Transcriptome for Photobiological Hydrogen Production Induced by Sulfur Deprivation in the Green Alga *Chlamydomonas reinhardtii* 

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Photobiological hydrogen production using microalgae is being developed into a promising clean fuel stream for the future. In this study, microarray analyses were used to obtain global expression profiles of mRNA abundance in the green alga *Chlamydomonas reinhardtii* at different time points before the onset and during the course of sulfur-depleted hydrogen production. These studies were followed by real-time quantitative reverse transcription-PCR and protein analyses. The present work provides new insights into photosynthesis, sulfur acquisition strategies, and carbon metabolism-related gene expression during sulfur-induced hydrogen production. A general trend toward repression of transcripts encoding photosynthetic genes was observed. In contrast to all other *LHCBM* genes, the abundance of the *LHCBM9* transcript (encoding a major light-harvesting polypeptide) and its protein was strongly elevated throughout the experiment. This suggests a major remodeling of the photosystem II light-harvesting complex as well as an important function of *LHCBM9* under sulfur starvation and photobiological hydrogen production. This paper presents the first global transcriptional analysis of *C. reinhardtii* before, during, and after photobiological hydrogen production under sulfur deprivation.

The development of new systems to produce zero CO2 emission fuels for the future is of major importance to combat the effects of climate change. Consequently, biofuel production using conventional crops has increased substantially in recent years with the aim of reducing our CO2 footprint. A downside using conventional crops has increased substantially in recent effects of climate change. Consequently, biofuel production sion fuels for the future is of major importance to combat the future. In this study, microarray analyses were used to obtain global expression profiles of mRNA abundance in the green alga *Chlamydomonas reinhardtii* at different time points before the onset and during the course of sulfur-depleted hydrogen production. These studies were followed by real-time quantitative reverse transcription-PCR and protein analyses. The present work provides new insights into photosynthesis, sulfur acquisition strategies, and carbon metabolism-related gene expression during sulfur-induced hydrogen production. A general trend toward repression of transcripts encoding photosynthetic genes was observed. In contrast to all other *LHCBM* genes, the abundance of the *LHCBM9* transcript (encoding a major light-harvesting polypeptide) and its protein was strongly elevated throughout the experiment. This suggests a major remodeling of the photosystem II light-harvesting complex as well as an important function of *LHCBM9* under sulfur starvation and photobiological hydrogen production. This paper presents the first global transcriptional analysis of *C. reinhardtii* before, during, and after photobiological hydrogen production under sulfur deprivation.
ments in H₂ production (8, 24). The degree of starch contri-

bution to the establishment of anaerobiosis, which is the re-

quirement for high hydrogenase activity. A recent study using NAD(P)H dehydrogenase inhibitors strongly suggested that a NAD(P)H dehydrogenase is involved in this pathway (34).

Given the metabolic complexity of inducing and producing H₂ under S-deprived conditions, further studies are required. Here, detailed transcriptional analyses of wild-type C. reinhardtii cultures sampled at different time points during the aerobic and anaerobic phase of the photobiological H₂ pro-
duction process are presented to provide new insights into the complex interplay between these biochemical pathways.

MATERIALS AND METHODS

Strains and growth conditions. Chlamydomonas reinhardtii wild-type strain CC124 was obtained from the Chlamydomonas Genetics Center. The alga was cultured in Tris-acetate-phosphate (TAP) medium (pH 7.3) (23) in sterilized conical 2-liter Erlenmeyer flasks under continuous white fluorescent illumination at 200 µmol photons m⁻² s⁻¹ on an orbital shaker at 80 rpm. For the microarray experiment, 3 liters of CC124 culture was inoculated from the same starter culture which was first grown to mid-logarithmic growth phase. An optical density at 750 nm [OD₇₅₀] of 1.5 and a chlorophyll concentration of 30 mg/liter). For the control sample, 100 ml of culture from each flask was harvested from the cultures described above by centrifugation at 3,000 × g for 1.5 min at 4°C. The resulting cell pellets were immediately frozen in liquid nitrogen and stored at −80°C for later RNA extractions. The remaining cultures were harvested for S deprivation and H₂ production. The cultures were washed three times to remove residual sulfur by centrifugation at 3,000 × g for 5 min at 4°C before resuspending the pellets in 100 ml of sulfur-free TAP medium. The washed cells were pelleted again under the same centrifugation conditions, resuspended, and then pooled together in a total of 2.7 liters of sulfur-free TAP medium, resulting in a sulfur-free suspension with an OD₇₅₀ of 1.2 and a chlorophyll concentration of ~22.8 mg/liter. The suspension was poured into four custom-built 650-ml photobiore-
actors (PBRs) for H₂ production and sample collection at later time points. The OD of the cultures stayed relatively constant after S deprivation, and no significant growth was observed. This experimental design allows direct comparisons between the samples taken after S depletion (time points 1 to 6 [T1 to T6]; see details described below) and the sample just prior to S deprivation (T0).

H₂ production conditions. The PBRs were tightly sealed and connected to custom-built gas collection cylinders placed on corner positions of a six-spot HP fluorescent light. In all cases, the light intensity measured at the surfaces of the PBRs ranged from 250 µE m⁻² s⁻¹ (on the darker side of the two PBRs) to 450 µE m⁻² s⁻¹ (on the side closer to the light source). The cultures were stirred continuously with magnetic bars at 150 rpm. The temperature, pH, and dissolved O₂ (DO) levels in the PBRs were recorded every 5 min by a D130 data logger (Consort, Belgium). The volume of gas produced by the cultures was constantly monitored once the DO level reached zero. The hydrogen gas content was analyzed as described previously (24). The samples were collected from the PBRs through a gas-tight septum attached to a sidearm by a syringe to ensure no O₂ was introduced into the system. Cells were immediately centrifuged (3,000 × g for 90 s at 4°C), and pellets were flash-frozen in liquid nitrogen and stored at −80°C before RNA isolations. When the gas volume in the collection cylinders was lower than 20 ml, nitrogen was used to flush the gas-collecting cylinders before sample collections to avoid diluting the cultures with the incoming distilled water used to submerge the cylinders’ outlet tubes. Samples were taken at six time points (T1 to T6). These correspond to the peak DO level (“peak oxygen” production; T1), the DO level at approximately half of its peak (“mid-oxygen” production; T2), the DO level at zero (“zero oxygen” production; T3), the hydrogen production rate at approximately half its peak (“mid-hydrogen” production; T4), the H₂ production rate at its maximum (“peak hydrogen” production; T5), and the H₂ production rate at near zero (“zero hydrogen” production; T6) (Fig. 2). The exact times for the T1 to T6 sampling were 6 h, 16 h, 21 h, 37 h, 52 h, and 86 h after the start of S deprivation, respectively (Fig. 2). The volume of culture collected was 20 ml each for T1, T2, T3, T4, and T5 and 50 ml for T6. The control/reference sample taken just prior to S deprivation allowed direct comparison between samples at different time points while minimizing the volume of culture taken from the PBRs.

RNA preparation. All samples were collected directly from the active PBRs using a syringe through an airtight sampling port. Samples were immediately transferred to 50-ml Falcon tubes and centrifuged at 3,000 × g for 90 s, and the supernatant was poured off and then transferred to liquid nitrogen. This sampling process was generally completed within 5 min; it was designed to

![Diagram](https://via.placeholder.com/150)
minimize the impact on the active PBRs and limit changes within the RNA. Frozen cells were stored at −80°C before RNA extraction. RNA was ex- tracted following the centrifugation protocol for plant tissues using the SV total RNA isolation system (Promega) without sample grinding. Purified RNA from each sample was eluted in 100 μl of Milli-Q H2O. RNA was quantified using a Nanodrop ND-1000 spectrophotometer. RNA integrity was checked by gel electrophoresis and quantitative reverse transcription-PCR (qRT-PCR). All the control RNA samples were pooled together. Purified RNA was further purified by using the RNeasy MinElute cleanup kit (Qiagen) before preparation of microarray probes. The RNA extracted from each time point was used for both microarray and qRT-PCR analyses.

