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RNA silencing of hydrogenase(-like) genes and investigation of their physiological roles in the green alga *Chlamydomonas reinhardtii*

James E. Godman, Attila Molnár, David C. Baulcombe and Janneke Balk [1]

Department of Plant Sciences, University of Cambridge, United Kingdom

[1] To whom correspondence should be addressed (email jb511@cam.ac.uk)

Corresponding author: Janneke Balk, Department of Plant Sciences, Downing Street, Cambridge CB2 3EA, United Kingdom. Tel: + 44 1223 330225; Fax: + 44 1223 339530; E-mail: jb511@cam.ac.uk

The genome of the green alga *Chlamydomonas reinhardtii* encodes two [FeFe]-hydrogenases, HydA1 and HydA2, and the hydrogenase-like protein HYD3. The unique combination of these proteins in one eukaryotic cell allows for direct comparison of their in-vivo functions, which have not been established for HydA2 and HYD3. Using a recently developed artificial miRNA silencing method, the expression of *HydA1*, *HydA2* and *HYD3* was specifically down-regulated. Silencing of *HydA1* resulted in 4-fold lower hydrogenase protein and activity under anaerobe conditions. In contrast, silencing of *HydA2* or *HYD3* did not affect hydrogen production. Cell lines with strongly (>90%) decreased *HYD3* transcript levels grew more slowly than wild-type. The activity of aldehyde oxidase, a cytosolic iron-sulphur (Fe-S) enzyme, was decreased in *HYD3* knockdown lines, while Fe-S dependent activities in the chloroplast and mitochondria were unaffected. In addition, the *HYD3* knockdown lines grew poorly on hypoxanthine, indicating impaired function of xanthine dehydrogenase, another cytosolic Fe-S enzyme. The expression levels of selected genes in response to hypoxia were unaltered upon *HYD3* silencing. Together, our data clearly distinguish the cellular roles of HydA1 and HYD3, and indicate that HYD3, like its yeast and human homologues, has an evolutionary conserved role in the biogenesis or maintenance of cytosolic Fe-S proteins.

Short title: Distinct in-vivo functions *of HYD* genes in Chlamydomonas

Key words: iron-sulfur, hypoxia, silencing, Moco enzymes, IOP, Nar1

The abbreviations used are: amiRNA, artificial micro RNA; AO, aldehyde oxidase; CIA, Cytosolic Iron-sulphur cluster Assembly; HIF, hypoxia-inducible factor; IOP, iron-only hydrogenase-like protein; Moco, molybdenum cofactor; qRT-PCR, quantitative reverse transcription PCR; TAP, Tris-acetate-phosphate; XDH, xanthine dehydrogenase.

INTRODUCTION

Proteins containing iron-sulphur (Fe-S) cofactors are ubiquitous in nature. They function predominantly in electron transfer and catalysis [1] and play crucial roles in photosynthesis, respiration and numerous metabolic pathways. In bacteria and selected eukaryotic algae, Fe-S dependent [FeFe]-hydrogenases catalyse the reversible reduction of protons to molecular hydrogen. The catalytic site is formed by the so called 'H-cluster', consisting of a [4Fe-4S] cluster coupled to a di-iron centre coordinated by cyanide and carbon monoxide ligands, and a dithiolate bridge [2]. The green alga *Chlamydomonas reinhardtii* expresses two hydrogenase genes, *HydA1* and *HydA2* [3], also annotated as *HYD1* and *HYD2*, however their relative contribution to hydrogen production is not known.

Although Fe-S clusters can self-assemble on proteins in a reaction tube, in vivo this process requires dedicated assembly proteins which are conserved from bacteria to multi-cellular organisms [4]. Photosynthetic eukaryotes have inherited two genetically distinct assembly systems through endosymbiosis. Studies on the model plant Arabidopsis have identified a complete set of six SUF (sulphur mobilisation) gene products in the plastids as well as additional scaffold proteins, while a complete ISC (iron sulphur cluster) pathway is located in the mitochondria (reviewed in [5]). The plant cytosol contains homologues of the Cytosolic Iron-sulphur cluster Assembly (CIA) pathway as identified in yeast, but with some differences. For example, the CFD1 scaffold protein is absent in the green lineage, and cluster assembly is thought to be mediated by a homodimer of the related NBP35 protein [6, 7].

Another protein of the CIA machinery, called Nar1 in yeast, is highly conserved in all eukaryotes, and is likely to have a monophyletic origin [8]. Chlamydomonas HYD3 belongs to this group. The protein bears striking similarity to [FeFe]-hydrogenases, not only in amino acid sequence (49%), but also in domain structure, cysteine ligands and 3D models of the active site [2, 9]. The Nar1 proteins from yeast and the model plant Arabidopsis can bind two Fe-S clusters [10-12]. However, these hydrogenase-like proteins are assumed not to have hydrogenase activity because they lack certain conserved residues (e.g. C_1 in H-cluster motif L1 [8]), and because most eukaryotes do not have the *HydEFG* genes for assembling the di-iron centre and its ligands, except algae that do have functional [FeFe]-hydrogenases [13]. Down-regulation of *NAR1* expression in yeast leads to a defect in the assembly of clusters on cytosolic and nuclear Fe-S proteins [10], but the precise molecular function of Nar1 remains unknown. Two Nar1 homologues are found in humans, IOP1 and IOP2 (iron-only hydrogenase-like protein). IOP1 was initially identified as a modulator of hypoxia-inducible factor (HIF)-1α activity [14], and was later shown to be required for cytosolic Fe-S protein biogenesis [15]. Mutants in plant and *Caenorhabtitis elegans* homologues displayed an oxygen-sensitive phenotype [12, 16], but whether the activity of cytosolic Fe-S enzymes is impaired in these mutants has not been investigated.

