Zinc deficiency impacts CO₂ assimilation and disrupts copper homeostasis in *Chlamydomonas reinhardtii*

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*Running title: Acclimation to zinc deficiency in *C. reinhardtii*

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CAPSULE
Background: Zinc is required for catalysis and protein structure.
Results: Zinc deficient Chlamydomonas lose carbonic anhydrases and cannot grow photoautotrophically in air. They also hyperaccumulate copper but are phenotypically Cu-deficient and therefore require Crr1, the nutritional Cu sensor.
Conclusion: Zn-deficiency impacts the carbon concentrating mechanism and disrupts copper homeostasis.
Significance: Cross-talk between Zn and Cu homeostasis pathways.

SUMMARY
Zinc is an essential nutrient because of its role in catalysis and in protein stabilization, but excess zinc is deleterious. We distinguished four nutritional zinc states in the alga Chlamydomonas reinhardtii: toxic, replete, deficient and limited. Growth is inhibited in zinc-limited and zinc toxic cells relative to zinc-replete cells, while zinc-deficiency is visually asymptomatic but distinguished by the accumulation of transcripts encoding ZIP family transporters. To identify targets of zinc deficiency and mechanisms of zinc acclimation, we used RNA-seq to probe zinc nutrition responsive changes in gene expression. We identified genes encoding zinc-handling components, including ZIP family transporters and candidate chaperones. Additionally, we noted an impact on two other regulatory pathways, the carbon concentrating mechanism (CCM) and the nutritional copper regulon. Targets of transcription factor Ccm1 and various CAH genes are up-regulated in zinc-deficiency, likely due to reduced carbonic anhydrase activity, validated by quantitative proteomics and immunoblot analysis of Cah1, Cah3 and Cah4. Chlamydomonas is therefore not able to grow photoautotrophically in zinc-limiting conditions, but supplementation with 1% CO2 restores growth to wild-type rates, suggesting that the inability to maintain CCM is a major consequence of zinc limitation. The Crr1 regulon responds to Cu limitation and is turned on in zinc deficiency, and Crr1 is required for growth in zinc-limiting conditions. Zinc deficient cells are functionally copper deficient, even though they hyperaccumulate copper up to 50-fold over normal levels. We suggest that zinc-deficient cells sequester Cu in a bio-unavailable form, perhaps to prevent mis-metallation of critical zinc sites.

INTRODUCTION
Zinc (Zn) is an essential nutrient required in abundance by organisms ranging from bacteria to humans. Over 300 known enzymes utilize Zn as a cofactor, and whole genome surveys estimate that 4-10% of all sequenced proteins from prokaryotes and eukaryotes contain Zn-binding domains (1). Excess Zn is believed to be toxic because it can compete for metal binding sites in other proteins and can indirectly generate damaging reactive oxygen species. Thus, intracellular Zn content must be regulated to ensure that Zn-containing proteins can function while excess Zn is avoided. The first level of control is at the step of assimilation, where intracellular Zn status controls the expression and presentation of low and high affinity transporters at the plasma membrane (2-7). Another level of control is by compartmentalization, where transporters can sequester Zn in the vacuole (in fungi), lysosome-related compartments (in C. elegans) or in zincoinsomes (in mammalian cells) in a situation of excess (8-10). Sequestered Zn can be mobilized by efflux transporters (11). The expression of each type of transporter is therefore critical for homeostasis, and there are multiple levels of control from transcription to trafficking to protein degradation (2,12).

Transporters in two families are important for Zn metabolism, the ZIP family (Zrt-, Irt-like proteins), whose members function primarily to move either Zn or iron (Fe) into the cytoplasm, and the CDF (cation diffusion facilitator) family, whose members function primarily to move Zn out of the cytoplasm ((13), reviewed in (14)). In plants, members of the CDF family are also called MTPs (metal tolerance protein) because their function in moving metals out of the cytoplasm is important for handling toxicity (15,16). The pattern of expression of individual members of these families in response to the concentration and type of metal nutrient provides a clue to their physiological functions.

Photosynthetic microorganisms rely on the carbon concentrating mechanism (CCM) to
concentrate CO\textsubscript{2} at the active site of Rubisco, the enzyme that catalyzes the first step in the CO\textsubscript{2} fixation pathway (reviewed in 17,18-20). Carbonic anhydrases are integral components in this pathway and contribute to the high productivity of some of these organisms. These enzymes generally rely on a Zn cofactor (to activate water) to catalyze the interconversion of bicarbonate and CO\textsubscript{2}. In a Zn-deficient marine environment, cobalt (Co) can replace Zn or an alternate enzyme that uses a cadmium (Cd) cofactor can substitute (21-23). The occurrence of these Zn-sparing mechanisms is indicative of the contribution of carbonic anhydrases to the cellular Zn quota, meaning the optimally desired Zn content of the cell in a Zn replete medium (24).

We have developed Chlamydomonas reinhardtii as a reference organism for understanding pathways of trace metal metabolism and homeostasis in algae and in the plant lineage, especially with respect to the impact of deficiency on bioenergetic pathways in the mitochondria and chloroplast (14,25). Chlamydomonas species have been found in various environmental niches distinguished by metal content, pH, and oxygen availability, suggesting that this genus has developed adaptive systems to deal with changing environmental conditions (26). In the laboratory, dilute Chlamydomonas cultures can reach stationary phase in 2 to 3 days in a simple salt-containing medium in which the trace elements are buffered by chelation with EDTA (27). The absence of serum or amino acid supplementation simplifies the provision of trace metal nutrients and the establishment of deficiency. We have been able to exploit this in previous work with Chlamydomonas cells experiencing Fe, copper (Cu), or manganese (Mn) deficiency, in which we noted that the assimilatory transporters are responsive to metal nutrition at the transcriptional level, including two genes encoding ZIP family members, which were named IRT\textsubscript{1} and IRT\textsubscript{2} because they responded to Fe-deficiency (28-31). Nevertheless, there are as many as a dozen members of the ZIP family in Chlamydomonas, and some of them are likely to be involved in Zn assimilation.