Preparation of fluorescent microarray probes using the indirect labeling method. Fluorescent microarray probes were prepared by using the indirect labeling method. Total RNA (30 to 40 μg) was denatured at 70°C for 10 min with 1 μg of oligonucleotide deoxyribosylthymine-12 in a reaction mixture volume of 15.3 μl. Master mix 2 was then added to the ice-chilled reaction mixture, containing 6 μl of SuperScript III reverse transcriptase buffer, 1.5 μl of 0.1 M dithiothreitol, 5 μl of deoxyxynucleoside triphosphate mix (0.9 mM dTTP, 3 mM dATP, 3 mM dGTP, 3 mM dCTP, and 1.8 mM aminoallyl- dUTP), and 2 μl of SuperScript III reverse transcriptase (200 U/μl; Invitro- gen). The 30-μl reaction mixture was incubated at 50°C in a Thermocycler for 50 min before RNA extraction. RNA was hydrolyzed by the addition of 15 μl of 1 M NaOH and incubation at 70°C for 15 min before the pH was neutralized by the addition of 15 μl of 1 M HCl. The amino-modified cDNA was purified using the Labeled Star purification kit (Qiagen) according to the manufacturer's directions and then concentra- ted to a 5-μl volume using a vacuum centrifuge. The concentrated cDNA was then labeled with either Alexa Fluor dye 555 or 647 (Invitrogen), 5 μl of cDNA with 3 μl of 0.3 M NaHCO3, and 2 μl of the Alexa Fluor succinimidyl ester dye in dimethyl sulfoxide and was incubated at room temperature for 1 h in the dark. The labeled cDNA from each time point following the initiation of S deprivation was combined with the labeled control cDNA. The labeled sample/control combined cDNA was purified with a Qiagen Label STAR purification kit. Purified cDNA was recovered in 25 μl of Milli-Q Ultrapure H2O. The incorporation of the fluorescent dyes into cDNA was quantified using a Nanodrop ND-1000 spectrophotometer. A direct labeling method using Alexa Fluor 555 5-amino-2-carboxyfluorescein-dUTP (aqua-dUTP) or Alexa Fluor 647 aha-dUTP (Invitrogen) was trialed at T2 (oxygen at approximately half its peak) but was found to be less efficient than the indirect method. The cDNA synthesis method was similar to the indirect labeling method described above but with the following changes. Master mix 2 consisted of the following: 6 μl of 5× buffer, 3 μl of 0.1 M dithiothreitol, 0.5 μl of deoxyxynucleoside triphosphates (10 mM dTTP, 25 mM dATP, 25 mM dGTP, and 25 mM dCTP), 2 μl of SuperScript II reverse transcriptase (200 U/μl), and 5 μl of 2 mM Alexa Fluor 555 aha-dUTP or Alexa Fluor 647 aha-dUTP. Hydrolysis, purification, and quantification were performed as described above.

Preparation, hybridization, washing, and scanning of the microarrays. Chlamydomonas microarray slides version 2 (9) were obtained from Arthur Grossman (Stanford, CA). The microarray slides were immobilized, prehybrid-
FIG. 3. Real-time qRT-PCR analysis of photosynthetic genes and other genes of interest that were identified by microarray analysis. Shown are averages ± the standard error of induction ratios of 57 genes from three independent biological replicates from six different time points before, during, and after hydrogen production. Full gene names and accession numbers/annotations are provided in Materials and Methods.
ber protein (XM_001697461); TTR, tetrafractide repeat (TTRP), domain contain-
ing protein (contig 81.15.2.1); PFL, pyruvate formate lyase (PFL) (A620191); ICL, isocitrate lyase (I18765.1); NAD+, nicotinamide adenine dinucleotide protein (1Y18786.1); SDB, scaffold attachment region DNA binding protein (contig 15.10.5.251); VSP3, VSP3 protein (L20029); AOX1, alternative oxidase (A1F873822); COX2, cytochrome c oxidase subunit 2a (XM_001697479); GPIH/PHO4, starch phosphorylase (XM_00170039); AMY2, alpha amylase 2 (XM_001695626); GRP, GTP binding protein (contig 11.12.0.15); NAD, NADH ubiquinone oxidoreductase (AY347459); Nucleolin (contig 7.4.1.5); HPP3, hypothetical protein 3 (XM_001692849); NAD-MDH, cytoplasmic malate dehydrogenase (AJ250844.1); NADP-MDH, chloroplast NADP-malate dehydrogenase (AJ277281.2); ARS2, arylsulfatase 2 (X52304); ECP88, extracellular protein 88 (AF359322); CAMK4, calcium/calmodulin-depend-
ent protein kinase 4 (contig 25.9.2.51); SRP, hypothetical serine-rich protein (XM_001700323); HPP1, hypothetical protein 1 (XM_001696966); HYDEF, iron hydrogenase assembly protein (AY582739); Unknown 1 (contig 116.19.1.5); Unknown 2 (contig 85.6.1.0); Unknown 4 (contig 4.8.5.1); Unknown 5 (XM_001697875); Unknown 7 (contig 134.13.3.11); Unknown 8 (contig 400.2.1.5); and Unknown 9 (XM_001703534).

Isolation of thylakoid membranes. For the analysis of differentially expressed LHCBM proteins during sulfur starvation, thylakoid membranes were isolated from a photobiological H₂ production experiment identical in design to that described above. All protein isolation procedures were performed under low-light conditions to prevent photodamage to the isolated thylakoid membranes. Cells were harvested by centrifugation (10 min at 2,200 g) and washed in 30 ml of buffer A (25 mM HEPES [pH 7.5], 1 mM MgCl₂, 0.3 M sucrose). The washed cells were again pelleted by the same centrifugation procedure and then resuspended in 30 ml of buffer B (5 mM HEPES [pH 7.5], 10 mM EDTA, 0.3 M sucrose), centrifuged (45 min at 4°C and 4,000 g), and finally resuspended in 2.65 ml of buffer B and 1 ml of buffer C (5 mM HEPES [pH 7.5], 10 mM EDTA, 2.2 M sucrose) to adjust the final sucrose concentration (not including the volume of the pellet) to 1.82 M. The sample was diluted with 5 volumes of buffer E (20 mM MES [morpholineethanesulfonic acid], pH 6.3, 5 mM MgCl₂, 15 mM NaCl, 10% [vol/vol] glycerol) to facilitate digitonin treatment of the purified thylakoid membranes upon centrifugation (20 min at 40,000 g) and then resuspended in a minimal volume (~1 ml) of MMNB buffer (25 mM MES, pH 6.0, 5 mM MgCl₂, 10 mM NaCl, 2 M betaine) and flash-frozen in liquid N₂ prior to storage at −80°C.

Pigment measurements. Pigments were extracted from C. reinhardtii cells in 80% acetone. The absorbance fraction was precipitated by centrifugation (20 min at 17,000 × g) before measuring the chlorophyll concentration and the chlorophyll a/b ratio according to Arnon (2).

SDS gel electrophoresis. Polyethylene glycol fractions from the sucrose density gradient were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% Tris-tricine gel. The stacking gel was made of 5% acrylamide (Bio-Rad), 0.7 M Tris, pH 8.45, 0.07% SDS, 0.1% AMPS (ammonium persulfate), 3.8 μl TEMED (N,N',N”-tetramethylethylene diamine). The resolving gel was made of 10% acrylamide (Bio-Rad), 0.9 M Tris, pH 8.45, 0.09% SDS, 12.5% glycerol, 0.05% AMPS, 3.8 μl TEMED. Samples from sucrose gradient fractions were concentrated from 2 ml to ~30 μl with Vivaspin 500 μl. Pigments were extracted from the protein samples with acetone in excess, dried with N₂ gas, and resuspended in H₂O. Solubilization was conducted in a buffer containing 0.2 M Tris, pH 6.8, 5 mM EDTA, 1 M sucrose, 0.4% diithiothreitol, 3.1% SDS, 0.8% glycerol, and bromophenol blue for approximately 15 min. Gel lanes were loaded with an equal amount of chlorophyll (10 μg). Gels were stained with 0.1% Coomassie blue R250 and G250 for protein visualization.

Identification of proteins by MS, MALDI-TOF, and MALDI-TOF-TOF. All bands from protein gels were excised and digested with trypsin (Promega) at 4 μg/ml. The standard preparation of the calibration mixture for matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) contained des-Argbranin (1.0 pmol/μl), angiotensin I (2.0 pmol/μl), Glu-hyricholic acid peptide II (1.3 pmol/μl), adrenocorticotropin (ACTH) 1-17 clip (2.0 pmol/μl), ACTH 18-39 clip (1.5 pmol/μl), and ACTH 73-38 clip (3.0 pmol/μl). A 10-fold dilution of the standard calibration mixture was used for calibration of the MALDI plates on which the tryptic digest samples were spotted. The sample matrix used was CHCA (a-cyanohexanocinnamic acid) at a concentration of (5 mg/ml) in 50% acetonitrile in 5 mM ammonium phosphate and 1% formic acid (aqueous). Samples were analyzed using a 4700 Proteomics analyzer MALDI-tandem TOF (MALDI-TOF-TOF) (Applied Biosystems, CA). The peptides were resuspended directly with 5 μl of CHCA in 60% acetonitrile/0.1% formic acid (aqueous) onto a MALDI target plate. All mass spectrometry (MS) spectra were recorded in the positive reflector mode at a laser energy setting of 4800. For MS data, 1,000 shots were accumulated for each spectrum using an MS-positive ion reflector mode acquisition method. All tandem MS (MS-MS) data from the TOF-TOF were acquired using the default positive ion, 1 kV collision energy, reflector mode, MS-MS method at a laser energy of 5500. MS-MS data acquisition was performed in a four-step process. First, MS spectra were recorded from each of the six calibration spots, and the default calibration parameters of the instrument and the plate model for that plate were updated. Second, MS spectra were recorded for all sample spots on the plate. Each spectrum was generated by accumulating the data from 1,000 laser shots, using the newly updated default calibration settings. Third, the TOF MS spectra were analyzed using the Peak Picker software supplied with the instrument. The 10 most abundant spectral peaks that met the threshold (a signal/noise ratio of >20) criteria and were not on the exclusion list were included in the acquisition list for the TOF-TOF. MS-MS portion of the experiment. The threshold criteria were set as follows: a mass range of 850 to 4,000 Da, a minimum cluster area of 500, a minimum signal/noise ratio of 20, and a maximum number of MS-MS spectra per spot of 10. A mass filter excluding matrix cluster ions and trypsin autolysis peaks was applied. An XML file was generated that contains the list of the precursor masses selected for MS-MS. Database searching of noninterpreted TOF-TOF and TOF-TOF MS-MS data was carried out using the Mascot search engine (Matrix Science) (44) and the MSDB 20040630 containing 1,501,893 sequences and 480,537,669 residues with the Vitis vinifera taxonomy. Proteins were identified from the MS-MS data with confidence of at least 95%. Confidence was based on the number of peptides found in MS and the ion score from MS-MS.

