Copper economy in *Chlamydomonas*: Prioritized allocation and reallocation of copper to respiration vs. photosynthesis

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Inorganic elements, although required only in trace amounts, permit life and primary productivity because of their functions in catalysis. Every organism has a minimal requirement of each metal based on the intracellular abundance of proteins that use inorganic cofactors, but elemental sparing mechanisms can reduce this quota. A well-studied copper-sparing mechanism that operates in microalgae faced with copper deficiency is the replacement of the abundant copper protein plastocyanin with a heme-containing substitute, cytochrome (Cyt) c_6. This switch, which is dependent on a copper-sensing transcription factor, copper response regulator 1 (CRR1), dramatically reduces the copper quota. We show here that in a situation of marginal copper availability, copper is preferentially allocated from plastocyanin, whose function is dispensable, to other more critical copper-dependent enzymes like Cyt oxidase and a ferroxidase. In the absence of an extracellular source, copper allocation to Cyt oxidase includes CRR1-dependent proteolysis of plastocyanin and quantitative recycling of the copper cofactor from plastocyanin to Cyt oxidase. Transcriptome profiling identifies a gene encoding a Zn-metalloprotease, as a candidate effecting copper recycling. One reason for the retention of genes encoding both plastocyanin and Cyt c_6 in algal and cyanobacterial genomes might be because plastocyanin provides a competitive advantage in copper-depleted environments as a ready source of copper.

copper homeostasis | acclimation | RNA-seq | metal | copper store

Copper occurs in two stable oxidation states in biological systems, Cu^{I} and Cu^{II}, which enables its use as a catalyst in various redox reactions, including electron transfer and oxygen chemistry. Its chemical repertoire parallels the chemical repertoire of iron because of the similar (albeit usually higher) redox potential of the Cu^{II}/Cu^{I} couple vs. the Fe^{III}/Fe^{II} couple. The overlap in chemical potential of these two metal ions is exemplified by the many instances of nonhomologous, isofunctional proteins where one family is solely dependent on copper for activity and the second on iron. Such instances include hemocyanin and hemoglobin (oxygen transport: Cu or heme), superoxide dismutases (superoxide detoxification: Cu-Zn or Fe), nitrite reductases (nitrite reduction: Cu or heme), electron transfer proteins ($1e^-$ transfers: Cu or heme), and various oxidases and oxygenases (1). In each of these cases, copper proteins are the more recent innovation; they evolved when the great oxidation event in Earth's history increased the bioavailability of copper while greatly decreasing the bioavailability of iron (2, 3). However, despite equivalent reactivity, iron was already established as a dominant redox cofactor in the ancient world, and, as a result, copper proteins are not as prevalent in the proteome as are iron-containing proteins (4). Nevertheless, because of its function in the aerobic world in fundamental metabolic pathways like photosynthesis and respiration, copper is an essential nutrient for nearly all forms of life.

Copper deficiency occurs naturally in geographic regions or local environments with poor copper content, in situations where high concentrations of other metals like zinc or cadmium interfere with copper metabolism, or where high concentrations of copper ligands like thiomolybdates reduce bioavailability (5–9). Genetic variation can also lead to a spectrum of phenotypes related to copper homeostasis and, in some cases, render organisms functionally copper-deficient, and there are cases of acquired copper deficiency in the clinical literature as well (10). The immediate biochemical consequence of biological copper deficiency is loss of function of cuproenzymes, and hence reduced operation of pathways relying on those enzymes (11–13). Many organisms can mitigate the consequences of suboptimal copper nutrition by facultative reduction of the copper quota, or "copper sparing" (14). Metal quota reduction is especially well-documented for microorganisms, particularly algae and cyanobacteria (15, 16). In a copper-replete environment, these oxygen-evolving phototrophs typically use a blue copper protein, plastocyanin, for photosynthetic electron transfer, and this

Significance

Many inorganic elements, because they are required for catalysis or as structural components in biomolecules, are essential nutrients for life. In a situation of poor nutrition, organisms economize on the use of these elements by choosing alternate chemistries to achieve the same function. The mechanism of copper economy in *Chlamydomonas* involves replacement of copper-containing plastocyanin with heme-containing cytochrome (Cyt) c_6. The copper that is saved by this substitution is used instead for Cyt oxidase biosynthesis. Copper recycling requires proteolysis of plastocyanin dependent on a copper-sensing transcription factor. A candidate protease has been identified.