The interaction of Zn and Cu homeostasis pathways is also likely in Chlamydomonas. Cu(I) is taken up by the CTR family of transporters whose members are components of the nutritional Cu regulon in Chlamydomonas. A Zn-containing SBP-domain transcription factor named Crr1 (copper response regulator) controls the expression of the CTR genes (28). Its functional homolog in Arabidopsis is SPL7 (32-34). Besides the CTR genes encoding the plasma-membrane localized assimilatory transporters, Crr1 regulates over 60 genes involved in acclimation to Cu-deficiency through associated Cu-response elements (CuREs), which are the target sites for the SBP domain (28,35-37). The best characterized of these target genes is CYC6 encoding cytochrome (Cyt) c\textsubscript{6}, which is a heme-containing replacement of the usual Cu-containing protein, plastocyanin, in the photosynthetic electron transfer chain. Therefore, although Cu-deficient Chlamydomonas cells do not accumulate plastocyanin, they remain photosynthetically competent (38). Decreased plastocyanin abundance and transcriptional activation of CYC6 are classic markers for the Cu-deficiency state.

In this work, we use growth and the expression of a subset of genes for ZIP family transporters as sentinels of Zn status to establish Zn-deficiency and Zn-limitation in Chlamydomonas, which are achieved by serial transfer of replete cells to medium lacking supplemental Zn. Transcriptome and proteome surveys identify the carbonic anhydrases and hence the carbon concentrating mechanism as a pathway impacted by poor Zn nutrition. We note also an impact on Cu homeostasis, and we suggest the existence of mechanisms that control the ratio of intracellular metal ions.

**EXPERIMENTAL PROCEDURES**

**Culturing and strains** – Chlamydomonas reinhardtii strains CC-4532 (wild-type, 2137), crr1-2 (referred to subsequently as crr1), and a rescued strain crr1-2::CRR1 (referred to subsequently as CRR1) was cultured under 50 – 100 µmol m\textsuperscript{-2} s\textsuperscript{-1} continuous illumination (2:1, cool white: warm white light) in Tris-Acetate-Phosphate (TAP) or Tris-Phosphate (TP) media with trace element supplements described in Kropat et al. (27). Briefly, stock solutions of 25 mM EDTA-Na\textsubscript{2}, 28.5 µM (NH\textsubscript{4})\textsubscript{6}Mo\textsubscript{7}O\textsubscript{24}, 0.1 mM Na\textsubscript{2}SeO\textsubscript{3}, 2.5 mM ZnSO\textsubscript{4} in 2.75 mM EDTA, 6 mM MnCl\textsubscript{2} in 6 mM EDTA, 20 mM FeCl\textsubscript{3} in 22 mM EDTA, and 2 mM CuCl\textsubscript{2} in 2 mM EDTA were made individually in Milli-Q-purified water...
and diluted 1 to 1000 in the final growth medium. For metal-free studies, all glassware was triple washed in 6N hydrochloric acid followed by at least six rinses in Milli-Q purified (Millipore) water. All media were made using Milli-Q water (39). For experiments other than the proteomic studies, cells were grown in nutrient replete medium, followed by one transfer into Zn-medium with no supplemental Zn, before inoculation into the experimental conditions. For the proteomic studies, cells were grown in replete medium and directly inoculated into the experimental conditions. For experiments involving CO₂ supplementation, cultures were bubbled with filtered air (control) or a mixture of 1% CO₂ with air. Cell density was measured by counting with a haemocytometer.

Fluorescence rise and decay kinetics – For CC-4532, room temperature fluorescence rise and decay kinetics were analyzed using a FluorCam 700MF (Photon Systems Instruments). Approximately 50 µL of concentrated mid-log phase liquid culture was spotted onto the lid of a plastic petri dish and dark adapted for 10 min. prior to a flash of saturating light and measurement of the Kautsky effect in continuous red light at 150 µmol m⁻² s⁻¹ PFD with 100% actinic light and 60% activity. For measurements of crr1 and CRR1 cells in Zn-replete and Zn-limited conditions, room temperature kinetics were measured using a laboratory-built instrument as described in Joliot and Joliot (40).

Cell size determination – Cell size was determined with a Beckman Coulter Laser Diffraction Particle Size Analyzer LS13 320. Approximately 100 mL of cultures in exponential phase were poured into the micro liquid module with a magnetic stir bar included to keep the cells in suspension. If necessary cultures were concentrated or diluted until the sample obscuration was between 8 and 12%.

Nucleic acid analysis – Total Chlamydomonas RNA was prepared as described by Quinn and Merchant (39). RNA quality was assessed on an Agilent 2100 Bioanalyzer and by hybridization to CBLP (also called RACK1) as described previously (39). A 915-bp EcoRI fragment from the cDNA cloned in pcf8-13 was used as the probe (41).

Quantitative reverse-transcriptase PCR (RT-PCR) – Genomic DNA was removed from the total RNA preparation by treatment with Turbo DNase (Ambion) according to manufacturer’s instructions with the following modifications: 3 U of enzyme was used per 10 µg nucleic acid, and incubation at 37 °C was increased to 90 min. Complementary DNA, primed with oligo(dT), was generated with reverse transcriptase (Invitrogen) according to manufacturer’s instructions. Amplification was carried out with reagents from the iQ SYBR Green Supermix qPCR kit (Bio-Rad Laboratories). Each reaction contained the vendor’s master mix, 0.3 µM of each primer, and cDNA corresponding to 20 ng input RNA in the reverse transcriptase reaction. The reaction conditions for the Opticon 2 from MJ Research were: 95 °C for 5 min, followed by cycles of 95 °C for 10 s, 65 °C for 30 s, and 72 °C for 30 s, up to 40 cycles. The fluorescence was measured at each cycle at 72 °C and 83 °C. The 2⁻∆∆CT method was used to analyze the database on the fluorescence at 83 °C (42). Melting curves were performed after the PCR reaction to assess the presence of a unique final product.