Microarray data accession numbers. The complete microarray data set has been submitted to Gene Expression Omnibus and can be accessed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=prszawvmoakvtsy&acc=GSE9165.

RESULTS

Global expression profiling in C. reinhardtii before, during, and after hydrogen production. Cultures of C. reinhardtii (CC124) were grown as independent biological replicates and induced for hydrogen production by sulfur deprivation. Samples for RNA extraction were taken from the PBRs at T1 to T6 corresponding to 6 h, 16 h, 21 h, 37 h, 52 h, and 86 h after the initiation of S depletion. The first three time points correspond with different DO levels in the PBRs, referred to as peak oxygen, mid-oxygen, and zero oxygen production rates (Fig. 2). The last three time points represent mid-hydrogen, peak hydrogen, and zero hydrogen production rates (Fig. 2). Quality control of all RNA samples was carried out by gel electrophoresis and real-time qRT-PCR to ensure that the RNA samples did not show any signs of degradation. Only a limited amount of quality RNA could be obtained from T6 samples due to cell death at this late time point. Consequently, microarray hybridizations were only conducted on samples collected during the first five time points (T1 to T5). All samples were compared to the control taken immediately prior to sulfur deprivation (T0). Stringent analyses of the data identified 166 genes that showed at least a twofold change in one or more of the five time points consistently across technical (duplicate spots on the array) and biological replicates of the experiment and that had shown statistical significance (Table 1). Based on their closest match to GenBank entries, genes were assigned to different groups according to their putative functions. These include photosynthesis (22 genes), sulfur metabolism (8 genes), carbon metabolism (4 genes), proteolysis (5 genes), etc.
### TABLE 1. Induction and repression ratios of 166 *C. reinhardtii* genes that showed at least a twofold change in transcript abundance in one or more time points (T1 to T5) before and during hydrogen production

| Gene function and GenBank accession no. or contig (no. of genes) | GAL file ID | Gene annotation description (closest match) | Peak O₂ | Mid O₂ | Zero O₂ | Mid H₂ | Peak H₂ |
|---------------------------------------------------------------|-------------|---------------------------------------------|---------|-------|-------|-------|-------|
| Photosynthesis (22)                                           |             |                                             |         |       |       |       |       |
| AB122117.1                                                    | 257.A       | LhcI-4 gene for light-harvesting chlorophyll a/b protein of PSI | 0.39    | 0.20  | 0.19  | 0.22  | NA    |
| AY171231                                                     | 262.A       | Light-harvesting complex I protein          | 0.36    | 0.29  | 0.25  | 0.31  | NA    |
| X15166                                                       | 250.A       | PsAK mRNA for 8.4-kDa subunit of PSI         | 0.31    | 0.28  | 0.32  | 0.29  | NA    |
| XM_001691032                                                 | 8094.D      | PSI reaction center subunit XI, chloroplast precursor | 0.47    | 0.45  | 0.45  | 0.45  | NA    |
| AB050007                                                     | 217.A       | CP26                                        | NA      | NA    | NA    | NA    | NA    |
| AF244524                                                     | 158.A       | Chlorophyll a/b binding protein              | NA      | NA    | 0.20  | 0.56  | NA    |
| AB051211                                                     | 159.A       | Light-harvesting chlorophyll a/b binding protein Lhcβ4 | 0.52    | 0.50  | 0.27  | 0.14  | 0.46  |
| AF170026.1                                                   | 426.A       | PSII reaction center W protein, chloroplast precursor | NA      | NA    | 0.50  | 0.34  | 0.50  |
| XM_001701652                                                 | 4465.A      | 4.1-kDa PSII subunit (PSBX)                  | NA      | NA    | 0.30  | 0.34  | NA    |
| J05524                                                       | 463.A       | Plastocyanin, chloroplast precursor          | 0.27    | 0.11  | 0.22  | 0.29  | NA    |
| X04472.1                                                     | 45.A        | RbcS2 for ribulose bisphosphate             | NA      | NA    | 0.12  | 0.13  | NA    |
| M36123                                                       | 352.A       | Phosphoribulokinase, chloroplast precursor (PRK) | NA      | NA    | 0.18  | 0.18  | NA    |
| AF107303                                                     | 458.A       | Open reading frame of intron 2 from chloroplast psbA gene | NA      | NA    | 1.48  | 1.91  | 2.74  |
| XM_001696073                                                 | 8770.D      | Chlorophyll a/b binding protein homolog LHCSR3 precursor | 4.89    | 1.29  | NA    | 1.29  | NA    |
| AF479778                                                     | 350.A       | Major light-harvesting complex II protein m9 (LHCBM9) | NA      | 11.26 | 17.41 | 109.03 | 22.30 |
| Sulfur assimilation (8)                                       |             |                                             |         |       |       |       |       |
| X52304                                                       | 73.A        | Arylsulfatase 2 precursor (ARS2)             | 15.39   | 7.62  | 18.10 | 5.63  | NA    |
| XM_0016977170                                                | 7778.D      | Sulfate transporter (SULTR2)                 | 3.61    | 2.90  | 2.68  | 3.16  | NA    |
| XM_001699252                                                 | 8577.D      | Sulfite reductase                           | NA      | NA    | 0.93  | 3.96  | NA    |
| X16719.1                                                     | 382.A       | Arylsulfatase 2 precursor                    | NA      | NA    | NA    | 7.25  | NA    |
| U57088                                                       | 468.A       | Probable sulfate adenyl transferase ATS1     | NA      | NA    | NA    | 2.86  | 2.22  |
| AF359251                                                     | 432.A       | Extracellular polypeptide Ecp76             | 2.19    | NA    | NA    | 4.27  | NA    |
| 199.8.2.31                                                   | 7885.D      | Extracellular polypeptide Ecp88             | 2.96    | 1.31  | 2.75  | 2.15  | NA    |
| AF359252                                                     | 167.A       | Extracellular polypeptide Ecp88             | 3.25    | NA    | 2.40  | 18.90 | 2.33  |
| Carbon metabolism (4)                                         |             |                                             |         |       |       |       |       |
| AF394513                                                     | 337.A       | 6-Phosphogluconate dehydrogenase             | 2.65    | NA    | 1.66  | 2.73  | NA    |
| U18765                                                       | 40.A        | Probable isocitrate lyase                    | 0.14    | 0.11  | 0.17  | 0.14  | NA    |
| X66410                                                       | 254.A       | Pyruvate formate lyase                       | NA      | NA    | 3.54  | 1.82  | NA    |
| U42979.1                                                     | 252.A       | Malate dehydrogenase, sodium acetate induced | 0.64    | NA    | NA    | 0.42  | 0.6   |
| Protein synthesis (5)                                        |             |                                             |         |       |       |       |       |
| X60626                                                       | 67.A        | UBI1 mRNA for ubiquitin fusion protein       | NA      | 1.47  | 3.18  | NA    | NA    |
| XM_001696123                                                 | 3750.C      | 268 proteasome regulatory complex, lid subcomplex, non-ATPase subunit RP3 (subunit 3) (PSMD3) | NA      | 3.63  | 2.77  | 0.76  | NA    |
| 72.37.1.5                                                    | 7281.C      | F15M7.13, ubiquitin carboxyl-terminal hydrolase, Arabidopsis | NA      | 4.01  | NA    | NA    | NA    |
| AY062935                                                     | 348.A       | Ubiquitin-conjugating enzyme E2 isofrom     | 2.98    | 2.09  | 6.00  | NA    | NA    |
| XM_001695251                                                 | 4464.C      | Putative serine carboxypeptidase precursor   | NA      | NA    | NA    | 2.48  | NA    |
| Amino acid synthesis (4)                                     |             |                                             |         |       |       |       |       |
| U36197                                                       | 135.A       | 5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase | NA      | NA    | 0.38  | 0.29  | NA    |