To investigate Fe-S protein biogenesis and its compartmentalization in the green lineage, we have previously catalogued putative Fe-S cluster assembly genes in the recently sequenced genome of the green algae *Chlamydomonas reinhardtii* [17]. Overall, Fe-S cluster assembly genes are well conserved from plants to algae, but are usually single copy in Chlamydomonas where Arabidopsis may have 2 – 3 copies. This is an advantage for reverse genetics studies, which can be cumbersome and time-consuming when no viable insertion mutant is available, the situation for many Fe-S cluster assembly genes in Arabidopsis.

The discovery of micro (mi) RNAs and characterisation of a miRNA-based silencing mechanism in Chlamydomonas has led to the development of tools for effective gene knock-down based on artifical miRNAs (amiRNAs) [18-21]. Here, we have used this

reverse genetics system to investigate the contribution of the three annotated *HYD* genes to hydrogen production, and to investigate whether *HYD3* has a role in Fe-S protein biogenesis and/or the hypoxia response.

EXPERIMENTAL

Chlamydomonas strains and culture conditions

The *C. reinhardtii* strain 325 *arg*$^-$ (*cw15 asl*$^-$ mt$^+$) was obtained from Michael Schroda, University of Freiburg, Germany. Cells complemented with the empty pChlamiRNA2 vector served as wild-type. Cells were cultured in Tris-acetate-phosphate (TAP) medium [22] supplemented with 100 µg ml^{-1} arginine if required, at 25ºC, 100 rpm and ~100 µmol m^{-2} s^{-1} light intensity. Anaerobic induction was performed by concentrating cultures to 100 µg/ml chlorophyll, and bubbling with N_2 gas for two hours. For analysis of hypoxanthine-dependent growth, cells were plated on TAP plates containing 2% (w/v) agar, or on TAP plates lacking NH_4Cl (7.5 mM) but with 2.35 mM hypoxanthine [23].

amiRNA silencing constructs

Artificial miRNA sequences were designed using the Web MicroRNA Designer at http://wmd3.weigelworld.org/. The 21-nucleotide amiRNA sequences to target *HydA1*, *HydA2* and *HYD3* (Protein IDs 183963, 24189 and 117241, respectively) were as follows (position in the coding sequence in brackets):
HydA1 miRNA1 (187-207) 5'- GCGCCTTTGAGTCATGTCCAG;
HydA1 miRNA2 (606-626) 5'- CAAGTCCTACCTAGCGGAAAA;
HydA2 miRNA2 (298-318) 5'- CTTGCCATTGCTGAGTCATTA;
HYD3 miRNA1 (141-161) 5'- AAAGGTGTCATTGCACGACTA;
HYD3 miRNA2 (100-120) 5'- CAGGTGAGCAGTCTCATTGCA.
Each amiRNA was synthesized, in forward and reverse orientation, as part of a 90mer oligonucleotide cassette: *Spe*I-amiRNA*-spacer-amiRNA-*Spe*I. Forward and complementary sequences of each 90mer were annealed and cloned into the *Spe*I site of the pChlamiRNA2 vector [19]. This places the amiRNAi cassette between flanking sequences of the cre-MIR1157 precursor and under the control of the *HSP70A-RBCS2* promoter. Transformation of Chlamydomonas and auxotrophic selection were as previously described [19].

Reverse transcription (RT)-PCR

Total RNA was extracted using TRIzol (Invitrogen). RNA concentration and quality (A_{260}/A_{280}) was determined using a ND-1000 spectrophotometer (Nanodrop, Delaware, USA). cDNA was synthesised using RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas, Maryland, USA), according to manufacturer's instructions, and an oligo dT primer, except for *HYD3*, for which a gene-specific primer was used (Supplemental Table 1). Quantitative reverse transcription PCR (qRT-PCR) was carried out using SYBR Green JumpStart Taq ReadyMix (Sigma, Missouri, USA), with 5% dimethyl sulfoxide added, using the Bio-Rad CFX96 Real-time PCR Detection system (Bio-Rad, California, USA), both according to manufacturer's instructions. PCR mixes were prepared as master mixes, with template DNA being added to the final reaction volume. Reactions were carried out in triplicate (biological or technical replicates) and *RACK1* (Prot ID 164254) amplification was used as a baseline. Changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method

[24]. Melting curve analysis was performed on all PCRs to ensure single DNA species were amplified, and the product sizes confirmed by agarose gel electrophoresis. Primer sets were designed to span an intron to distinguish PCR products derived from cDNA (Supplemental Table 1).

Enzyme activity measurements

In vitro hydrogenase activity was measured as described in [25]. Rates of respiratory oxygen consumption in the dark and photosynthetic oxygen evolution under saturating light conditions (250 µmol photons m^{-2} s^{-1}) were measured in 1.5-ml cell culture volume (25 µg/ml chlorophyll) using an oxygraph oxygen electrode (Hansatech, King's Lynn, UK).