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protein constitutes a large fraction of the copper quota (17, 18). In a copper-deficient situation, plastocyanin function can be replaced by cytochrome (Cyt) c_6, a functionally equivalent heme (iron)-containing *c*-type Cyt. The replacement makes the copper protein nonessential for photosynthesis and reduces the copper quota (19).

The copper-sparing pathway has been well-elucidated in the reference organism *Chlamydomonas* (20). The *PCY1* gene encoding plastocyanin is constitutively expressed, but the protein accumulates only in copper-replete cells; in copper-deficient cells, the protein is rapidly degraded (19, 21). The *CYC6* gene encoding Cyt c_6 is transcribed exclusively in copper-deficient cells to cover the loss of plastocyanin function (22). *CYC6* expression occurs via transcriptional activation dependent on copper-response elements and a copper-sensing transcription factor, copper response regulator 1 (CRR1), which binds to such elements associated with the *CYC6* promoter (23, 24).

A survey of the *Chlamydomonas* genome revealed dozens of candidate cuproproteins, but transcriptome and proteome analyses indicate that the copper quota of the cell is dependent largely on a few (25). *PCY1* mRNA is one of the most abundant transcripts in the cell (top 10 of >17,000 genes), consistent with the abundance of plastocyanin ($\sim 8 \times 10^6$ molecules per cell). Other abundant transcripts include *FOX1*, encoding a ferroxidase required for iron assimilation, and hence more heavily expressed in iron-deficient cells; *COX2A* and *COX2B*, encoding one of two copper-binding subunits of Cyt oxidase, which is essential for respiration in the mitochondria; and *UOX1* encoding urate oxidase, which functions in nitrogen metabolism. Quantitative proteomics of soluble fractions confirmed the dominance of plastocyanin over other cuproproteins (e.g., 100-fold higher than urate oxidase), which underscores its dominant contribution to the total cellular copper quota (26).

In this study, we manipulate the cellular copper quota by growing cells under conditions where one of three major copper-using pathways is prioritized (photosynthesis with plastocyanin, iron assimilation with ferroxidase, and respiration with Cyt oxidase) to probe the dynamics of copper distribution to these pathways. Copper is preferentially allocated to Cyt oxidase (whose proton-pumping function cannot be replaced by a copper-independent enzyme) over plastocyanin and, in the absence of a nutritional supply, can be recycled from plastocyanin in a CRR1-dependent pathway.

Materials and Methods

Strains and Culture Conditions. *Chlamydomonas reinhardtii* WT strain CC-4532, WT strain CC-124, *dum19* mutant strain CC-3400, *crr1-2* mutant strain CC-3960, and the complemented *crr1-2* (*CRR1*) strain CC-5071 were cultured in copper-deficient (20 nM) or copper-supplemented (400 nM) tris-acetate-phosphate (TAP) medium with Hutner's trace elements as described previously (27). Cultures were incubated at 24 °C with 180 rpm agitation and 120 μmol of photons per $m^{-2} \cdot s^{-1}$ of continuous illumination under 4,100-K cool-white fluorescent bulbs. Where indicated, copper-deficient medium was supplemented to the desired amount with EDTA-chelated $CuCl_2$. For iron-deficient cultures, cells were precultured to stationary phase in deficient medium (1 μM Fe) (28). For photoautotrophic growth, acetate was eliminated from TAP medium and the pH was adjusted to 7.4 with HCl. For heterotrophic growth, flasks were wrapped in aluminum foil.

Subfractionation of Cells. *Chlamydomonas* cultures were collected by centrifugation (1,000 × *g* for 5 min) and washed twice with ultrapure 4 mM sodium phosphate, pH 7.0. Quantitative fractionation of cells into soluble and membrane components is described by Howe and Merchant (29). After the first separation, the pellet was washed with an equal volume of phosphate buffer and reseparated by centrifugation. The supernatants were combined and further clarified three times by centrifugation. The pellet fractions at each step were combined. Immunoblot analyses confirmed that the membrane fraction was free of plastocyanin or Cyt c_6 and the soluble fraction was free of Cyt oxidase. For samples that were subsequently analyzed for metal content, the cells were washed with 1 mM EDTA before the washes with phosphate buffer and fractionation. All tubes used in these experiments were washed with soap, rinsed with deionized water (Biocel A10; Millipore), and soaked for 7 d in 10% (wt/vol) nitric acid, followed by a second rinse with deionized water.