RNA-seq – RNAs were sequenced by Illumina on a GAIIx system for estimating transcript abundance. The reads were aligned using Bowtie (43) in single-end mode and with a maximum tolerance of 3 mismatches to the Au10.2 transcripts sequences (http://www.phytozome.net/chlamy), corresponding to the version 4.0 assembly of the Chlamydomonas genome. Expression estimates were obtained for each individual run in units of RPKMs (reads per kilobase of mappable transcript length per million mapped reads, see (44)) after normalization by the number of aligned reads and transcript mappable length. The transcript coverage across the genome was visualized on a local installation of the UCSC browser (http://genomes.ucsc.edu/cgi-bin/hgGateway). Differential expression analysis was performed in R with the DESeq package (45) and p-values were adjusted to control for false discovery rate (FDR) with the Benjamini-Hochberg method (46). Sequence files are publicly available at NCBI’s Gene Expression Omnibus, accession numbers GSE25622 and GSE41096.

Protein isolation for immunoblot analysis – Chlamydomonas cultures were collected by centrifugation (1000 ×g, 5 min.) and washed twice with 10 mM sodium phosphate, pH 7.0. The total
protein fraction was further subfractionated into soluble and membrane components as described in Howe and Merchant (47). Protein concentration of soluble fractions was determined using the Pierce BCA Protein Assay Kit following manufacturer’s instructions. Samples were diluted to 4 µg per µL. Membrane fractions were normalized by resuspending them to a volume that was equivalent to the final soluble fraction volume.

**Immunoblot analysis** – Protein samples were denatured by addition of 5% β-mercaptoethanol and boiling for 10 min before separation on denaturing polyacrylamide gels and transferring in a semi-dry blotter onto nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 0.0004% SDS (w/v), and 20% (v/v) methanol). The membrane was blocked overnight with 1% dried milk in Tris-buffered saline (10 mM Tris-Cl, 150 mM NaCl, pH 7.5) + Tween 20 (0.05% [w/v]) before incubation in primary antiserum for 4-12 h. Gel concentrations (acylamide monomer) and dilutions for each primary antibody were: plastocyanin, 16%, 1:1000 dilution; Cyt c₆, 16%, 1:1000 dilution; Cah1, 12%, 1:2,000 dilution; Cah3, 12%, 1:2000; Cah4, 12%, 1:15000 dilution; ferredoxin 15%, 1:10000 dilution; and CF₁, 10%, 1:20000 dilution. Secondary goat anti-rabbit horseradish peroxidase (Pierce Biotechnology) was used at a 1:5000 dilution.

**Sample preparation and analysis for quantitative proteomics** – Soluble protein was extracted from CC-4532 cells growing in 2.5 µM Zn and the first round of 0 supplemental Zn. For each condition, label-free, data independent quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS or “MS²”) was performed as previously described with slight modifications (35,48). In brief, cells were collected at late exponential phase from replete Zn conditions or from the first transfer to Zn-deplete medium by centrifugation. Cells were broken by slow freezing and thawing at −80 °C and 24 °C, respectively. Insoluble material was removed by centrifugation at 16,000 × g for 10 minutes at 4 °C followed by centrifugation at 253,000 × g for 20 minutes at 4 °C. Approximately 30 µg of protein (per lane) was separated by gel electrophoresis on 4-12% NuPage Bis-Tris gels (Invitrogen) and visualized by staining with Coomassie blue (Bio-Rad). Gel lanes were divided into ~3 mm bands and individual bands were subjected to in-gel trypsin digestion (sequencing grade modified trypsin; Promega). Digested peptides were extracted into 50/50 water/acetonitrile solution containing 2.5% formic acid and lyophilized. Peptides were then resuspended into a 25 fmol/µL of bovine serum albumin (BSA) digest and quantification standard (Waters Corporation). LC-MS² was performed using a nanoAcquity UPLC (Waters) system coupled to a quadrupole time-of-flight mass spectrometer (Waters Xevo QTOF). Protein Lynx Global Server (PLGS version 2.4; Waters) was used to process the LC-MS raw data and determine protein identification and quantification. The quantification of protein levels was achieved via the addition of an internal protein standard (BSA trypsin digest standard) to which the data set was normalized. Our criteria were that the difference in protein abundance between growth conditions must be statistically significant (p < 0.05 by Student’s t test) and at least 2-fold or greater in magnitude in order to define a change in protein abundance (48).

**Stoichiometric measurements of cytochrome:P₇₀₀ and PSI:PSII** – Spectroscopic measurements were performed using a JTS-10 spectrophotometer (Biologic, France). Light-induced absorption changes were measured as absorption of flashed light at discrete times. Changes in the amount of functional photosynthetic complexes were evaluated measuring the electrochromic shift (ECS) spectral change, a shift in the pigment absorption bands that is linearly correlated to the number of light-induced charge separations within the reaction centers. Functional PSI and PSII content was estimated from changes in the amplitude of the fast phase of the ECS signal (at 520 nm - 546 nm) upon excitation with a saturating laser flash (520 nm, 5 ns duration). PSII contribution was calculated from the decrease in the signal amplitude upon addition of DCMU (20 µM) and hydroxylamine (2 mM) to irreversibly block PSII charge separation. Conversely, PSI was estimated as the fraction of the signal that was insensitive to these inhibitors (49). Cytochrome redox changes were calculated as the difference between the absorption at 554 nm and a baseline drawn between 545 and 573 nm and corrected for the contribution of the electrochromic signal (50). P₇₀₀ (the primary electron donor to PSI) was measured
at 705 nm. To evaluate the relative cytochrome:P700 stoichiometry, measurements were performed in continuous saturating light (1100 µmol photons m⁻² s⁻¹) and in the presence of saturating concentrations of DBMIB (10 µM). This is required to ensure full inhibition of cytochrome b₆f reduction by light generated plastoquinol, and therefore a maximum oxidation of P700 and of the cytochrome.

