPL10a (Cre02.g101350)</td>
<td>GCTCCAGACCTTCTGGTCTGAGC</td>
<td>CAGCTGCGCCAGCGCAGAGCC</td>
</tr>
<tr>
<td>HYDA1 (Cre03.g199800)</td>
<td>GCCACAGTAGGATGCTGCTGAGC</td>
<td>CAGCTGCGCCAGCGCAGAGCC</td>
</tr>
<tr>
<td>HYDG (Cre06.g296750)</td>
<td>GCCACAGTAGGATGCTGCTGAGC</td>
<td>CAGCTGCGCCAGCGCAGAGCC</td>
</tr>
<tr>
<td>PFL1 (Cre01.g448800)</td>
<td>GCCACAGTAGGATGCTGCTGAGC</td>
<td>CAGCTGCGCCAGCGCAGAGCC</td>
</tr>
<tr>
<td>FDX5 (Cre17.g700950)</td>
<td>GCCACAGTAGGATGCTGCTGAGC</td>
<td>CAGCTGCGCCAGCGCAGAGCC</td>
</tr>
<tr>
<td>CRR1 (Cre02.g125250)</td>
<td>GCCACAGTAGGATGCTGCTGAGC</td>
<td>CAGCTGCGCCAGCGCAGAGCC</td>
</tr>
</tbody>
</table>

The gene and locus names refer to the annotation on Phytozome.
putative additional factor(s) binding to the HYDA1 promoter might throw light on the orchestrated Cu- and O₂-responsive signaling in C. reinhardtii.

MATERIALS AND METHODS

Organisms and Growth Conditions

Chlamydomonas reinhardtii strains CC-124, CC-425, and crl1-2 mutant strain CC-3960 (Eriksson et al., 2004; Knapht et al., 2005) were obtained from the Chlamydomonas Resource Center, University of Minnesota. Strain MR9 was generated as described before (Stemberg and Happe, 2004). It resulted from cotransformation of C. reinhardtii HYYDA1::CRLUC transformants generated as described below by growing the cells once in TAP ENEA2 medium as reported previously (Ferrante et al., 2008). Then, a main culture of colonies growing on TAP agar without paromomycin and incubated in low light (15 mol photons m⁻² s⁻¹) was achieved by growing the cells once in TAP ENEA2 medium, in which sulfate salts were replaced by chloride compounds. Starvation of the cells was done using methyl viologen as an artificial respiratory O₂ consumption by the cells. When indicated, mercuric chloride (HgCl₂) was added to the cells to a final concentration of 5 µmol L⁻¹. S. starvata was grown until the cells had been incubated for 2 h.

Transformants with Impaired HYDA1 Promoter Activity

Transformation of C. reinhardtii

C. reinhardtii cells were transformed using the glass bead method (Kindle, 1990). Strain MR9 was transformed using 1 µg of the APHVIII-encoding paromomycin resistance cassette derived from plasmid pSL18 (Sizova et al., 2001) by KpnI and SfiI digestion. After transformation, cells were streaked on TAP agar plates without paromomycin and incubated in low light (15 mol photons m⁻² s⁻¹) for 24 h. The agar blocks were transferred to petri dishes containing filter paper soaked with a paromomycin solution that resulted in a final concentration of 5 µmol L⁻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 Scal-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 µmol L⁻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 µL of a 3-mm solution of the artificial arylsulfatase substrate X-SO₄ were dropped on each colony. Twenty-four hours later, colonies were checked for a blue staining, which would result from cleavage of X-SO₄ 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 5 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 A02 (5'-GCGGCGTTCC-3') and Arps (5'-CATACGCACGACCATCAAAGGCAAGGCAAAAAG-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 µL of 20-fold-diluted ligation mixture in a 25-µL 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'-GAGTGTTCCGGCGGCTGCC-3') and 5'GSP1 (5'-CATACGCACGACCATCAAAGGCAAGGCAAAAAG-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 2,011-bp fragment that included the CRRI gene and a fragment of an unknown gene (GI JGI v4.0 ID519527, au3g93051). 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 1,290-bp fragment carrying only the CRRI gene flanked by 100 bp of genomic DNA at the 5' and 3' end. This fragment was cloned into pKpnI and EcoRI digested pBluescript II SK+ resulting in plasmid pMP2. Plasmid pMP2 was then used for cotransformation of C. reinhardtii strain 41-6 with Scal-linearized plasmid pHyg, which contains a hygromycin resistance cassette (Berthold et al., 2002). Transformants were selected on agar plates containing 5 µmol L⁻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 +118 relative to the transcription start site) was amplified from genomic DNA by PCR using Pfu polymerase (Stratagene) and oligonucleotides HYDA1_rev and CL33, listed for 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 Naisbett, 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 2, and 2 and B, respectively (Table II). Oligonucleotides 1 and 2 were used for amplifying all constructs. Oligonucleotides A and B contained the
resulted in the generation of construct CL44, and template BB01 led to proximal GTAC motif of the 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 pHYDA1: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 (Transonic T 460, Ehrna) and then frozen in liquid nitrogen until analyzing luciferase activity. The latter was determined in 200-μl 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 μL of 2 mM coelenterazine (pk-gmb-H) in methanol was added to cell lysates and light emission was again recorded for 10 s. In Cu-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 H2 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. EMSAs 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 (Schlos et al., 1984), except that RNA was precipitated with 1 volume of 20 mL 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 μg of DNase-treated RNA using oligo(dT)_18 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 S and 5X Enhancer solution included in the kit in 20 μL reactions. The latter contained 2 μl 10-fold diluted cDNA, 2 μl 10X SYBR-mix (0.1% [v/v] SYBR Green I 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 μL Taq (1 unit/μL). 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 20 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 (Pfaf, 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 JUTPs (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'-GAGAGAAGAGCGAGAAGA-3') and hydA1-3-1883 (5'-TTAGCGTAGTACTGGCA-3'). The RPL10a fragment was generated applying oligonucleotides L10a-1-3-445 (5'-GAACTGCAATGATCCT-3') and L10a-1-3-596 (5'-CAGCTTCGGCAGTICTC-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 5× SDS-PAGE sample buffer (0.25% Tris-HCl pH 8.0, 25% [v/v] glycerol, 7.5% [v/v] SDS, 0.25 mg mL⁻¹ b-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 (Henschmeier et al., 2008), 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 FluoroChem 8800 system and quantified with AlphaEase FCTM Software version 3.12 (a 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 database by accession numbers XP_001691972 (ARS2), XP_003701729 (CPX1), XP_001693259 (CRD1), XP_001695062 (Cre13.g125200), XP_001694890 (CRKI), XP_001698242 (CYC6), XP_001691603 (FDX5), XP_001693576 (HYDA1), XP_001694050 (HYDA1), XP_001691465 (HDFE), XP_001691319 (HYDG), XP_001692889 (PET1), XP_001697479 (VLI3), XP_003701208 (PFR1), XP_001698605 (RCK1), and XP_001699807 (RPL10).

**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 (cr1-1-3).

**Supplemental Figure S2.** Determination of the integration frequency of the poramycin resistance cassette in strain 41-6 (cr1-1-3).

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

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

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Regulation of Chlamydomonas HYDA1 Gene by CRR1

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