Preparation of COX2B Antiserum. A 447-bp cDNA fragment corresponding to amino acid residues 4–153 of COX2B (GenBank accession no. AF305543) was amplified using the primers 5′-gcatggattccAAGGACCAGCTGAAGGAGAAG-3′ and 5′-gtacgaattcCTGAATCCATTCCTGACGTA-3′ in a 50-μL PCR assay consisting of 1 μg of plasmid DNA, 32 pmol of each primer, 10 nmol of dNTPs, and 2.5 units of Taq polymerase. The amplification conditions were 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.5 min. The amplified fragment was cloned into the BamHI and EcoRI sites of the pGEX4T-1 vector (GE Healthcare) and introduced into *Escherichia coli* for isopropyl-β-D-thiogalactopyranoside–inducible expression. The expressed GST-fusion protein was found in inclusion bodies and was purified by preparative SDS gel electrophoresis. Rabbits were immunized via s.c. nodal injection (200 μg of purified fusion protein with Freund's complete adjuvant) at Covance Research Products, Inc., followed by additional boosts (2 × 250 μg and 1 × 200 μg with Freund's incomplete adjuvant). Antiserum specificity was demonstrated by immunoblotting of protein extracts from *C. reinhardtii* WT CC-124 and the *dum19* mutant (strain CC-239, which lacks Cyt oxidase) and by signal competition with antigen. All antibodies used in this work are available at Agrisera AB.

Immunoblot Analysis. For immunoblot analysis, proteins were separated on an SDS-containing polyacrylamide gel (7.5% monomer for FOX1; 10% monomer for CF_1; and 15% monomer for plastocyanin, Cyt c_6, and COX2B) and transferred at room temperature onto a nitrocellulose membrane (0.1 μm; Whatman GmbH) using a semidry blotter under constant power (4 W) for 30 min (plastocyanin, Cyt c_6, and COX2B), 1 h (CF_1), or 1.5 h (FOX1) in 25 mM Tris, 192 mM Gly, and 20% (vol/vol) methanol with 0.01% SDS (FOX1 and CF_1) or without 0.01% SDS (plastocyanin, Cyt c_6, and COX2B). All membranes were blocked overnight with 1% calf serum (HyClone) in PBS (9.5 mM phosphate, 8 mM sodium, 4.5 mM potassium, and 140 mM NaCl). Primary antibody concentrations were 1:300 (FOX1), 1:500 (plastocyanin and Cyt c_6), 1:5,000 (COX2B), and 1:10,000 (CF_1) dilution in 1% nonfat milk in PBS with 0.3% Tween 20 (wt/vol; Fisher). A secondary antibody, goat anti-rabbit alkaline phosphatase (1:3,000 dilution; Southern Biotech), was used for colorimetric detection with NBT and BCIP. Unless stated otherwise, protein extracts from a fixed number of cells were analyzed (5×10^6 cells for Cyt c_6 and FOX1, 3×10^6 cells for plastocyanin, 2×10^6 cells for COX2B, and 3×10^5 cells for CF_1). Loading based on equal cell numbers is valid because the cell size distribution in the cultures, although broad, did not change in the various conditions used. Those amounts were chosen for a linear range for detection of each protein. To permit a fair comparison between samples within an experiment, all individual gels were transferred onto a single membrane and processed simultaneously.