Aldehyde oxidase (AO) activity was analysed using an in-gel activity assay as previously described for Arabidopsis [26], adapted for Chlamydomonas. Fifteen ml cell culture at mid-logarithmic phase was centrifuged and the cell pellet was resuspended in 150 µl 100 mM KPi pH 7.5, 5 mM dithiothreitol, 20 mM EDTA on ice. All further procedures were carried out at 4ºC. Samples were sonicated (Soniprep 150, MSE, London, UK) for 3 x 20 s, with 1 min between sonication steps, and centrifuged for 30 min at 13,000 rpm. The supernatant was recovered, and mixed with one volume of loading buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 10% (v/v) glycerol and 0.1% (w/v) bromophenol blue. Twenty µl (50 µg protein) was separated on a standard 12% (w/v) polyacrylamide gel (without SDS) at 150V for 2 hours. Gels were equilibrated for 15 min in 100 mM KPi pH 7.5, and AO activity was visualised using 1 mM 1-napthaldehyde and 1 mM indole-3-carboxyaldehyde as substrates and coupling of the reaction to the formation of purple formazan precipitate.

Immunolabelling

Cells from 0.5 ml mid-log phase culture were harvested by centrifugation and resuspended in 90 µl loading buffer (0.125 M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue). Mixtures were boiled for 3 min and debris pelleted at 13,000 rpm for 10 min. Proteins were separated by SDS-PAGE, blotted onto nitrocellulose membrane (Protran BA 83, Whatman), and labelled with the following antibodies: histone 3 (AbCam, Cambridge, UK); HydA1 kindly provided by Peter J Nixon (Imperial College London); PsaA (Agrisera, Vännäs, Sweden).

Other methods

Protein was quantified using the Bio-Rad Protein Assay (Bio-Rad, Hercules, California, USA); Chlorophyll was determined in 100% (v/v) acetone extracts [27]; Densitometry was performed using ImageJ software [28].

RESULTS

Specific amiRNA-mediated silencing of hydrogenase(-like) genes in Chlamydomonas

amiRNA were designed following criteria described in Molnár et al. [19], against unique 21 nucleotide target sequences identified in the 5' regions of *HydA1*, *HydA2* and *HYD3* coding sequences, see Figure 1A. The *HydA1* and *HydA2* coding sequences are 69% identical, whereas *HYD3* shares only 12% and 16% in nucleotide sequence with *HydA1* and *HydA2, respectively*. Two independent miRNAs were designed for each target gene in

order to determine whether any effects seen were specific. The amiRNAs were cloned under the control of the constitutive promoter *HSP70A-RBCS2* into the Chlamydomonas expression vector pChlamiRNA2 [19]. Transformants were selected on TAP plates lacking arginine, and genomic insertion of the amiRNA cassette was confirmed by PCR. More than 100 positive transformants were obtained for each amiRNA construct. Because genomic insertion is random in Chlamydomonas, causing positional effects on the expression of transgenes, independent transformants were screened by RT-PCR. Considerable variation in transcript abundance was observed and only 4 out of 20 transformed lines showed strongly decreased levels of the target transcript. No significant silencing was observed for lines bearing HydA2 miRNA2, and this miRNA construct was not used in further investigations.

To confirm the specificity of each amiRNAs to its target transcript, the expression levels of *HydA1*, *HydA2* and *HYD3* were quantified in wild-type and selected amiRNA lines using qRT-PCR. *HydA1* and *HydA2* are known to be expressed at very low levels under aerobic conditions, but induced in the absence of oxygen or sulphur [3, 29]. The expression of *HYD3* has not been investigated, but RNA-seq data indicate that transcripts are of low abundance (S. Merchant, *pers. communication*). We therefore analysed expression of all three genes in cultures grown in air and after 2 h of anaerobic treatment. Under these conditions, *HydA1* and *HydA2* transcript levels were induced approximately 4-fold in wild-type cells (Figure 1B and C), but *HYD3* expression did not change (data not shown). In lines transformed with miRNA1 or miRNA2 against HydA1, the induction of *HydA1* expression was only 1.4 and 2.5-fold, respectively (Figure 1B), while *HydA2* induction was as wild-type (Figure 1C). Vice versa, the induction of *HydA2* expression was abbrogated in cells harbouring HydA2 miRNA1, but not in cells with HydA1 amiRNAs (Figure 1B and C). *HYD3* mRNA was specifically down-regulated to 7 ± 12% by HYD3 miRNA1, and to 23 ± 10% by HYD3 miRNA2 (Figure 1D and E). These qRT-PCR results show that amiRNA gene silencing in Chlamydomonas is effective to down-regulate the expression of inducible and low-abundance genes.

HydA1 represents the major hydrogenase activity under anaerobe conditions

Next, we analysed the effect of silencing each of the hydrogenase(-like) genes on hydrogenase protein levels and activity. Wild-type and amiRNA lines were sampled before and after 2 h anaerobic induction for immunoblot analysis using antibodies raised against the 16 kDa C-terminal fragment of HydA1 (kindly provided by Peter J Nixon, Imperial College London). This region of HydA1 is 71% identical (82% similar) in amino acid sequence to HydA2, and cross-reactivity with HydA2 protein is likely, but remains to be confirmed. However, only HydA1 knock-down lines showed decreased accumulation of hydrogenase protein both in aerobic conditions and after 2 hours of anaerobic treatment compared to wild-type samples, Figure 2A. Quantification of the signal by densitometry showed that HydA1 knock-down lines accumulated about one quarter of wild-type hydrogenase protein level. In HydA2 and HYD3 knock-down lines, HydA1 protein accumulation was similar to wild-type cells. Coomassie staining (lower panel) confirmed equal protein loading.