Measurement of intracellular metal content – Cells were collected by centrifugation at 1700 × g for 5 min. Pellets were washed once in 1 mM EDTA to remove cell surface-associated metals and once in Milli-Q water. The washed cell paste was overlaid with nitric acid corresponding to a final concentration of 24% in 1 mL and digested at 65 °C. To obtain a corresponding blank, the volume of the cell paste was replaced by deionized water and treated as described above. Total metal and phosphorous content was measured by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7500).

**RESULTS**

Identification of four distinct Zn nutrition states. Chlamydomonas grows in media containing a wide range of supplemental Zn concentrations (51). Elemental analysis of wild-type Chlamydomonas cells grown in standard Tris-acetate-phosphate (TAP) medium supplemented with Hutner’s trace elements, which contains about 80 µM of EDTA-chelated Zn, indicated a Zn quota of approximately 2-3 × 10⁷ atoms per cell. We calculated that a minimum concentration of 0.85 µM Zn ions would be necessary to support Zn-replete growth to stationary phase (~ 2 × 10⁷ cells/ml). After allowing for variation in Zn quota in situations of altered physiology (e.g. low pCO₂ when carbonic anhydrases would be induced), we chose 2.5 µM Zn ions in a revised micronutrient solution (27,51,52). Indeed, this concentration of Zn is more than sufficient to support normal growth and a typical intracellular trace metal (Cu, Fe, Mn) quota; when the Zn content of the medium is reduced to 0.25 µM, there is no impact on growth (Figure 1A). Therefore, 2.5 µM Zn ions is considered metal replete for laboratory growth of Chlamydomonas.

To generate Zn limitation, we inoculated cells (to a density of 10⁵ cells per ml) from a replete culture into growth medium without any Zn supplementation (labeled 0 supplemental Zn in the figures). Although all media constituents were prepared using high purity salts and all glassware was freshly acid washed (39), there remained residual Zn in the medium, which we estimated at approximately 10 nM based on ICP-MS analysis. When this culture (referred to as the 1st round in Zn-depleted medium) reached early stationary phase (~ 1 x 10⁷ per ml), the cells were used to re-inoculate fresh Zn-“free” medium or medium with various amounts of supplemented Zn (Figure 1A). A growth phenotype was identified in medium supplemented with no (0) or very low (10 to 25 nM, data not shown) Zn, and this correlated with a reduced intracellular Zn content of these cultures (varying from 25% to 50% of the Zn in the replete situation in individual experiments) (Supplemental Figure S1). The Zn content of cells transferred twice to the 0 Zn medium was only slightly less than or nearly the same as that of cultures transferred once to 0 Zn medium. Further subculturing was therefore not necessary, and we routinely used two transfers to 0 Zn medium as the baseline for poor Zn nutrition. We also tried to generate Zn-deficiency by reducing Zn bioavailability with introduction of Zn(II)-specific chelators, like N, N, N', N'-tetrakis (2 pyridylmethyl) ethylenediamine (TPEN) into the medium, but this did not enhance the phenotype of cells in the second round of growth in 0 Zn medium. TPEN was also not effective in generating a Zn-deficient (see below) or -limitation condition when it was added at higher concentrations to Zn-replete medium. Therefore, we concluded that, in the absence of a transporter-defective mutant, sequential transfer into defined medium with 0 supplemental Zn(II) is the only effective way to generate Zn-limitation in Chlamydomonas. Analysis of the fluorescence induction curves shows that PSII activity is retained by cells growing in all concentrations of supplemental Zn, including those from cultures with no supplemental Zn, and that electron transfer between the two photosystems still operates under all of these conditions (Figure 1B). Light microscopy indicated that Zn-limited cells are larger than the replete ones, and this is confirmed by measuring the size distribution of cells in batch culture. The average diameter of Zn-limited cells is 11 µm compared to the 9 µm size of Zn-replete
cells. In addition, we noted the occurrence of smaller bodies of size ~2.5 µm in the Zn-limited culture. Their identity is not known, but they may represent stress-induced cell fragmentation (Figure 1C).

At the other extreme, Zn-excess is defined as high Zn ion concentrations at which Zn starts to become toxic and the growth rate is inhibited relative to the maximum observed growth rates. In TAP medium, growth is inhibited when the medium contains more than 125 to 250 µM chelated (EDTA) Zn ions (Figure 1A, red curve), and this defines the Zn excess situation.

In previous work on Fe nutrition, we distinguished the deficiency state from the limitation state. The Fe deficiency state is characterized by the absence of a growth phenotype or other visual symptoms, but the presence of a molecular signature, which in the case of Fe nutrition is the expression of the Fe assimilation pathway (31). Therefore, we sought to identify biomarkers for Zn-deficiency. As putative Zn transporters, members of the ZIP family are excellent candidates for Zn assimilation proteins. Previously, 14 members of this family were identified in Chlamydomonas based on homology to Arabidopsis, yeast, and human sequences (14,53). We assessed the patterns of mRNA accumulation for each of these as a function of Zn nutrition status to distinguish a) which of these might respond to Zn nutrition and b) whether their expression might be an early gauge of the Zn status. RNAs were isolated from cells transferred to medium containing the indicated amounts of Zn (after 1 round in 0 supplemental Zn) and analyzed by real-time RT-PCR for the expression of each of the ZIP genes. Among these, 5 Zn-nutrition responsive genes were identified and named ZRT1, ZRT2, ZRT3, ZRT4, and ZRT5 for zinc-responsive transporter (Figures 2A and 2B). Based on the magnitude of the change, we chose ZRT1 and ZRT3 as sentinel genes and defined 25 nM as the boundary between Zn-deficiency and Zn-limiting laboratory conditions (Figure 2C).