Continued on following page
| Gene function and GenBank accession no. or contig (no. of genes) | GAL file ID | Gene annotation description (closest match) | Peak O₂ | Mid O₂ | Zero O₂ | Mid H₂ | Peak H₂ |
|---------------------------------------------------------------|------------|---------------------------------------------|---------|--------|---------|--------|--------|
| **Transcription and translation (10)**                         |            |                                             |         |        |         |        |        |
| XM_001696696                                                  | 5869.C     | PRP3, pre-mRNA processing factor 3          | NA      | NA     | NA      | 2.53   | NA     |
| XM_001691400                                                  | 9120.E     | Eukaryotic initiation factor 4A-10-like protein | NA      | NA     | NA     | 2.84   | 1.45   |
| XM_001691255                                                  | 4469.C     | SF3A3 splicing factor 3a, subunit 3 (SPL3)  | NA      | NA     | NA     | 2.78   | 4.11   |
| XM_001696696                                                  | 9339.E     | Aspartate-semialdehyde dehydrogenase (ASSD1) | NA      | 0.80   | 0.74   | 0.40   | 1.17   |
| **Redox cycling (3)**                                         |            |                                             |         |        |         |        |        |
| XM_001693558                                                  | 9401.E     | Similar to vanadium chloroperoxidase         | 2.72    | 1.28   | NA      | 3.22   | 7.71   |
| 15.82.2.11                                                    | 9635.E     | Putative oxidoreductase *Oryza sativa* subsp. *japonica*, 82.3% identity | 0.67    | NA     | 3.11   | 1.90   | NA     |
| X78822                                                        | 17.A       | Thioredoxin H-type                          | NA      | 2.91   | NA      | 0.60   | NA     |
| **Other pathways and processes (27)**                         |            |                                             |         |        |         |        |        |
| U36196                                                        | 161.A      | Protein kinase, 48K                         | 0.45    | NA     | NA      | 0.46   | NA     |
| XM_001691558                                                  | 4567.C     | WNK protein kinase (WNK1)                   | NA      | NA     | 2.86    | 1.37   | 0.74   |
| XM_001702027                                                  | 516.B      | CDK inhibitor kinase (WEE1)                 | NA      | NA     | NA      | 3.98   | NA     |
| XM_001692562                                                  | 506.B      | Rhodanese domain phosphatase (RDP3)         | 5.99    | NA     | 2.96    | 1.94   | 2.32   |
| XM_001696853                                                  | 645.C      | G protein-coupled seven transmembrane receptor | NA      | 3.51   | NA      | 0.64   | NA     |
| XM_001703235                                                  | 9249.C     | PWR motif protein (PWR1)                    | NA      | 0.45   | 2.85    | 6.72   | NA     |
| XM_001703036                                                  | 3414.C     | Selenium binding protein (SBD1)             | 5.17    | NA     | 2.48    | 2.39   | 4.54   |
| XM_001698919                                                  | 4207.C     | Conserved protein similar to EMP70/nonaspanin | NA      | 0.55   | NA      | 0.55   | NA     |
| AF399653.1                                                    | 389.A      | Ezy2                                        | NA      | 2.91   | NA      | 0.60   | NA     |
| L29029                                                        | 186.A      | VSP-3                                       | NA      | 0.33   | 3.31    | 1.90   | NA     |
| XM_001694402                                                  | 9383.E     | Hydroxylamine reductase, hybrid cluster protein HCP4 | 2.18    | 3.42   | NA      | 1.32   | NA     |
| XM_001690300                                                  | 6995.C     | NOP58 structural component of C/D snoRNPs   | NA      | 4.38   | NA      | 0.46   | NA     |
| XM_001703004                                                  | 8375.D     | Type II NADH dehydrogenase (NDA7)           | 2.42    | NA     | NA      | NA     | NA     |
| XM_001702995                                                  | 8352.D     | Dehydrogenase with pyroloquinoline quinone as a cofactor | NA      | 3.84   | NA      | 1.34   | NA     |
| XM_001690491                                                  | 4875.C     | Endoplasmic reticulum-located HSP11/SSE-like protein | NA      | 2.67   | 1.46   | NA     | NA     |
| XM_001701229                                                  | 2968.C     | Flagellar-associated protein                | NA      | NA     | 3.04    | NA     | NA     |
| AF133672                                                      | 311.A      | Coproporphyrinogen III oxidase precursor    | NA      | NA     | NA      | 3.53   | 2.40   |
| XM_001696259                                                  | 2161.C     | Mu1-adaptin (AP1M1)                         | NA      | NA     | 1.85    | 2.44   | NA     |
| XM_001693906                                                  | 1657.C     | 1-Deoxy-d-xylulose 5-phosphate reductoisomerase (DXR1) | 0.40    | 0.56   | 0.46    | 0.55   | NA     |
| X76117                                                        | 212.A      | Zygote-specific protein                     | 0.38    | 0.37   | 0.28    | NA     | NA     |
| XM_001699620                                                  | 9223.E     | TH4 regulatory protein                      | 0.25    | 0.47   | 0.44    | NA     | NA     |
| XM_001693730                                                  | 166.A      | Acyl carrier protein (ACP2)                 | 0.53    | 0.59   | 0.48    | 0.28   | NA     |
| XM_001690256                                                  | 9591.E     | Cell wall protein pherophorin C2 (PHC2)     | 0.59    | 0.25   | 0.36    | NA     | NA     |
| XM_001697946                                                  | 6272.C     | Flagellar-associated protein (FAP218)       | 0.53    | 0.43   | 0.35    | NA     | NA     |
| XM_001697963                                                  | 4463.C     | Cell wall protein pherophorin C3 (PHC3)     | 0.52    | 0.35   | 0.35    | NA     | NA     |
| Unknown (83)                                                  |            |                                             |         |        |         |        |        |
| XM_001690783                                                  | 1650.C     | Low-CO₂-inducible protein (LC130)           | NA      | 2.31   | NA      | 1.35   | 0.39   |
| AF015661                                                      | 477.A      | Low-CO₂-inducible protein (LC13)            | 0.71    | NA     | 0.35    | 0.20   | NA     |
| 141.1.1.0                                                     | 5077.C     | Phenylcoumaran benzylic ether reductase homolog TH6 | NA      | NA     | NA      | NA     | 6.82   |
| 25.9.2.51                                                     | 6103.C     | CAMK4, calcium/calmodulin-dependent kinase IV *Homo sapiens*, 62.2% identity | NA      | 3.21   | NA      | 0.68   | NA     |