Since the lower hydrogenase protein levels in HydA1 miRNA1 (Figure 2A, lane 4) could be due to an air leak and associated failure of induction, the expression of *Fdx5*, an anaerobically induced ferredoxin [30] was assessed by qRT-PCR using RNA extracted from the same samples. *Fdx5* expression was induced 12 to 15-fold in all anaerobic cultures, including the HydA1 miRNA1 line (data not shown, and Figure 6).

To investigate the relative contribution of HydA1 and HydA2 to hydrogenase production in Chlamydomonas, the amiRNA lines were treated anaerobically for 2 h,

followed by an in-vitro assay to determine hydrogenase activity. In brief, cell extracts were incubated with methyl viologen as electron donor in a closed vial, and gas samples were removed for analysis by gas chromatography. The HydA1 miRNA1 line showed a 76 ±15% decrease in hydrogenase activity compared to wild-type (Figure 2B), which correlates well with HydA1 protein levels (Figure 2A). Lines containing the amiRNA targeting *HydA2* did not show any significant decrease in hydrogenase activity, even though *HydA2* transcript levels were decreased by 97±4% under these conditions (Figure 1E). Silencing of *HYD3* expression also did not affect total hydrogenase activity. Together, these data show that HydA1 is the major hydrogenase induced by anaerobic conditions in Chlamydomonas.

HYD3 is required for normal growth

To further investigate the function of HYD3, we analysed growth of the amiRNA lines. Cultures of wild-type and two independent HYD3 knock-down lines were grown concurrently in triplicate under aerobic conditions, and the chlorophyll content of standard culture volumes was determined as a measure for cell density and therefore growth, Figure 3. To control for chlorophyll differences, per-cell chlorophyll concentrations were calculated for wild-type and HYD3 amiRNA lines and were not observed to differ significantly (Figure 3, inset), nor was there an obvious difference in cell size (not shown). The HYD3 amiRNA1 line displayed clear growth defects, lagging after 4 days post-inoculation compared to wild-type, and reaching saturation after 5 days at approximately 80% of the cell density of wild-type cultures. HYD3 amiRNA2 cell cultures initially grew more slowly, but reached similar levels of cell density as wild-type by day 5 – 7. The difference in growth between the amiRNA1 and amiRNA2 lines corresponded to the different degree of silencing of *HYD3* in these two lines (Figure 1D and E).

The growth of HydA1 and HydA2 knock-down lines was also assayed, but the growth curves were identical to wild-type under aerobic conditions (not shown), as reported for mutants in the hydrogenase assembly genes HydEFG [13]. In contrast, the observed growth defects of the HYD3 amiRNA lines fits with the expectation that *HYD3* is an essential gene, like its yeast homologue *NAR1* [10].

HYD3 is required for the activity of cytosolic Fe-S enzymes, but not organellar Fe-S enzymes

Next, we investigated whether HYD3, like the yeast homologue Nar1 and the human IOP1 protein, is required for the activity of cytosolic Fe-S proteins, but not for mitochondrial Fe-S proteins. The mitochondrial respiratory chain contains Fe-S proteins in Complex I, II and III, therefore dark respiration rate was measured using an oxygen electrode. As expected, HYD3 knock-down lines had similar oxygen consumption rates as wild-type, Figure 4A. In photosynthetic eukaryotes like plants and algae, Fe-S proteins are also abundant in the plastids, for instance in Photosystem I, which has 3 [4Fe-4S] clusters. Oxygen evolution in the light was assayed to detect any lesions in photosynthetic electron transfer, but no decrease was observed in HYD3 miRNA lines, Figure 4B. In agreement, the levels of PsaA, a core subunit of Photosystem I binding the F_x [4Fe-4S] cluster, were as wild-type (Figure 4C). In contrast, PsaA is degraded in Fe-S protein assembly mutants in Arabidopsis chloroplasts [31, 32]. Together with our result that knockdown of *HYD3* has no effect on the chloroplast-localized hydrogenase activity, these data indicate that *HYD3* is not required for the assembly of organellar Fe-S proteins.

While Fe-S proteins are abundant and well studied in the endosymbiotic organelles, they are generally of low abundance and poorly characterized in the cytosolic/nuclear compartment. Moreover, the cytosolic enzymes isopropylmalate

isomerase (Leu1) and sulfite reductase, which are commonly used as marker Fe-S enzymes in Baker's yeast, are located in the plastid. Searching the genome of Chlamydomonas, we identified a gene encoding aldehyde oxidase (AO, Protein ID 518045), which has been used as a marker for cytosolic Fe-S enzyme activity in Arabidopsis [33, 34]. A native gel assay using synthetic aldehydes as substrates revealed a single AO activity in Chlamydomonas cell extracts. The intensity of the activity staining was linear with the amount of protein (Supplemental Figure 1), indicating that this assay is at least semi-quantitative. In HYD3 miRNA lines, the AO activity is much decreased compared to wild-type, and the electrophoretic mobility is poor, indicating that the protein is partly folded (Figure 5A). Densitometric analysis of the staining intensity indicated that AO activity is more than 70% lower in the HYD3 knock-down lines. In contrast, transcript levels of AO were equal to wild-type, Figure 5B, supporting a post-transcriptional defect in AO assembly. The activity of AO is also dependent on FAD and molybenum cofactor (Moco). However, HYD3/NAR1/IOP have not previously been implicated in the well-studied FAD or Moco biosynthesis pathways. Moreover, hydrogenase-like proteins are conserved in organisms that lack Moco enzymes altogether, suggesting that the lower AO activity in HYD3 knock-down lines is due to a lack of Fe-S clusters.