Thus, we established growth conditions to generate each of 4 states of Zn nutrition: 0-10 nM Zn (no added Zn) for Zn-limiting conditions, 25 nM Zn for deficient conditions, 2.5 µM Zn for replete conditions, and 250 µM for Zn-toxic conditions.

Transcriptome analysis of cells growing in limited vs. replete Zn conditions. To understand the basis for growth inhibition and to discover new Zn homeostasis factors, we used RNA-Seq for an exploratory transcriptome of Zn-limited CC-4532 (wild-type) cells. The cells were generated by transfer from the first round of growth in medium with no supplemental Zn into TAP medium supplemented or not with 2.5 µM Zn-EDTA. Cells were collected from all cultures at mid-exponential phase. RNA was prepared and validated for physiology by RT-PCR for sentinel gene expression and analyzed by sequencing of cDNA libraries on the Illumina platform. The reads were aligned to the Augustus 10.2 gene models on the version 4 assembly of the Chlamydomonas genome and were analyzed to quantify abundance of transcripts as described previously. Using a cutoff of 4-fold change and a p-value of < 0.05, we identified 533 genes that showed increased transcript abundance and 119 genes that showed decreased transcript abundance in Zn-limiting conditions relative to Zn-replete conditions (Supplemental Dataset 1).

Total transcript abundance for the ZRT genes dramatically increased in Zn-limited conditions relative to Zn-replete conditions (Figure 3). However the proportion of each individual ZRT transcript in the total pool is different in the Zn-limited vs. replete state. For instance, ZRT1 and ZRT3 are not prominent in replete cells but become more prominent in Zn-limited conditions, suggesting that they may encode the primary high affinity assimilative Zn transporters in a situation of deficiency. ZRT5 may encode a lower affinity form that can function with higher extracellular Zn availability.

Evident in the dataset are genes belonging to 3 physiological categories: transcripts that were shown previously to increase in CO2-limited cells (54), transcripts that increase in Cu-deficient cells (35) and transcripts encoding candidate Zn transporters and Zn homeostasis factors. The CO2-responsive genes HAP3 (Cre03.g177250.t1.1) and a gene encoding an unknown hypothetical protein (Cre10.g436450.t1.1) are among those whose transcripts increase most dramatically (approximately 103-fold), and the same is true for genes in the Cu regulon, like CYC6 (Cre16.g651050.t1.1) encoding Cyt c6 and a gene encoding a hypothetical protein.
The degree of regulation is as dramatic as that for the putative Zn transporters ZRT1 (Cre07.g351950.t1.2) and ZRT2 (Cre13.g573950.t1.2) and two genes encoding COG0523 domain-containing proteins that are implicated in Zn homeostasis (55). We named the two COG0523-domain proteins Zcp1 and Zcp2 (for Zinc-responsive COG0523 domain-containing protein). Zcp2 corresponds to Cre02.g118400.t1.2, but Zcp1 is missing in the version 4 assembly of the Chlamydomonas genome. Nevertheless, when the reads are aligned to the version 3 assembly, the increase in transcript abundance for Zcp1 (Protein ID 117458) is dramatically evident.

The Zn-responsiveness of two previously identified limiting CO2-inducible genes prompted us to compare the genes regulated by Zn starvation to those regulated by low CO2, including targets of the transcription factor Ccm1 (54) (Supplemental Dataset 2). We found that 82 genes were similarly Zn-responsive and CO2-responsive, with 54 of them being predicted Ccm1 targets. Among extensively-studied low CO2-induced genes that are also Zn-responsive are CAH4, CAH5, CCP1, HLA3 and LCIA. When we compared the genes regulated by Zn starvation to those regulated by Cu starvation, we found a similar situation. There are 23 instances of genes responding similarly to deprivation of either micronutrient (Supplemental Dataset 3), with 16 increasing in transcript abundance and 7 decreasing, and of the 23, 11 (or about half) are Crr1 targets, which is the same fraction as that of Crr1 targets among genes that respond to Cu nutrition (35). The reason that we pick up only a fraction of the Cu regulon and Crr1 targets is because the Cu regulon is not as highly activated in Zn-deficiency as it is in Cu-deficiency and some of the transcript changes did not meet our criteria for inclusion. A few (8) genes respond in an opposite fashion to Zn vs. Cu nutrition and two of these are Crr1 targets, pointing to additional Crr1-independent controls at the transcriptional level for these two genes. When we checked the reproducibility of a subset of the changes in RNA abundance by real time RT-PCR on RNAs isolated from triplicate cultures, we confirmed the findings (data not shown).

Therefore, we conclude that a) there is a Zn nutrition-sensing regulatory pathway in Chlamydomonas that operates in part to control the abundance of transcripts for Zn transporters of the ZIP family and Zn homeostasis factors, and b) there may be an impact of Zn starvation on Cu homeostasis and CO2 assimilation.

When we used the Algal Functional Annotation Tool (10), with selected gene ontology terms from Arabidopsis thaliana to overcome the limitation of available annotations for Chlamydomonas, to identify metabolic functions or pathways that might be associated with the Zn-responsive genes identified from transcriptome profiling, we noted three meaningful (p-value<0.01) biological processes for transcripts whose abundance increases: Zn and other ion transport (17 hits, scores range from 2 × 10^{-3} to 5 × 10^{-4}), Zn and other ion homeostasis (4 hits, scores range from 8 × 10^{-3} to 2 × 10^{-4}), and the response to arsenic (2 hits, score = 1 × 10^{-3}). For transcripts that decrease, we found genes related to organelle and peroxisome organization, organic acid metabolism, fatty acid metabolism, and regulation of cell division and DNA replication, probably a consequence of the role of Zn ions as a structural cofactor in proteins involved in nucleic acid transactions.