Continued on following page
| Gene function and Gene annotation description (closest match) | Peak O₂ | Mid O₂ | Zero O₂ | Mid H₂ | Peak H₂ |
|-------------------------------------------------------------|---------|--------|---------|--------|---------|
| 44.35.1.5 Fibroin heavy chain precursor [Bombyx mori], 6.8% identity | NA      | NA     | 3.75    | NA     | NA      |
| 688.1.1.0 Large tegument protein [Herpes simplex virus], 10.7% identity | 2.49    | NA     | NA      | 1.19   | NA      |
| 13.36.2.11 SRP40, suppressor protein SRP40 [Saccharomyces cerevisiae], 68.7% identity | 4.62    | NA     | 0.56    | NA     | NA      |
| 26.39.1.0 EBNA-1 nuclear protein [Human herpesvirus 4], 27.0% identity | NA      | NA     | 0.38    | NA     | NA      |
| 96877 Hypothetical luminal protein precursor, chloroplast | 0.57    | 0.54   | 0.34    | 0.40   | 0.72    |
| XM_001695499 Predicted protein (SAA15) with TPR, ANK, and zf-MYND domains | NA      | NA     | 3.72    | 1.38   | NA      |
| XM_001700193 Predicted protein CAP_ED, effector domain of CAP transcription factor | NA      | 2.56   | 1.44    | 0.63   | NA      |
| 3.43.1.0 Putative glucan synthase [Arabidopsis thaliana], 72.8% identity | NA      | NA     | 2.76    | 1.49   | NA      |
| 16.97.1.5 Y25C1A.3 protein [Caenorhabditis elegans], 57.9% identity | NA      | NA     | 3.96    | 1.68   | NA      |
| 121.14.1.5 Vegetative cell wall protein gp1 precursor [C. reinhardtii], 30% identity | NA      | NA     | 2.27    | NA     | NA      |
| XM_001694590 Hypothetical protein | NA      | 2.30   | NA      | NA     | NA      |
| 111.18.4.11 Cullin 1B (Nicotiana tabacum), 99.7% identity | NA      | 2.87   | NA      | NA     | NA      |
| 7.42.1.5 Nucleolin (protein C23) [Mesocricetus auratus], 67.8% identity | NA      | 6.30   | NA      | 0.14   | 0.50    |
| XM_001697504 Hypothetical protein | NA      | 0.49   | 0.39    | 0.33   | NA      |
| 152.14.15 CrE protein (Shigella flexneri), 55.4% identity | NA      | 2.74   | 1.29    | NA     | NA      |
| 58.41.1.5 Fibroin heavy chain precursor (Fib-H) (H-fibronin) [B. mori], 10.5% identity | NA      | 2.20   | 0.89    | NA     | NA      |
| 576.2.1.0 Hypothetical protein | 0.61    | NA     | 0.52    | 0.33   | 1.81    |
| 356.1.3.11 Hypothetical protein | 0.56    | NA     | 0.52    | 0.12   | NA      |
| 46.52.2.11 CG2839 protein | 3.73    | 0.86   | 0.79    | NA     | NA      |
| XM_001689556 Hypothetical protein, homology to Phytophthora infestans CRN1 | NA      | 1.35   | 2.16    | NA     | NA      |
| XM_001696388 Hypothetical protein | NA      | 1.39   | 2.23    | 1.57   | NA      |
| 116.19.1.5 C_1160006 [158018:161045] | 2.53    | 2.55   | 1.56    | 0.44   | NA      |
| XM_001700674 Hypothetical protein 45% identity to A. thaliana UDP glucosyl transferase | NA      | NA     | 1.62    | 2.68   | NA      |
| XM_001694976 Hypothetical protein | NA      | 2.14   | 3.64    | 2.48   | NA      |
| Conif Ig 85.6.1.0 | 0.85    | 6.61   | 2.42    | 0.10   | NA      |
| XM_001693665 CAS_like, clavaminic acid synthetase-like | 5.31    | NA     | 2.64    | 3.94   | 3.02    |
| XM_001689728 Hypothetical protein with ring finger domain | NA      | 3.79   | 2.39    | 1.31   | NA      |
| 1.83.11 HC_10364 [926397:929841] | 1.73    | NA     | 5.22    | 4.16   | NA      |
| 400.2.1.5 | 0.47    | NA     | 0.69    | NA     | NA      |
| 8.83.1.5 Tax1d=9666 splice isoform 1 of Q15149 plectin 1 | NA      | 2.39   | 0.59    | NA     | NA      |
| XM_001698598 Hypothetical protein | NA      | 2.85   | 1.10    | NA     | NA      |
| XM_001699124 Hypothetical protein | NA      | 2.63   | 1.11    | NA     | NA      |
| 117.7.1.0 | 3.64    | NA     | 1.25    | NA     | NA      |
| 30.47.1.5 Y25C1A.3 protein | NA      | 3.26   | 1.32    | NA     | NA      |
| 205.4.1.5 | 4.02    | NA     | 1.41    | 1.40   | NA      |
| XM_001691394 Hypothetical protein | NA      | 3.46   | 1.73    | 1.33   | NA      |
| 103.28.2.31 Hypothetical protein | NA      | 6.32   | 0.47    | NA     | NA      |
| 667.1.1.0 | 4.04    | NA     | 0.51    | NA     | NA      |
| 17.108.1.0 C_1740018 [20231:20880] | NA      | 4.90   | 0.61    | NA     | NA      |
| XM_001699240 TPR repeat protein | NA      | 4.17   | 0.78    | NA     | NA      |
| 26.58.1.5 | 4.40    | NA     | 0.81    | NA     | NA      |
| 26.55.1.5 | 6.04    | NA     | 0.12    | NA     | NA      |
| 3.101.2.11 MGC33630, hypothetical protein MGC33630 | NA      | 4.03   | 0.38    | NA     | NA      |
| XM_001698756 Hypothetical protein | NA      | 3.91   | 0.33    | NA     | NA      |
| 8.45.1.0 | 4.64    | NA     | 0.20    | NA     | NA      |
| XM_001692849 Hypothetical protein, 26% identity to DNA-dependent RNA polymerase E' (ISS) [Ostreococcus tauri] | NA      | 3.88   | 0.45    | 0.74   | NA      |
| XM_001697999 Hypothetical protein | 1.76    | NA     | 2.64    | 1.37   | NA      |
| XM_001692679 Hypothetical protein | NA      | NA     | 2.18    | NA     | NA      |
| 1.59.2.11 | 2.70    | NA     | 1.28    | NA     | NA      |
| 10.23.2.11 | 2.78    | NA     | 2.68    | NA     | NA      |
| 12.107.1.5 | 824.7   | NA     | NA      | NA     | 0.42    |

Continued on following page
### TABLE 1—Continued

| Gene function and GenBank accession no. or contig (no. of genes) | GAL file ID | Gene annotation description (closest match) | Peak O₂ | Mid O₂ | Zero O₂ | Mid H₂ | Peak H₂ |
|---------------------------------------------------------------|------------|---------------------------------------------|---------|--------|---------|--------|---------|
| XM_001702460                                                  | 8328.D     | 44% identity to molecular cochaperone STI1 (ISS) [O. tauri] | NA      | NA     | NA      | 2.24   | NA      |
| XM_001694296                                                 | 5751.C     | 45% identity to PBCV-1 prolyl 4-hydroxylase (ISS) [O. tauri] | NA      | NA     | NA      | 0.17   | NA      |
| 145.2.2.11                                                   | 5700.C     |                                              | NA      | NA     | NA      | 2.40   | 1.38    |
| XM_001695405                                                 | 4344.C     | 37% identity to PEX22 (peroxin 22), protein binding [A. thaliana] | NA      | NA     | NA      | 2.51   | NA      |
| XM_001702135                                                 | 1572.C     | Hypothetical protein                         | NA      | NA     | NA      | 2.18   | NA      |
| XM_001691387                                                 | 2256.C     | Tax_ID=9606                                  | NA      | NA     | NA      | 2.33   | NA      |
| XM_001701095                                                 | 5542.C     | LMETM1-like protein                          | NA      | NA     | NA      | 3.31   | 1.40    |
| XM_001699679                                                 | 2712.C     | CG10555 protein                              | NA      | NA     | NA      | 3.12   | NA      |
| XM_001702888                                                 | 6018.C     | CG1517 protein                               | NA      | NA     | NA      | 2.24   | NA      |
| 53.15.3.11                                                   | 8244.D     |                                              | NA      | NA     | NA      | 2.16   | NA      |
| 59.52.1.0                                                   | 1456.C     | Tax_ID=10090, Ensembl_locations:7-3323606 | NA      | NA     | NA      | 0.47   | NA      |
| 6.97.1.0                                                   | 9930.E     | C_60084 [69134694926]                        | NA      | NA     | NA      | 3.75   | 1.74    |
| XM_001703534                                                 | 3420.C     | Hypothetical protein, 31% identity to ERD4-related membrane protein | NA      | NA     | NA      | 0.38   | NA      |
| 93.32.2.31                                                   | 3081.C     |                                              | NA      | NA     | NA      | 2.20   | NA      |
| 117.16.2.11                                                  | 9185.E     | C_1170030 [9358399018]                       | NA      | NA     | NA      | 3.75   | 1.51    |
| XM_001696892                                                 | 5915.C     | SPRY superfamily                             | NA      | NA     | NA      | 2.46   | 1.56    |
| XM_001690875                                                 | 8243.D     | Hypothetical protein                         | NA      | NA     | NA      | 2.43   | 1.62    |
| 69.8.2.31                                                   | 652.C      | Hypothetical protein                         | NA      | NA     | NA      | 4.76   | 1.63    |
| 3.16.8.1.0                                                   | 5052.C     | Hypothetical 38.7-kDa protein, 65.6% identity | NA      | NA     | NA      | 2.51   | 1.65    |
| XM_001701276                                                 | 9373.E     | Hypothetical protein                         | NA      | NA     | NA      | 6.36   | 5.95    |
| XM_001701229                                                 | 2968.C     | Flagellar-associated protein [Chlamydomonas reinhardtii] | NA      | NA     | NA      | 3.04   | NA      |
| 5.123.2.31                                                   | 5749.C     |                                              | 2.94    | 1.21   | NA      | NA      | NA      |
| 329.4.1.5                                                   | 4269.C     | C_3290004 [3392636211]                      | NA      | 1.63   | NA      | 0.33   | NA      |
| XM_001699578                                                 | 4127.C     | Hypothetical protein                         | NA      | 1.77   | NA      | 2.16   | 1.17    |
| XM_001699290                                                 | 5393.C     | Hypothetical protein                         | 0.79    | NA     | NA      | 2.03   | 3.39    |
| 341.1.5                                                      | 6015.C     |                                              | NA      | 1.22   | 2.08   | 3.40   | NA      |
| XM_001703283                                                 | 2535.C     | 31% identity to 2OG-Fe(II) oxygenase         | 1.92    | 2.79   | NA      | NA      | NA      |
| 81.50.2.11                                                   | 9274.E     | [Frankia sp. strain EAN1pec]                 | NA      | 1.48   | 2.70   | 2.44   | NA      |

*Shown are averages obtained from microarray data using two to three independent biological replicates. Ratios of at least twofold induction or repression that passed all stringent criteria for data analysis, including Student’s t test (P < 0.05), are bold. Student’s t test was carried out on normalized microarray signals against control signals to establish significant differences. A complete list, including results for all genes used for microarray analysis, is shown in Table S1 in the supplemental material. NA, data points that did not pass the background cutoff or other stringent analysis criteria; GAL file ID, GenePix array list file identification number.