Xanthine dehydrogenase (XDH) is similar in structure and cofactors to AO. The activity of XDH has been shown to depend on Fe-S cluster assembly pathway in mammals [35, 36] and plants [34]. Unfortunately, XDH activity could not be detected in total cell extracts from Chlamydomonas. However, Chlamydomonas can use hypoxanthine as a nitrogen source [37], which depends on a functional XDH. Wild-type and HYD3 miRNA lines were plated on standard TAP, containing ammonium chloride, or on TAP with hypoxanthine as the only nitrogen source (Figure 5C). On TAP plates, the HYD3 miRNA1 line showed a clear growth defect as previously shown in liquid culture, but cells were viable. On hypoxanthine plates, in contrast, HYD3 knock-down lines turned chlorotic. This phenotype is not linked to slow growth, since it was also evident in HYD3 miRNA2 lines, which reach similar cell densities as wild type (Figure 3, 5C). Taken together, these data indicate that HYD3 is required for the activity of the cytosolic Fe-S containing enzymes AO and XDH, but not for Fe-S proteins in the chloroplast or mitochondria.

HYD3 is not involved in the hypoxia response

In mammalian cells, plants and nematodes, IOPs have been implicated in the hypoxia response and/or oxygen sensing. Knockdown of *IOP1* in mammalian cells increased the mRNA and protein levels of HIF-1α, the α-subunit of the hypoxia response regulator, under both normoxic and hypoxic conditions. The expression of both endogenous HIF-1α target genes and a hypoxia-response-element reporter gene were induced [14]. The hypoxia response in Chlamydomonas is well characterized, and the levels of more than 500 transcripts are known to increase significantly, including *HydA1*, *HydA2* and the ferredoxin *Fdx5* [29]. We therefore investigated the transcript levels of these three genes in *HYD3* knockdown lines before and after 2 h anaerobic induction using qRT-PCR. HYD3 miRNA1 cells were able to induce *HydA1* and *HydA2* 4 – 5 fold under these conditions, and *Fdx5* expression was induced 12-15-fold, similar to wild-type cells, Figure 6. The induction of HydA1 protein levels and hydrogenase activity are also not affected in HYD3 knock-down lines, see Figure 2. These data suggest that HYD3 in Chlamydomonas is not involved in regulating the transcriptional or post-transcriptional response to low oxygen.

DISCUSSION

The recently sequenced genome [17] and amiRNA-mediated gene silencing tools [19, 21] have significantly added to the already existing advantages of Chlamydomonas as a model system, being a haploid, uni-cellular organism. In this study, we show that amiRNA-mediated gene silencing can be highly specific in the case of *HydA1* and *HydA2*, which are 69% identical in their coding sequence. In theory, degradation of a transcript could lead to multiple 21-nt fragments that, when converted to double stranded RNA, could amplify the silencing effect and knock down the expression of highly similar genes. However, no RNA-dependent RNA polymerases have been identified in the Chlamydomonas genome therefore it is unlikely that the targeted mRNAs would be involved in amplification and spread of the silencing signal.

Tracing the silencing effect from RNA to protein, we show that the levels of *HydA1* transcript (~36%), protein (~25%) and enzyme activity (~25%) correlated well. In our system, the amiRNAs are constitutively expressed under the control of the *HSP70A-RBCS2* promoter. The expression levels of the amiRNA are sufficient to down-regulate both uninduced as well as induced transcript levels (Figure 1, Figure 2 and data not shown). In the case of *HydA1* mRNA, the induction under anaerobiosis was not completely abbrogated by its cognate amiRNA (Figure 1B), which could explain the residual hydrogenase activity (Figure 2B). In contrast, *HydA2* transcript levels were not induced at all in the presence of HydA2 amiRNA1. It may be that HydA2 amiRNA1 is more effective (Figure 1E), or that *HydA2* expression levels are generally much lower than those of *HydA1*. The latter possibility is supported by RNA-seq data (Sabeeha Merchant, *pers. communication*) and by a quantitative proteomics study in which HydA1 and HydG, but not HydA2, were identified in isolated chloroplasts from cells grown under anaerobic conditions [38].

The *HYD3* gene product is not predicted to be targeted to an organelle (Predotar 1.03; TargetP 1.1; WoLF PSORT). It is therefore reasonable to assume that HYD3 is limited to the cytosol, as shown for yeast Nar1 [10]. Also similar to Nar1 and its human homologue IOP1, Chlamydomonas HYD3 is required for the activity of cytosolic, but not organellar, Fe-S proteins. Using ^{55}Fe labelling studies in yeast, Nar1 was shown to function down-stream of the cytosolic scaffold proteins Nbp35/Cfd1 [39]. Interestingly, Nar1 was not needed for cluster transfer in vitro. Therefore, it is thought that Fe-S clusters are directly transferred from Nbp35/Cfd1 to recipient proteins (including Nar1 which needs clusters for its own function), and that Nar1 plays an essential role in the cluster tranfer process.

Perhaps remarkable is that several Nar1 homologues have been identified linked to oxygen sensing [12, 14, 16]. Also, the growth phenotype of Nar1-depleted yeast cells is dependent on oxygen levels [16]. One possible explanation is that the hydrogenase-like proteins function as oxygen-dependent regulators of Fe-S cluster transfer in the CIA pathway. An example of a sensing 'enzyme' derived from a strictly catalytic isoform is seen in NiFe hydrogenases: The H_2-sensing hydrogenase in *Ralstonia eutropha* orchestrates a transcriptional response when hydrogen becomes available as an energy source [40]. However, we found that the transcriptional response of *HydA1*, *HydA2* and *Fdx5* in the HYD3 knockdown line was normal under (an)aerobic conditions. Moreover, oxygen-sensing systems are described in yeast (Hap1) and mammals (HIF1α), but are not conserved [41], whereas hydrogenase-like genes are highly conserved.