Proteomic analysis. To determine if changes in the transcript abundance of some genes were recapitulated at the level of the soluble proteome, we analyzed soluble protein samples by quantitative LC-MS/MS from cells growing in medium supplemented with 2.5 µM Zn or in an initial round of medium with no supplemental Zn (see also (48)). We chose to use only one round of growth in medium lacking Zn in an attempt to distinguish between the primary effects of Zn deficiency vs. more general stress markers, which are likely to be more prevalent after sustained growth (i.e. two transfers) in medium with 0 supplemental Zn. Among the proteins identified as the most abundant and differentially regulated in Zn-limiting conditions are the hypothetical protein Cre07.g352000.t1.1 and both Zcp1 and Zcp2. In addition, we identified and showed an increase in the abundance of Cgl78/Ycf54 and Fea1, whose transcripts increase in both Zn-limitation and Cu-deficiency (Supplemental Dataset 3).

Carbonic anhydrases and CO2 requirements during photoautotrophic growth. The carbon concentrating mechanism allows algae and cyanobacteria to grow photoautotrophically at air levels of CO2, and carbonic anhydrases (CAs) are key enzymes in this mechanism (56-58).
Chlamydomonas there are 12 genes predicted to encode Zn-containing carbonic anhydrases (59). The up-regulation of the CCM genes suggested to us that the activities of one or more of these enzymes might be compromised in Zn-limitation leading to a functional low CO₂ phenotype despite the presence of acetate, which normally suppresses the low CO₂-activated genes. Therefore, we surveyed the expression of the CAH genes that contribute to the bulk of the CA activity or that are known to be required for operation of the CCM. Cah1 is the major contributor to carbonic anhydrase activity in whole cell extracts in the presence of light and low (i.e. atmospheric levels) CO₂, although no phenotype has been associated with its absence. Differences in transcript accumulation for CAH1 in response to Zn ion concentration were small but suggested a slight decrease in expression in response to decreasing Zn (Supplemental Dataset 1). Immunoblot analysis revealed a more dramatic decrease in Cah1 accumulation in Zn-limiting medium (Figure 4), and this result was recapitulated in proteomic studies (48). Cah3 is localized to the chloroplast and is believed to be the primary carbonic anhydrase required for maintenance of the CCM. Transcripts for CAH3 increase in Zn limitation but again immunoblot analyses showed that Cah3 abundance is decreased in Zn deficiency. Two other carbonic anhydrases, Cah4 and Cah5 (nearly identical in sequence), are located in the mitochondria. The transcripts encoding these enzymes increase under Zn limitation, but immunoblot analysis revealed the decreasing abundance of Cah4 in Zn-limitation as well. We conclude that Zn limitation has a major impact on carbonic anhydrase activity, as documented already for diatoms, which led to the hypothesis that the decreased growth rate under phototrophic conditions might result from impaired CCM.

To test this hypothesis, we grew Zn-limited and Zn-replete cultures in TP medium, which lacks acetate as a carbon source, and bubbled the flasks with either air, representing limiting CO₂ conditions, or air with 1% CO₂, representing high CO₂ conditions, where CA activity is not required. In air-bubbled flasks, Zn-limited cultures displayed limited growth relative to Zn-replete cultures (Figure 5). When flasks were bubbled with 1% CO₂, growth in Zn-limited cultures was comparable to growth in Zn-replete cultures although overall growth in both cultures decreased slightly. We attribute the slight change in both cultures to a change in the pH of the medium caused by the introduction of 1% CO₂. The Zn-limitation phenotype in phototrophic medium is therefore rescued by high CO₂, establishing a causal connection between CCM and the Zn-limitation phenotype resulting from impaired CA activity. The importance of back-up CAs in diatoms for productivity in low Zn environments is evident.

Cu deficiency and Crr1 function in Zn-limited cells. The up-regulation of the Crr1 regulon and Cu-deficiency responses suggests that the Zn-deficient cells may be functionally Cu-deficient, reminiscent of the connection between Fe and Cu, where Cu-deficient cells are secondarily Fe deficient (60). To test this idea, we monitored the abundance of plastocyanin and Cyt c₆ as a function of Zn concentration by immunoblotting (Figure 4). Indeed, plastocyanin is decreased in Zn-deficient cells, and a concomitant increase in cytochrome c₆ is evident. If excess Cu is provided, we can restore plastocyanin content in proportion to the added Cu, but much greater amounts of Cu are required to maintain plastocyanin in Zn-limited cells compared to Zn-replete ones (Figure 6). This supports the idea that Zn-limited cells are functionally Cu-deficient. Therefore, we tested whether Crr1 might be required for Chlamydomonas to acclimate to Zn limitation. In Zn-limiting and toxic conditions, growth of crr1 mutant cultures was lower than growth of the complemented strain, CRR1 (Figure 7A). Growth of the mutant was also slightly decreased in replete conditions, consistent with the role of Crr1 in all states of Cu nutrition.

Measurement of fluorescence rise and decay kinetics in crr1 vs. CRR1 cells revealed that the mutant maintains a high steady state level of fluorescence emission in Zn-limitation (Figure 7B). This is likely due to the inability of the mutant to transfer electrons downstream of Photosystem II (PSII) to Photosystem I (PSI) in the photosynthetic electron transport chain, most probably as a result of the inability to upregulate Cyt c₆. To support this, we estimated the stoichiometry of Cyt c₆ to P₇₀₀ (PSI) in Zn-limited crr1 and CRR1 cultures by quantifying the ratio of redox active Cyt:P₇₀₀ using the electrochromic shift assay (Figure 7C). For the complemented...
strain, Cyt:P700 ratios increased from 0.15 in Zn replete conditions to 0.25 in Zn-limitation, whereas the mutant showed a slight decrease in the Cyt:P700 ratio from 0.15 in Zn replete conditions to ~0.05 in Zn-limitation (Figure 7D). PSI:PSII ratios were approximately 1:1 for both strains in both Zn-replete and Zn-limited conditions (Figure 7D). These results support the idea that the increase in Cyt c is necessary to maintain photosynthesis in Zn-limited Chlamydomonas cells, even when Cu is available in concentrations that should be sufficient to support plastocyanin maintenance.