Genes), amino acid synthesis (4 genes), transcription and translation (10 genes), redox cycling (3 genes), other pathways and processes (27 genes), and a large number of genes with unknown functions (83 genes).

**Photosynthesis genes.** The majority of transcripts encoding proteins involved in photosynthesis declined soon after S deprivation and were consistently suppressed throughout the experiment (Table 1, photosynthesis). These included transcripts encoding a ferredoxin; cytochrome b₅₆; plastocyanin; an oxygen-evolving enhancer (OEE1/PSB1) 1, 2, and 3; subunits of PSI and PSII; and light-harvesting complex proteins (LHCA, LHCb4, and LHCb5). Surprisingly, there was an increase in transcript abundance of the genes encoding the major light-harvesting complex 9 (LHCBM9) and two different stress-related chlorophyll a/b binding proteins (LHCSR1 and LHCSR3) (39, 40). In addition, the transcripts of *PRK* encoding phosphoribulokinase and the open reading frame containing *psbH* intron 2 increased more than twofold during mid-hydrogen production. Together with the increase of *LHCbM9* and *LHCSR* transcripts, this result indicates that photosynthetic activity and its regulation did not undergo a simple decrease but underwent a more complex adjustment during photochemical hydrogen production (PBHP) (see Discussion).

**Sulfur assimilation and carbon metabolism genes.** As expected, the microarray data showed strong and consistent responses to S deprivation for genes involved in S assimilation. Expression was strongly induced at all four time points for genes encoding asparaginase (*ARS2*) and the sulfate transporter (*SULTR2*). Genes encoding sulfite reductase and extracellular proteins ECP76 and two different ECP88 proteins were induced in at least one time point. It was apparent that major changes also occurred in the carbon metabolic pathways after the initiation of S depletion. There was a marked and consistent decrease in the transcript levels of genes encoding isocitrate lyase and malate dehydrogenase, two enzymes in the glyoxylate cycle and the citric acid cycle (Table 1, carbon metabolism). On the other hand, transcript levels of the genes encoding 6-phosphogluconate dehydrogenase, two enzymes in the pentose phosphate pathway) and FPL (belonging to the fermentative PFL pathway) increased during the H₂ production phase.

**Proteolysis and amino acid synthesis.** There were also indications of increased protein degradations in *C. reinhardtii* cells...
during PBHP. Genes involved in protein degradation that showed an increase in transcript abundance encode a 26S proteasome subunit S3, a putative serine carboxypeptidase, a putative ubiquitin, an E2 ubiquitin-conjugating enzyme and a ubiquitin carboxyl-terminal hydrolase (Table 1, proteolysis). Furthermore, transcript levels of genes involved in amino acid synthesis, such as those encoding methionine synthase and aspartate β-semialdehyde dehydrogenase, were consistently decreased (two- to threefold), especially at mid-H₂ production (T4). This is consistent with a previous report by Melis et al. (30), demonstrating that significant protein degradation occurred during H₂ production. The only exception was the induction of a gene encoding the putative cytochrome-synthesizing O-acetylsereine lyase precursor which could also be classified to the sulfur assimilation category.

**Genes involved in transcription, translation, and regulation.** Among the more than twofold differentially expressed genes, 10 were classified as having a putative role in transcription or translation. Four of these were upregulated during the hydrogen production phase, including the genes encoding pre-mRNA processing factor 3 (PRP3), the eukaryotic initiation factor 4A-10 (eIF4A-10), splicing factor 3a subunit 3 (SP3a3), and the chloroplast 30S ribosomal protein L11 (rps11). The genes encoding the remaining ribosomal proteins were mostly repressed throughout the course of the experiment. Other genes also potentially involved in regulation or signal transduction include, for example, genes encoding protein kinases (WNK1 and WEE1), phosphatase (RDP3), or the G protein-coupled transmembrane receptor (Table 1, other pathways and processes).

**Genes involved in other pathways and processes and unspecified genes with unknown functions.** A total of 27 genes were identified with differential expression during hydrogen production that are likely to be involved in other general cell maintenance, regulation, and developmental processes not specified above (Table 1). Interestingly, these include a relatively large number of genes (11) that were induced at early time points (T1 or T2). A large number of genes (83 genes) could not be assigned to any putative function. Although their sequences were used for BLAST searches to check against GenBank entries (3), none returned any significant match to any other gene or protein with known functions.

**Expression profiles using real-time qRT-PCR.** Real-time qRT-PCR was carried out on cDNA from all *C. reinhardtii* CC124 samples (three independent biological replicates) and using all six time points (T1 to T6). Among the 166 genes that were found by microarray analysis to be significantly (P < 0.05) differentially expressed by more than twofold, 30 were chosen for further expression profiling by qRT-PCR using specific primers. In addition, another 27 genes of particular interest were subjected to further qRT-PCR analysis (Fig. 3). These genes were grouped according to their functions (e.g., photosynthesis) or sorted by similar expression patterns. As shown in Fig. 3A and C and in agreement with the microarray data, most photosynthesis genes analyzed by qRT-PCR were consistently downregulated, with the exception of *LHCSR1* (encoding a light-induced chlorophyll a/b binding protein), *LHCBM9* (both strongly upregulated in all time points), and the gene encoding the chloroplast Rieske Fe-S precursor protein (*PETC*; T6 only). The upregulation of *LHCBM9* was further investigated (see below). Other repressed genes include isocitrate lyase (*ICL*), light-harvesting gene translation suppressor (*NABI*), and the scaffold attachment region DNA binding protein (*SDB*), as shown in Fig. 3H. Genes that were notably induced include, as expected, the hydrogene-encoding genes *HYDA1, HYDA2*, and *HYDEF* as well as genes encoding a putative glycerol-3-phosphate acyltransferase (*GPAT*) involved in glycerol lipid biosynthesis, the first step in phospholipid biosynthesis. Also induced were *TPR, PFL*, the gene encoding the variable flagellar number protein *VFL*, and an unknown gene termed "Unknown 2" (Fig. 3E to G). Apart from *LHCBM9*, strong induction (up to several thousand-fold) throughout the time course experiment was found for the genes encoding arylsulfatase *ARS2*, the extracellular 88-kDa polypeptide *ECP88*, and calcium-dependent calmodulin kinase *CAMK4* (not in T1), suggesting that these genes may play important roles during S starvation and PBHP (Fig. 3F). For the same genes, the microarray results display the same induction trend although at a much lower magnitude (Table 1, sulfur assimilation). A gene termed “Unknown 7” and genes encoding the coproporphyrinogen III oxidase precursor *CPX1* and a putative NADPH oxidoreductase (*OXR*) showed a strong induction from T3 onwards when anabiosis started (Fig. 3D), making them potential targets for hydrogen production improvements. Genes that were differentially regulated in an antagonistic fashion during the early S starvation (T1 and T2) and the late-hydrogen-production phase (T5 and T6) include a lipoxygenase (*LOX*), cytochrome *b₉* complex small subunit *PETG* and *PETC* as well as an unknown gene (“Unknown 4”); these were generally repressed in T1 to T4 and then induced in T5 and/or T6 (Fig. 3B and G). The opposite expression pattern (early induction followed by repression during H₂ production) was observed for a range of genes, notably those encoding alternative oxidase 1 (*AOX1*), cytochrome *c* oxidase subunit II (*COX2A*), glycogen/starch phosphorylase (*GLPV*), and the gene encoding alpha amylose 2 (*AMY2*), which showed a strong induction of a gene encoding a putative S deprivation and PBHP. Genes that were notably induced in earlier time points) (Fig. 3A to C).