Another possible explanation is that oxygen sensitivity in *nar1* mutants is merely a consequence of decreased Fe-S cluster assembly. Oxygen is known to damage Fe-S clusters, which then require a higher rate of de novo assembly compared to hypoxic conditions. Also, decreased levels of cytosolic Fe-S proteins are more likely to cause

lethality, unlike disrupted oxygen sensing. Further studies on the precise molecular function of the hydrogenase-like proteins, as well as on Fe-S cluster turnover in the presence and absence of oxygen, is needed to understand the evolution of hydrogenase-like proteins and their structural similarities to [FeFe]-hydrogenases.

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FIGURE LEGENDS

Figure 1 Gene-specific silencing of *HydA1*, *HydA2* and *HYD3* expression by amiRNAs.
(**A**) Positions of the amiRNA target sites (vertical gray bars) and qRT-PCR primers (arrow heads) in the coding sequences of *HydA1*, *HydA2* and *HYD3* (black lines, v.4 models). (**B,C**) Relative induction of *HydA1* (B) and *HydA2* (C) transcript levels after anaerobic treatment in wild-type and amiRNA-transformed lines. Transcript levels before and after 2 h of anaerobic growth were determined by qRT-PCR, and normalised to *RACK1* transcript levels (receptor of activated protein kinase C1), a constitutively expressed gene. Data represent the average of three experiments ± S.E. (**D**) Relative *HYD3* transcript levels in wild-type and amiRNA-transformed lines under standard conditions. Data were normalised to *RACK1* expression, and are the average of three experiments ± S.E. (**E**) Expression levels of the target genes in amiRNA transformed lines compared to wild-type, after anaerobic induction for *HydA1* and *HydA2*, in standard conditions for *HYD3*. Data are calculated from (B), (C) and (D).

Figure 2 Hydrogenase protein levels and activity are decreased in *HydA1* knock-down lines.
(**A**) HydA1 protein levels in wild-type and amiRNA lines, in the presence of oxygen (+) and after anaerobic induction for 2 h (-). Total protein extracts (15 µg) were separated by SDS-PAGE, blotted and labelled with polyclonal antibodies raised against the C-terminal 16 kDa of HydA1. Coomassie staining of the gel after transfer served to confirm equal protein loading. The results are representative of two independent experiments. (**B**) Hydrogenase activity in wild-type and amiRNA lines after 2 h of anaerobic induction. Activity was measured in total cell extract using methyl viologen as electron donor. The hydrogen production rate was normalised to culture chlorophyll concentration. Data shown are the average of three experiments ± S.E.

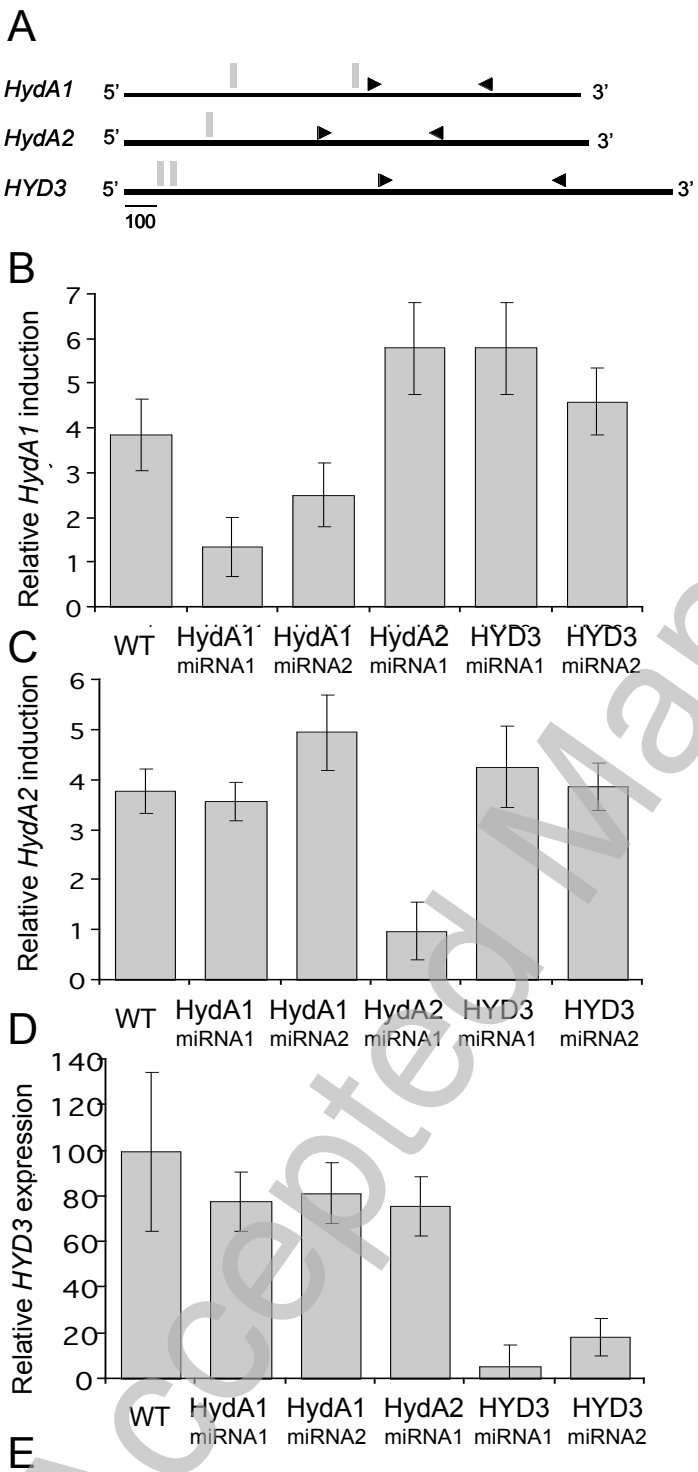
Figure 3 Growth of *HYD3* knock-down lines is decreased compared to wild-type. Growth of wild-type and HYD3 amiRNA lines, expressed as chlorophyll content per culture volume. Values shown are the average ± S.E of three cultures grown in parallel. Cultures were sampled daily until saturated. Inset: Chlorophyll content (Chl) ± S.E of the used cell lines, calculated from chlorophyll analysis and haemocytometer cell counts.