Trace metal quota. To validate the impact of Zn nutrition on Cu content, we measured the metal content of Chlamydomonas cells over a range of supplemental Zn concentrations (Figure 8). In the range of concentrations from 25 nM to 250 µM, the Zn quota is maintained at approximately 3 × 10^7 atoms/cell with some variation that tracked changing external Zn concentrations. When Zn in the medium was less than 25 nM, the quota decreased to ~1 × 10^7 atoms/cell, consistent with symptoms of Zn deficiency and limitation. In contrast, the abundance of Fe and Cu ions (and possibly also Mn ions) increased with decreasing Zn ion supplementation. This might be attributed to up-regulation of ZIP transporters and the relatively high concentrations of these divalent cations in the medium. In fact, we note that sentinel genes for Fe assimilation are down-regulated in Zn-deficiency, presumably because of higher intracellular Fe content. Unexpectedly, given the molecular phenotype of the Cu regulon (see above), the Cu quota was increased most dramatically. Its intracellular abundance increased by over an order of magnitude from ~1-2 × 10^7 atoms/cell in Zn-replete conditions to as much as 4 × 10^8 atoms/cell in Zn-limiting conditions. This contrasts strikingly with the tight regulation of Cu content observed in previous work (28) and presents a conundrum with respect to the molecular phenotype. We conclude that normal Cu homeostasis requires Zn.

DISCUSSION

Though studies investigating acclimation strategies to Zn deficiency have been performed in yeast and other organisms (61), the impact of Zn deficiency on photosynthetic organisms is under-investigated. Here, we have defined and characterized distinct stages of Zn nutrition in Chlamydomonas because of an excellent infrastructure for understanding trace element homeostasis developed through our prior studies (25). We have distinguished a stage where growth is inhibited and we refer to that as Zn limitation vs. a stage where the Zn-sensing signal transduction pathway is operational to turn on assimilation genes but growth is not affected, and we refer to that as deficiency. As is the case for other organisms, excess Zn results in toxicity, which is also distinguished by growth inhibition (Figure 1).

In Saccharomyces cerevisiae and Arabidopsis, a primary strategy for the regulation of Zn homeostasis involves the Zn-responsive expression of low and high affinity Zn transporters (62,63). Therefore, these seemed like excellent candidates for targets of nutritional Zn signaling as well. A genome survey of ZIP and CDF family transporters (14) led to the identification of 5 putative Chlamydomonas Zn transporters whose transcripts accumulate in Zn-limiting conditions (Figure 2). The regulation of these genes was used to identify Zn concentrations that induce a change in the abundance of the corresponding RNAs, and this corresponds to concentrations at which the Zn content is less than approximately 2 × 10^7 atoms/cell, which defines the typical Zn quota for Chlamydomonas. Very low Zn content in the medium (Zn-limitation) results in physiological stress, as suggested by the change in cell size (Figure 1) as well as by the accumulation of Nile Red-staining bodies under Zn-limiting conditions (27).

Based on fluorescence measurements of photosynthetic ability, the impact of Zn limitation did not affect the photosynthetic apparatus per se (Figure 1B). However, the known role of Zn in DNA-binding proteins and carbonic anhydrases (CAs) participating in the carbon concentrating mechanism (CCM) suggested that photosynthetic growth might be affected indirectly as a result of Zn deficiency. RNA-seq technology has been employed and rigorously evaluated in Chlamydomonas, showing this technology to be as quantitative as real-time PCR to evaluate changes in transcript abundance in response to external conditions (35). Therefore, we used it as an exploratory tool to discover pathways that might be impacted by poor Zn nutrition. We identified...
nearly 700 genes whose expression was significantly changed in Zn limited vs. replete cells (Supplemental Dataset 1).

**Photoautotrophic growth at air levels of CO₂ and carbonic anhydrases:** The finding that genes described/discovered in previous studies as being targets of the CCM pathway or Crr1 pointed to inorganic carbon assimilation and Cu homeostasis, both of which affect photosynthetic performance, as being affected by poor Zn nutrition. In both cases, we substantiated this by rescuing the Zn starvation phenotype by provision of high CO₂ or excess Cu (Figures 5 and 6, respectively). Consistent with an impact on the CCM pathway, we found that photoautotrophic growth of Zn-limited cells was inhibited relative to Zn-replete cells at air levels of CO₂ (low CO₂ conditions) but provision of 1% CO₂ increased photoautotrophic growth rates in Zn-limited cells. The **LHCSR1**, **LHCSR3.1**, and **LHCSR3.2** genes encoding a sub-type of light harvesting chlorophyll binding protein are regulated by CO₂ as well, but they are inversely regulated in response to Zn limitation relative to CO₂ limitation (64). This suggests that these genes are under the control of multiple regulatory factors because they respond differently to low CO₂ than they do to other nutrient deprivation regimes, including nitrogen and sulfur starvation (65).

The most obvious targets of poor Zn nutrition are the carbonic anhydrases, and we characterized their expression profiles relative to Zn nutrition. Transcripts for some carbonic anhydrases are increased in Zn-limitation, however immunoblots for Cah1, Cah3, and Cah4/5 revealed that these proteins decreased in Zn-limiting conditions. We could not detect Cah2 because its abundance is quite low (5%) relative to that of Cah1, and indeed it is not detected in cells grown in uninduced conditions (i.e. high CO₂) (59). The discrepancy between transcript and protein accumulation may suggest post-transcriptional regulation of these genes or a compensatory upregulation of the genes in response to decreased activity. It is possible that the proteins are indeed synthesized, but they might be degraded in the absence of the essential Zn cofactor or they might be actively degraded as part of a Zn sparing program (24,48). Carbonic anhydrases are important for photosynthesis under low CO₂ conditions based on the effect of enzyme inhibitors (acetazolamide and DBS) and based on the phenotype of a mutant lacking luminal carbonic anhydrase Cah3 (66), and the expression of several **CAH** genes is increased in cells grown at air levels of CO₂ as part of the CCM (59). The conditional CO₂-repressed phenotype of Zn-deficient Chlamydomonas cells establishes the carbonic anhydrases as key targets of Zn deficiency and therefore, in an environmental context, emphasizes the importance of the ability of diatoms to substitute Co or Cd in the active site of CAs (22,67) to maintain photosynthetic growth in face of Zn deficiency.