**PSII light-harvesting complex is remodeled during S deprivation and PBHP.** Our microarray analyses showed a strong increase of *LHCBM9* transcripts before (T2 and T3) and during PBHP with induction ratios up to 109-fold (mid-H₂ production; T4). To further investigate this, we carried out detailed expression profiling of LHCBM-encoding genes, followed by biochemical analyses of LHCBM isoforms. The Lhc family of light-harvesting proteins in *C. reinhardtii*, which capture the light required for normal photosynthesis and PBHP, consists of 11 highly conserved proteins (LHCBM1 to LHCBM6, -8, -9, and -11; LHCBM4 and LHCBM5) that are predominantly associated with PSII (10), and nine proteins (LHCA1 to LHCA9) that are more closely associated with PSI (43). Although the Lhc genes and proteins share a high degree of sequence similarity, using specific primers designed in the 5' untranslated region, qRT-PCR allowed us to distinguish individual members of this gene family (35). Results obtained by qRT-PCR confirmed that *LHCBM9* was massively induced (up to 3,000-fold) immediately after S deprivation (T1) and
FIG. 5. Identification of \( LHCBM9 \) in \( C. \) reinhardtii CC124 following anaerobiosis by \( N_2 \) bubbling in comparison to sulfur depletion under aerobic or anaerobic conditions. \( N_2 \) bubbling, transcripts measured at zero \( O_2 \) production and under anaerobic \( H_2 \) production conditions; \( S \) deplete aerobic, \( S \)-depleted cultures bubbled with air to maintain aerobic conditions; \( S \) deplete anaerobic, anaerobiosis (microoxic conditions) induced by \( S \) depletion. Transcript levels were determined by qRT-PCR and compared to T0 from two to three independent biological replicates (shown are averages ± the standard error; hours show time after \( S \) depletion).

then decreased but maintained high levels along the course of PBHP (Fig. 3A). While \( LHCBM9 \) was highly induced throughout the experiment, there was strong suppression of the other genes encoding LHCBM isoforms (Fig. 3A). Interestingly, under normal conditions, \( LHCBM9 \) transcript abundance was very low (~0.25% of that of \( LHCBM1 \)). However, during \( S \) deprivation, its abundance rose to as high as twice the combined abundance of all the other LHCBM-encoding genes under normal conditions (data not shown).

To test whether remodeling of the antenna system of PSII was linked not only to \( S \) deprivation but also to anaerobiosis or PBHP, we induced PBHP solely by anaerobiosis using \( N_2 \) bubbling (“microoxic \( H_2 \) production”) rather than by \( S \) starvation. In addition, we carried out \( S \) deprivation under aerobic and anaerobic conditions. In this experiment, transcriptional regulation of \( LHCBM9 \) in cells of \( C. \) reinhardtii wild-type strain CC124 was analyzed under the following three conditions: (i) anaerobiosis was induced by purging the culture with \( N_2 \); (ii) cells were deprived of sulfur but compressed air was bubbled to keep the culture oxygenated; and (iii) cells were deprived of sulfur, the culture became anaerobic, and \( H_2 \) production was induced. As shown in Fig. 4, \( LHCBM9 \) induction (sixfold) was also observed during PBHP, following anaerobiosis independently of \( S \) starvation. It was also about 45-fold induced at 72 h following aerobic sulfur deprivation and up to 470-fold during anaerobic sulfur deprivation. This suggests that \( LHCBM9 \) regulation depends on both sulfur deprivation and the microoxic \( H_2 \) production process. Although \( LHCBM9 \) transcript levels increased during anaerobic \( H_2 \) production conditions induced by \( N_2 \) bubbling, \( S \) deprivation had a more marked effect. The strongest upregulation was observed during anaerobic conditions induced by \( S \) depletion at all time points analyzed (Fig. 3A and 4). This result therefore suggests that although \( LHCBM9 \) is upregulated during \( H_2 \) production, its induction is regulated mainly by sulfur depletion. In an independent time course experiment under aerobic conditions, \( LHCBM9 \) showed initial induction at 12 h, as expected, as a response to sulfur deprivation; however, this was followed by a steady decrease in transcript abundance until 96 h (data not shown).

To directly analyze the protein composition of the \( C. \) reinhardtii Lhc family of light-harvesting proteins under normal and under \( S \) starvation-induced PBHP conditions, we isolated thylakoid membrane fractions (same samples as those shown in Fig. 4) and subjected the thylakoid proteins to one-dimen-

sional SDS-PAGE. As shown in Fig. 5, a difference in intensity could be observed for proteins around 27 kDa. To further identify the proteins, bands corresponding to \( C. \) reinhardtii before/after \( S \) thylakoid membrane proteins were excised, digested with trypsin, and analyzed by MS-MS. The proteins that were identified with a confidence greater than 95% are listed in Fig. 5, most notably \( LHCBM9 \) and ECP88, which were identified only in thylakoid fractions after \( S \) induction. This confirms the marked upregulation of expression for both genes observed in the microarray and qRT-PCR studies. Although the homology between LHCBM proteins is very high (66 to 95% amino acid sequence identity, with an average of 77% (43), the differentiation between LHCBM9 and other LHCBM proteins was possible thanks to a specific precursor ion which is unique to LHCBM9, which has an observed mass of 2,540.25 Da. Among the SDS-PAGE-resolved proteins, LHCBM iso-

forms were by far the most dominant, followed by \( \alpha \) and \( \beta \) subunits of ATP synthase.

DISCUSSION

Following microarray experiments and stringent data analysis, we were able to confidently measure the regulation of 7,124 transcripts from \( C. \) reinhardtii CC124 during sulfur de-
privation at five time points: peak O₂ production, mid-O₂ production (consumption), zero O₂ production, mid-H₂ production, and peak H₂ production. Among the characterized genes, 166 showed more than a twofold change in transcript abundance. Out of these, 18 genes were confirmed by qRT-PCR. 4 were not differentially regulated, and 8 did not confirm the microarray results (Fig. 3; Table 1; see Table S1 in the supplemental material). Different results obtained by both methods are likely to be caused by weak signals and cross-hybridization events to other genes, a common problem with microarray hybridizations that are carried out at relatively low temperatures (5, 14, 18, 23, 41), suggesting that further confirmations by qRT-PCR may be required for certain genes of interest. Nevertheless, our microarray data from three biological replicates that passed all stringent criteria for data analysis, including Student’s t test, will provide a useful platform for gene discovery in *C. reinhardtii* during PBHP. The Blast search homology matches in GenBank provide a guide for putative functions of *C. reinhardtii* genes (listed in this paper after their closest match), and the following paragraphs discuss the possible roles of genes during PBHP, keeping in mind that further experimentation may be required to determine the exact functions.

Expression patterns in *C. reinhardtii* cells during initial phases of PBHP reveal commonalities to responses to sulfur stress. A large portion of the expression signature presented here shows overlaps to results from S starvation experiments (51), suggesting that S starvation presents a main factor for gene expression changes in our experiment. PBHP in *C. reinhardtii* using sulfur depletion is a complex process. From the start of the experiment to peak H₂ production, *C. reinhardtii* cells undergo two major physiological changes: S starvation and then anaerobiosis. In the first two phases (oxygen evolution phase and oxygen consumption phase), both the cell’s structure and its metabolic system must adapt to the lack of sulfur supply. Consequently, the cell has to conserve S by minimizing usage and maximizing scavenging efforts. Such adaptation has been studied in detail (37, 42, 51). Evidence at the transcript level and some evidence at the protein level have shown that during this phase, major cellular activities such as photosynthesis and protein synthesis decrease, while a significant increase in the abundance of transcripts encoding stress-responsive proteins was observed. A steep decline in photosynthetic activity at 20 h after sulfur deprivation was confirmed by pulse amplitude modulation measurements in four independent PBRs (data not shown). The following three phases (anaerobic phase, hydrogen production phase, and termination phase) have not been analyzed for transcriptional changes until now. During these phases, in addition to S starvation, cells also suffer a lack of oxygen. As a result, carbon metabolism shifts from respiration to fermentation and reduced hydrogen is released as H₂ gas to circumvent NAD(P)H production (17). Therefore, a large number of sulfur starvation and anaerobic-responsive genes mask the process of hydrogen production. The results showed that photosynthetic activities were reduced under S starvation. With a few exceptions, the transcript level of most photosynthesis genes decreased significantly throughout the first 40 h (Fig. 3A and C). Previous studies showed that the concentration of functional PSII (Qₐ), cytochrome b₅₆, and PSI (P700) as well as the chlorophyll concentration in cultures undergoing PBHP decreased steadily after the initiation of S deprivation (30). In contrast to those of most photosynthesis genes, transcripts of *C. reinhardtii* sulfur assimilation genes increased significantly during PBHP in response to the lack of sulfur in the medium to increase the capacity for S scavenging (37, 42, 51). All transcripts encoding sulfur assimilation genes presented in this study have been described as sulfur acclimation-specific genes (37). Our data show that cells undergoing PBHP also display a downregulation of genes required for amino acid synthesis and an upregulation of genes involved in protein degradation, as expected for cells that do not multiply but maintain only their basic functions for survival. This is in agreement with Melis et al. (30), who reported that under PBHP conditions, the protein concentration of the culture decreased steadily over time.