Figure 4 Photosynthesis and respiration are unaffected by *HYD3* knock down. (**A**) Respiratory oxygen consumption rates from wild-type and amiRNA lines. Data is expressed per µg chlorophyll and is the average of three independent cultures ± S.E. The rates are corrected for KCN (1 mM)-insensitive oxygen consumption. (**B**) Photosynthetic oxygen evolution rates from wild-type and amiRNA lines. Data is expressed per µg chlorophyll and is the average of three independent cultures ± S.E. (**C**) Immunolabelling of PsaA, an Fe-S cluster binding subunit of Photosystem I, in protein samples (15 µg) of wild-type and amiRNA lines. Antibodies against histone 3 were used as a control for protein loading. The results are representative of two independent experiments.

Figure 5 Aldehyde oxidase and xanthine dehydrogenase are less active in HYD3 knock-down lines. (**A**) Aldehyde oxidase (AO) activity staining in protein extracts (50 µg) from wild-type and amiRNA lines separated by native PAGE. Protein levels were confirmed by separating equal volumes on SDS-PAGE followed by Coomassie staining. (**B**) AO mRNA levels in wild-type and amiRNA lines, determined by RT-PCR (25 cycles). Primers for *RACK1* were used as a control. RNA was extracted from the same cell cultures as used in (A). (**C**) Growth of wildtype and amiRNA lines on standard TAP medium containing 7.5 mM NH_4Cl (top panel), and on TAP medium with 2.35 mM hypoxhanthine as the only nitrogen source (bottom panel). Cells were plated as drop spots from 5 x serial dilutions of algal culture and grown for 13 days. Three independent HYD3 amiRNA2 transformant lines were spotted to assess the variation in growth with respect to the nitrogen source.

Figure 6 *HYD3* knock-down lines respond transcriptionally to hypoxia. *HydA1*, *HydA2* and *Fdx5* transcript induction in wild-type and HYD3 amiRNA lines after anaerobic treatment as determined by qRT-PCR. Data shown are the average of three experiments ± S.E.

Figure 1



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	Relative transcript level	
	miRNA1	**miRNA2**
HydA1	36±20%	45±10%
HydA2	3±0.4%	N/A
HYD3	7±12%	23±10%

Figure 1 Gene-specific silencing of *HydA1*, *HydA2* and *HYD3* expression by amiRNAs

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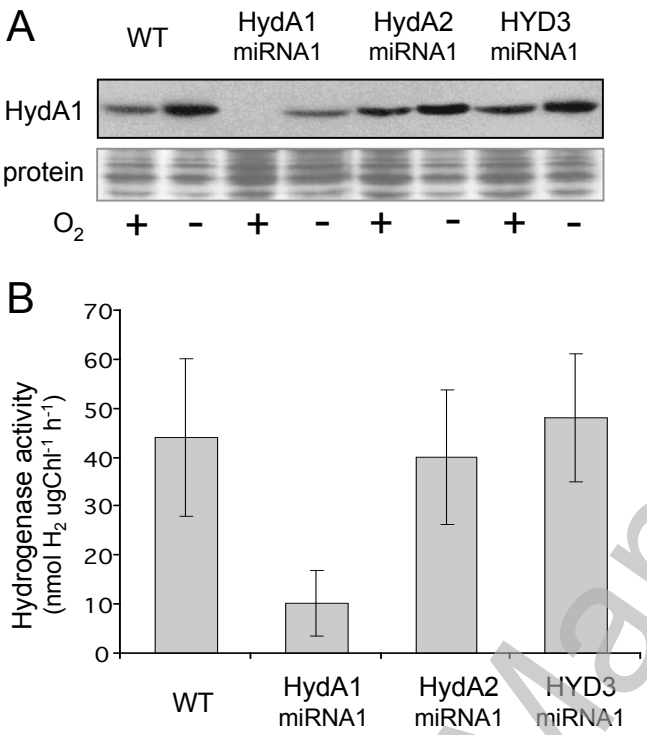
Figure 2