Metal Measurement. *Chlamydomonas* cells grown to the indicated cell density or to stationary phase (>1×10^7 cells per milliliter) were collected and treated as described (30), with the exception that trace metal grade nitric acid (OPTIMA; Fisher) was used to a final concentration of 2.4% for solubilizing the cells. For measurement of the metal content of soluble or insoluble protein extracts, extracts derived from 2×10^8 cells were analyzed. Soluble extract was cleared of any remaining insoluble material by sequential centrifugations at 20,000 × *g* for 5 min until no insoluble material was present anymore. The insoluble material from each step was combined and washed once with phosphate buffer; after centrifugation, the resulting supernatant and pellet fractions were combined with the initial soluble or insoluble fractions. For measurements of the metal content of the spent medium, cells were collected in acid-washed tubes and part of the remaining medium was carefully transferred into fresh acid-washed tubes. Residual cells were removed by centrifugation (two times sequentially at 20,000 × *g* for 10 min) before the addition of nitric acid. The metal content was analyzed using a PE-6100DRC ICP-MS system (PerkinElmer) (*SI Text*).

RNA-Sequencing Analysis. RNA-sequencing (RNA-seq) libraries were prepared from total RNA and sequenced in 35-nt reads at Illumina. The resulting sequence data were filtered to remove the 3′ adaptor sequence with scythe (https://github.com/vsbuffalo/scythe) and low-quality nucleotide calls (<15) with sickle (https://github.com/vsbuffalo/sickle). Next, reads were aligned to version 5 of the *C. reinhardtii* reference genome (phytozome.jgi.doe.gov/) using TopHat2 (31). The quality of the alignment was evaluated by visualization with the Integrative Genomics Viewer browser (32). Gene expression estimates were calculated in terms of fragments per kilobyte of transcript

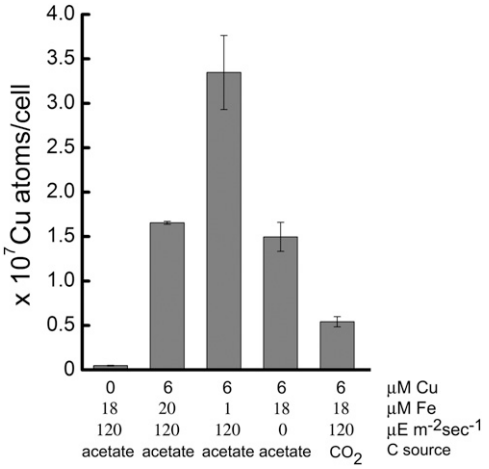

Fig. 1. Copper quota is determined by the metabolic demand for copper-using pathways. WT strain CC-4532 was grown photoheterotrophically under copper-deficient or copper-replete conditions, with or without additional iron deficiency (1 µM Fe), heterotrophically (0 µmol of photons per $m^{-2}{\cdot}s^{-1}$) on medium containing acetate or phototrophically (CO_2). All cultures were bubbled with air. The metal content was measured by inductively coupled plasma (ICP)-MS. The values represent the average and SD of three independent experiments.

per million mapped reads (FPKMs) by cuffdiff (33). Genes whose expression was 10 FPKMs or greater and whose expression increased eightfold or more, or were coexpressed with *CYC6*, were identified with the cummeRbund package of Bioconductor and plotted as a clustered heat map using the R programming language (34).

Results

We can demonstrably manipulate the copper quota of *Chlamydomonas* by changing physiological demand for one of three abundant cuproproteins (Fig. 1). The biogenesis of the photosynthetic apparatus does not require light, but it is promoted by illumination coincident with a two- to threefold increased capacity for photosynthesis (Table 1). The capacity for oxygen consumption, on the other hand, is increased (about twofold) when cells are provided with acetate, whose utilization requires respiration. The abundance of the copper proteins plastocyanin and Cyt oxidase in these bioenergetic pathways parallels the change in pathway capacity (Fig. 2) and contributes to the variation in cellular copper quota. The quota is further increased in iron-poor medium (Fig. 1) when the expression of a high-affinity pathway consisting of a copper-requiring ferroxidase coupled to a ferric transporter is induced (35).

Each of these cuproproteins is located in a different compartment in the cell and uses distinct pathways for copper delivery. The ferroxidase (encoded by *FOX1*) is anchored to the plasma membrane and has an extracellular active site with six copper atoms, which are loaded via the copper chaperone ATX1 (Cre09.g392467) and a Cu^+-transporting P-type ATPase (Cre16.g682369) through the secretory pathway (35, 36). Plastocyanin, with a single copper at its active site, is located in the thylakoid lumen; Cu(I) delivery to this compartment requires the related Cu^+-transporting ATPases PAA1 and PAA2 (CTP2/Cre10.g424775 and CTP4/Cre10.g422201 in *Chlamydomonas*) in the envelope and thylakoid membranes, respectively (37). Cyt oxidase is localized to the inner mitochondrial membrane and has three copper atoms associated with integral membrane subunits COX1 and COX2. In *Chlamydomonas*, COX2 consists of two domains, a membrane component COX2A and a soluble extramembrane domain COX2B, whose accumulation is used as a proxy for the whole complex (38).