Changes in carbon metabolism. From the beginning of S deprivation, carbon metabolism in *C. reinhardtii* changed markedly. Our results indicate a strong suppression of the glyoxylate and Calvin cycles. Both cycles are coordinately regulated at the enzyme level to balance the energy-yielding metabolism and glucogenesis from acetate. In one glyoxylate cycle, one succinate and one NADH are produced for every two acetyl coenzyme A (acetyl-CoA) and one NAD⁺. When enough energy is produced through the glycolytic and citric acid cycles, acetate is funneled into the glyoxylate cycle for the production of glucose, amino acids, or nucleotides. Although reducing power is generated as NADH, probably the process is not favorable due to the lack of energy for synthesizing acetyl-CoA and due to the decreased demand for succinate under S-depleted conditions. It is expected that both cycles are downregulated during PBHP, since energy supply from respiration is very limited toward the hydrogen production phase of PBHP (29). In contrast, the pentose phosphate pathway was induced. Such changes are expected because the glyoxylate cycle functions only when the cell has enough energy from glycolysis and respiration, which is not the case under microoxic or anaerobic conditions due to S starvation. Although acetate is ubiquitous in the S-deprived culture, acetyl-CoA is not readily available for the glyoxylate cycle. The following conversion of acetate to acetyl-CoA catalyzed by acetyl-CoA synthase enzyme requires ATP: acetate + ATP + CoA → acetyl-CoA + ADP.

The pentose phosphate pathway produces two NADPH, one CO₂, and one ribose-5-phosphate from one glucose-6-phosphate. The five-carbon sugar can be used for nucleotide synthesis or recycled into glucose-6-phosphate. In the latter case, the pentose phosphate can provide protection from oxidative damage to the cell by recycling the five-carbon-sugar product ribose-5-phosphate. This generates primarily NADPH, whose oxidative damage protection role is rather well known (e.g., in erythrocytes). S starvation studies have shown that cells suffer from oxidative stress under these conditions (51). Moreover, the NADPH produced from the pentose phosphate pathway potentially can feed electrons into the chloroplast electron transport chain (ETC) via one of the putative NAD(P)H dehydrogenases for hydrogen production and therefore maintains ATP production (34). An increase in transcript abundance of phosphoglucuronate dehydrogenase was also reported in S-deprived culture (51). The increase in the pentose phosphate pathway indicates that *C. reinhardtii* cells already suffer from redox stress at the beginning of S starvation. Although no
microarray data were available for the alternative oxidase genes, qRT-PCR data showed a substantial increase in the transcript abundance of AOX1 at peak and mid-O_2 production levels before gradually decreasing to below that of the control level at mid-H_2 and zero H_2 production, indicating that C. reinhardtii cells were under redox stress during the initial phases of the experiment (Fig. 31). Zhang et al. (51) reported that AOX1 was repressed after 24 h of S deprivation (their first point of measurement), which is earlier than that in our experiment. This may be attributed to slightly different conditions used in our experimental setup that led to more severe stress (gas-tight conditions and sulfur depletion under high-light conditions). During the oxygen evolution phase, the rate of photosynthetic O_2 evolution rapidly slows down. At peak O_2 levels where sample T1 was harvested, the respiration rate began to exceed that of O_2 evolution, causing O_2 shortage and redox stress in the photosynthetic ETC (Fig. 2). More evidence came from the increase in the transcript abundance of a gene similar to the Yersinia pestis hydroxylamine reductase (HCR)-encoding gene soon after the initiation of S deprivation (Table 1). HCR has been reported to increase under redox stress (1), and more recently, HCR was shown to be strongly induced shortly after the initiation of dark anaerobiosis in C. reinhardtii (33). There was also evidence that fermentative pathways increased from the start of anaerobiosis. Our data show that the transcript encoding formate acetyltransferase (PFL) increased substantially at T0 O_2 and mid-H_2 production. At those points, the culture had used up all the DO available for respiration. Consequently, fermentation was the only way to generate ATP at the substrate level, with an additional mechanism being through cyclic electron transport. By catalyzing the conversion of pyruvate to formate, ethanol, and acetate with the production of ATP, PFL helps the cell to generate energy without the requirement of O_2. This result agrees with previous studies of the role of the PFL pathway in C. reinhardtii carbon metabolism under anoxic conditions (3, 17).

Surprisingly, our qRT-PCR data showed that transcripts encoding starch degradation enzymes starch phosphorylase (GLPV) and alpha amylase (AMY2) were upregulated very early after S depletion, when starch accumulation patterns are normally observed. Prevailing evidence at the substrate level demonstrated that C. reinhardtii cells accumulate starch in the early phase (20 to 30 h) of both S starvation only and in combination with closed conditions for H_2 production (4, 11, 21, 30, 44, 49, 50). Similar results to ours were also observed in at least two other shorter time course studies of sulfur (24 h) and phosphate (48 h) deprivation (32, 51). In these studies, genes encoding starch phosphorylase and alpha amylase were significantly upregulated at the same time as the gene encoding the granule-bound starch synthesizing enzyme starch synthase (STA2). Our data further show that GLPV (PHOA) and AMYA2 were suppressed later during H_2 production (Fig. 31) when starch is consumed (21). While transcriptional regulation may not always be linked to enzymatic regulation, Moseley et al. (32) suggested that starch degradation was required for starch synthesis. Another possible explanation for the spatial separation of simultaneous degradation and synthesis of starch can also be excluded, since starch-degrading activities in C. reinhardtii are located solely in the chloroplast (19, 26), unlike in many higher plants where it also occurs in the cytosol. This may suggest that GLPV and AMYA2 are involved in starch accumulation rather than degradation. Supporting evidence from Yu et al. (48) showed that alpha amylase was not required for transitory starch breakdown in Arabidopsis spp. leaves, while a more recent study of starch phosphorylase postulated that PHOA, which is identical to GLPV, also has starch synthase activities (7).

The trigger for HYDA4 transcription is not just anaerobiosis. Although anaerobiosis is a requirement for hydrogenase activity, it is not the only trigger for HYDA transcription. Our qRT-PCR results clearly show that HYDA4 transcription was induced soon after S starvation, long before anaerobiosis in the medium was established, although anaerobiosis did enhance the transcription of HYDA4. After the first 16 h of our experiment (peak O_2 and mid-O_2 production), the DO levels were at ~9 mg liter^{-1}, which is far above the induction level for hydrogenases reported by others, although fully aerobic sulfur-deprived culture showed repression of HYDA transcription during the first 24 h (51). Therefore, it is possible that the induction of HYDA1 and HYDA2 was influenced by the effect of higher levels of light in our experiment (250 to 450 μE compared to only 100 μE used by Zhang et al. [51]).

Remodeling of PSII during S-deprived PBHP. It is not surprising that a large number of differentially expressed genes can be classified as S starvation responsive. This group of genes is either constitutively induced or suppressed throughout the experiment. Belonging to this group are most of the photosynthesis, sulfur assimilation, and acquiring genes and some of the genes involved in carbon metabolism, protein synthesis, and degradation (Table 1; Fig. 3). To this end, our data confirm previous findings about the impacts of S starvation on the activities of photosynthesis, S scavenging, protein degradation, and synthesis in C. reinhardtii (51). Furthermore, we provide strong evidence of the remodeling of PSII-LHC where LHCMB9 and LHCSC-encoding genes were constitutively induced up to thousands-fold in spite of the dramatic repression of genes encoding other LHCBM isoforms. This was also confirmed by our biochemical analyses of LHCBM isoforms (Fig. 5). The induction of different LHCSC isoforms is in agreement with previous studies which suggest that LHCSC proteins protect the cell from photodamage induced by low-CO_2 and high-light conditions (10, 39). The massive induction of LHCBM9 against other LHCBM isoforms is more surprising. They share a striking number of nucleotide and polypeptide similarities, but their individual functions are not known. Confirmed by the biochemical analyses of the PSII-LHC, our data suggest that LHCBM9 becomes the dominant LHCBM isoform during Lhc remodeling under S starvation. This could be explained by the fact that LHCBM9 displays the smallest S contents (only four atoms) compared to all other LHCBM proteins that contain either six or seven S atoms each.

Remodeling of light-harvesting complexes, for example via the state transition process, is a common adaption of photosynthetic organisms in response to environmental changes (36), in particular to optimize light energy capture and distribution to PSI and PSII and dissipate excess energy to minimize photooxidative damage to the PSI reaction centers. For example, under high-light conditions, LHClI proteins can be phosphorylated, causing them to dissociate from PSII and to migrate to PSI where they are functionally integrated. By
using pulse amplitude modulation to obtain fluorescence yield measurements, Melis (28) and Wykoff et al. (47) showed that the maximal fluorescence ($F_{\text{max}}$) of S-starved cells was only 76% that of unstarved cells, indicating a larger amount of light energy absorbed by LHClI was dissipated as heat in starved cells. However, it is unlikely that LHCm9 replaces other energy absorbed by LHCII was dissipated as heat in starved cells. Moreover, LHCm9 polypeptides to increase the energy dissipation capacity, and A. R. Grossman. 2000. A genome’s-eye view of the light-harvesting poly peptides of Chlamydomonas reinhardtii. Curr. Genet. 49: 543–548.

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