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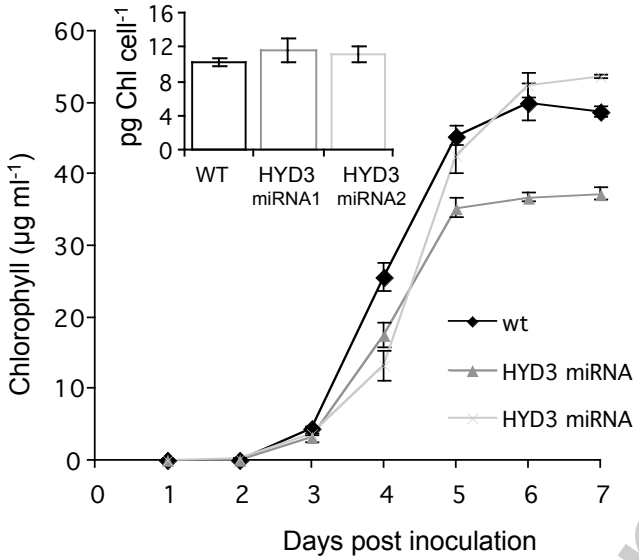

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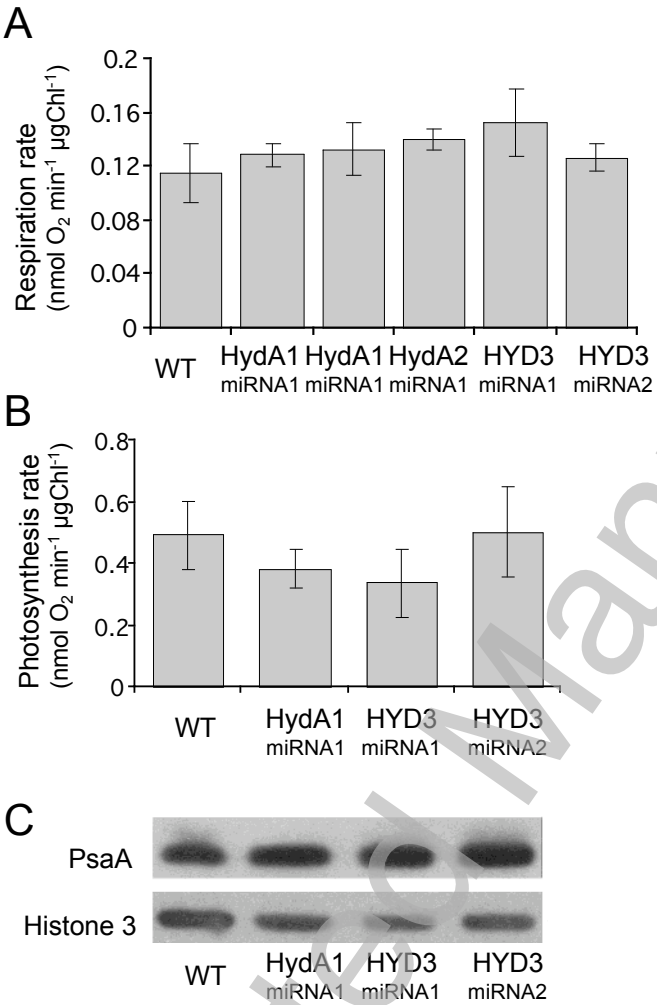

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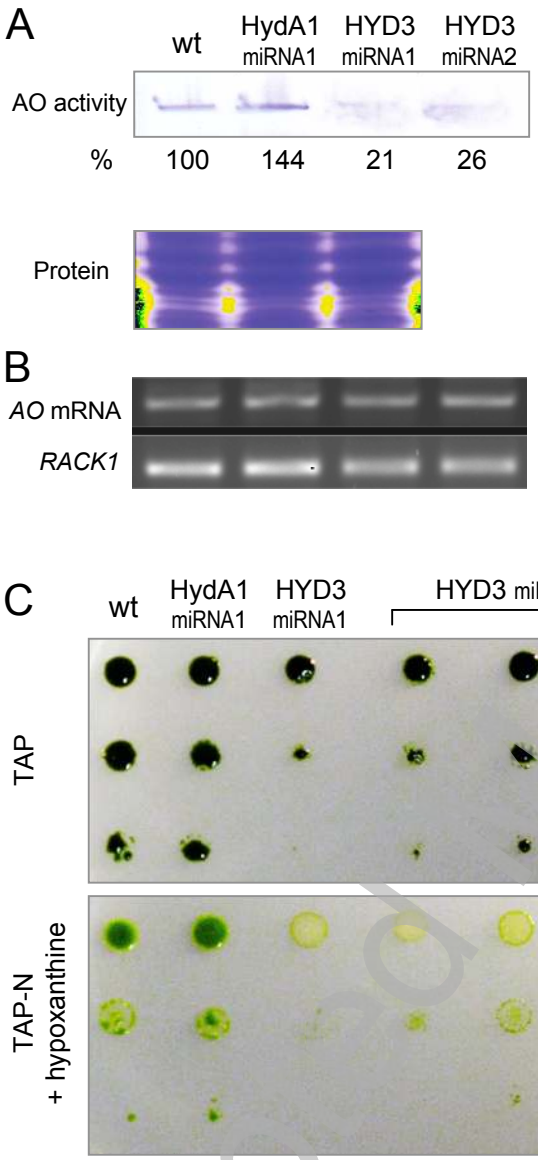
Figure 5



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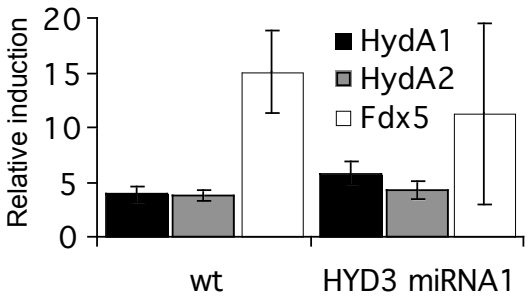

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