We monitored the abundance of these proteins (plastocyanin, ferroxidase, and COX2B) as a function of copper availability (0–400 nM supplemental copper) in illuminated cultures of WT cells in iron-poor medium in photoheterotrophic conditions (i.e., with acetate supplementation) (Fig. 3). These conditions maximize the expression of all three copper proteins, which is reflected by the high copper quota (Fig. 1), and hence maximum demand for copper nutrition. A clear hierarchy of copper distribution is evident, with priority being given to Cyt oxidase over ferroxidase, followed by plastocyanin (Fig. 3). This hierarchical priority is maintained even when cells are grown phototrophically (Fig. S1), which is indicative of the metabolic importance of Cyt oxidase under these conditions. The loss of plastocyanin does not have a negative impact on the capacity for photosynthetic growth because of the compensatory induction of Cyt c_6 (*Discussion*). In iron-poor medium, additional copper is required to maintain plastocyanin to compensate for the draw on the intracellular copper pool caused by ferroxidase synthesis (Fig. S1). The higher nutritional requirement for copper in iron-poor medium is more evident in cells grown in photoheterotrophic conditions (on acetate), where the ferroxidase is even more highly induced (presumably because of the increased demand for iron in respiratory chain components) (Fig. S2).

When copper is supplied below the quota, the driving force for in vivo copper protein synthesis (39) results in complete depletion from the medium (Table 2). For instance, 20 nM copper is inadequate to support plastocyanin accumulation at a density of 6×10^6 cells per milliliter, yet the abundance of Cyt oxidase is maintained as the culture divides to reach a density of 12×10^6 cells per milliliter (Fig. 3). Maintenance of Cyt oxidase occurs coincident with the complete loss of plastocyanin, implicating plastocyanin as the source of copper for Cyt oxidase synthesis (<3%; Fig. S3).

To demonstrate the importance of plastocyanin as the source of copper for de novo Cyt oxidase synthesis, we quantitatively monitored copper during growth of a batch culture in a situation of limiting copper nutrition, analogous to an algal bloom in nature. We chose 20 nM copper because it was sufficient for plastocyanin accumulation at early log phase but inadequate at late log phase (Fig. 3). The medium contained ammonium (which minimizes urate oxidase expression) and was supplemented

Table 1. Light and carbon source affect capacity for photosynthesis and respiration

Growth condition		Respiratory rate, nmol of O_2 per minute per 10^7 cells	Photosynthetic rate, nmol of O_2 per minute per 10^7 cells	Cu content, $\times 10^7$ atoms per cell
Light	Acetate	35.6 ± 7.0	42.9 ± 6.7	1.9 ± 0.2
Light	CO_2	19.0 ± 1.3	33.1 ± 4.0	1.3 ± 0.4
Dark	Acetate	34.7 ± 3.2	12.0 ± 1.8	2.3 ± 0.1

For photoheterotrophic growth, WT strain CC-4532 was grown in acetate containing medium in the light to a density of 3×10^6 cells per milliliter. For heterotrophic or phototrophic growth, the strain was grown in the dark or without acetate, respectively. Two milliliters of culture was removed; after addition of 34 mM acetate, oxygen consumption in the dark was analyzed with a ChloroLab2 electrode from Hansatech. The rate of oxygen production was measured after the addition of 10 mM potassium hydrogen carbonate and illumination with 300 µmol of photons per $m^{-2}{\cdot}s^{-1}$ in 2 mL of culture. After measurement of activities, the cells were collected and the copper content was determined by inductively coupled plasma (ICP)-MS. The values represent the mean and SD of three independent experiments.