**Influence of Zn on Cu nutrition:** The similar regulation of 23 Cu-responsive genes, including 11 Crr1 targets, suggested a connection between Cu and Zn metabolism. The fraction of Crr1 targets (50%) is about the same as the fraction of the responding genes in Cu-deficient cells (35). Since most of these are genes that are highly responsive and highly expressed, we concluded that Zn-limited cells are signaling a slightly Cu-deficient status. Nevertheless, when we measured intracellular metal content, we noted the opposite – that is, several fold higher Cu content in Zn-deficient cells (Figure 8). We conclude therefore that cell-associated Cu is somehow not accessible to the Cu sensor. Further, the Cu also seems to be inaccessible to plastocyanin. The biosynthetic pathway for plastocyanin is normal because when excess Cu is added to Zn-deficient cells, this Cu can be used for holoplastocyanin synthesis and the expression of **CYC6** is decreased. Therefore, cell-associated Cu is generally not accessible for cuproprotein biosynthesis. The biosynthesis of Cyt c₆ would, in this case, become essential, which may explain the non-photosynthetic phenotype of Zn-deficient **crr1** cells (Figure 7).

The advantage, if any, of Cu-sequestration is not known. The effects of Zn nutrition on organisms is known to be complicated by the fact that interactions exist between Zn and Cu homeostasis pathways. *In vivo*, *Drosophila* metallothionein (MT) genes are induced by both Cu and Zn, suggesting that MTs bind both metals (68). Among humans, serum Cu:Zn ratios were significantly higher in patients with malignant lung tumors than in healthy patients and may serve as a diagnostic test in lung cancer patients (69). The Cu:Zn ratio is also significantly increased in
disabled elderly patients relative to healthy elderly people and was associated with levels of systemic oxidative stress (70). In the case of patients with Wilson Disease, Zn supplementation has been used to counteract Cu hyperaccumulation. Mechanistic explanations for this effect are not readily available.

Crr1 and Ccm1 are both Zn-dependent transcription factors (37,71). The maintenance of transcription factors Crr1 and Ccm1 in Zn limitation indicates that Zn remains available for their function even though carbonic anhydrase function is compromised, indicative of hierarchical rules governing the allocation of Zn ions. This mechanism has already been documented previously for Cu, Mn and Fe in Chlamydomonas (24,72). It is tempting to speculate that the COG0523 domain proteins, Zep1 and Zep2, may play a role in metal allocation and sparing. The corresponding genes are as highly up-regulated (~10^3-fold and ~7x10^3-fold, respectively) and as highly expressed (~6x10^2 RPKM) as are some of the genes encoding ZRT proteins (e.g. ZRT1 and ZRT3). These are excellent candidates for discovery of the mechanism of regulation or the Zn sensor in Chlamydomonas. There are also a number of proteins of unknown function that are also highly regulated by Zn-deficiency, indicative of room for new discovery in Zn homeostasis.
REFERENCES


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Footnotes
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1To whom correspondence may be addressed: 607 Charles E. Young Drive East, Los Angeles, CA 90095-1569. Fax: 310-206-1035; E-mail: merchant@chem.ucla.edu
2 The elemental abbreviations will be used throughout the article without indication of speciation, ionic or oxidation state, unless relevant.
FIGURE LEGENDS

FIGURE 1. Phenotypes of Zn-deficient Chlamydomonas. Strain CC-4532 was grown in TAP medium with different amounts of supplemental Zn (in µM): 0 (black), 0.25 (blue), 2.5 (green), 80 (purple), and 250 (red). (A) Growth measured by counting cells, (B) Kautsky fluorescence rise and decay kinetics, (C) diameters of cells growing in medium containing 2.5 µM Zn (left) and 0 supplemental Zn (right). Growth curves represent the averages of biological triplicates from three separate inocula (s.d. not shown for clarity). For fluorescence measurements and cell size determination, representative data from experimental triplicates are shown.

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FIGURE 3. Abundance of Chlamydomonas ZRT transcripts. Pie charts show the contribution of each ZRT transcript during growth in Zn-limited (no supplemental Zn) and Zn-replete (2.5 µM Zn) media. The size of each pie chart represents the sum of all ZRT transcripts in that condition relative to the other.

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**FIGURE 8.** Zn-deficient Chlamydomonas cells accumulate other metal ions. Intracellular metal ion concentrations of Zn (A), Cu (B), Mn (C), and Fe (D) were quantified as a function of supplemental Zn. Chlamydomonas strain CC-4532 was grown in TAP medium supplemented with 0, 0.025, 0.25 (Experiment 1), 2.5 25, 80, 125, or 250 μM (Experiment 2) Zn. For analysis, cells were collected by centrifugation. The cell pellet was washed with 1 mM EDTA to remove extracellular bound metal and dissolved in 30% nitric acid by incubation at 65°C. The metal content was measured by ICP-MS. Data points represent the average of experimental triplicates.
Table 1. Subsets of CO$_2$, Cu, and Zn-responsive genes are highly upregulated in Zn-limitation. Comparison of mRNA abundances among genes that are the most highly upregulated in Zn-limited relative to Zn-replete photoheterotrophic wild-type cells identifies genes known previously to be regulated by CO$_2$, Cu, and Zn. Protein ID Augustus 10.2 = loci identified in the Augustus update 10.2 annotation of the JGI assembly v4 for each gene. RPKM = reads per kilobase of transcript mappable length per million mapped reads. Fold change is presented as the ratio of $-\text{Zn}/+\text{Zn}$. Differential expression statistics are provided in terms of p-values.

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