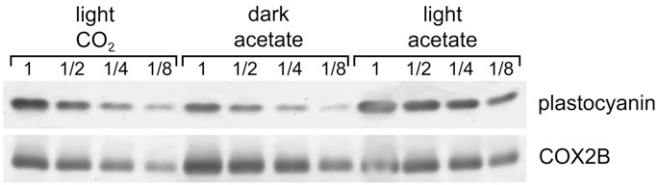

Fig. 2. Plastocyanin and Cyt oxidase abundance per cell is dependent on light and acetate. For photoheterotrophic growth, WT strain CC-4532 was grown in acetate-containing medium with 6 μM copper in the light to a density of 6×10^6 cells per milliliter. For heterotrophic or phototrophic growth conditions, the strain was grown in the dark or without acetate, respectively. Cells were collected by centrifugation, and plastocyanin and COX2B abundances in an equal number of cells (3×10^6 cells and 2×10^6 cells, respectively) were analyzed by immunoblotting. A representative blot of a dilution series from an experimental triplicate is shown.

with excess iron (200 μM) to repress ferroxidase accumulation; thus, in these conditions, plastocyanin and Cyt oxidase represent the bulk of the cellular cuproprotein content. We measured the copper content of the spent medium vs. intracellular copper separated into the supernatant fraction containing plastocyanin and the membrane fraction containing Cyt oxidase. Freeze–thaw rupture of cells followed by centrifugation allowed quantitative separation and recovery of plastocyanin into the supernatant fraction and Cyt oxidase into the pellet fraction, which were also probed by immunoblot analyses for plastocyanin, Cyt c_6, and COX2B (Fig. 4). In parallel, the culture was also sampled to assess its capacity for photosynthesis (O_2 evolution) and respiration (O_2 consumption) (Table 3).

At an early stage in the bloom, when the cells are in a copper-replete state, the copper contents of plastocyanin and Cyt oxidase increase with increasing cell density ($0.5–1 \times 10^6$ cells per milliliter), whereas the spent medium shows a corresponding decrease in copper. At 2×10^6 cells per milliliter, the medium is completely depleted of copper. However, Cyt oxidase content and the copper content of the membrane fractions continue to increase as the cells grow and divide. The increase occurs at the expense of plastocyanin (Fig. 4*E*) and the copper content of the supernatant fraction (Fig. 4*B*). Quantitative mass recovery of the copper content of the partitioned samples (Fig. 4*D*) is consistent with plastocyanin as the only source of copper for de novo Cyt oxidase synthesis. This scavenging mechanism allows the cells to maintain the capacity for respiration (Table 3). The loss of plastocyanin does not compromise photosynthetic capacity, even at the switch point, because Cyt c_6 accumulation occurs concomitant with plastocyanin loss (4×10^6 cells per milliliter) (Fig. 4*E* and Table 3). Because the immunoblot assay for plastocyanin is linear over a 30-fold concentration range (Fig. S3), the disappearance of plastocyanin in a single round of cell division (Fig. S3 vs. Fig. 3 and Fig. 4*E*) is consistent with active degradation of the protein (40).

To reveal the factors in copper mobilization during an algae bloom in a copper-limited situation, we profiled the *Chlamydomonas* transcriptome by RNA-seq during the transition from copper-replete (0.5×10^6 and 1×10^6 cells per milliliter) to copper-deficient (4×10^6 and 8×10^6 cells per milliliter) conditions (as in the experiment shown in Fig. 4) in cultures supplemented with 20 nM copper. To control for changes in gene expression that are dependent on cell density, we monitored, in parallel, cultures supplemented with 400 nM copper, which is saturating for the synthesis of all copper proteins (Fig. 3 and Fig. S2). Sequenced reads were aligned to the *Chlamydomonas* genome and used to estimate gene expression in terms of FPKMs. In previous work based on a two-way comparison of WT and *crr1* cells acclimated to copper deficiency, we had identified 63 genes that were likely targets of CRR1 (25). Only 56 of these genes were mapped forward to the latest version of the *Chlamydomonas* genome, and 90% of these genes (50 of 56) show increased expression as cells divide in 20 nM copper but not as they divide in 400 nM copper (Fig. 5). Among these genes are *CYC6* and *CPX1*, which are sentinel genes of the copper nutrition signaling pathway and, not surprisingly, a number of candidate metal transporters. The *CTR*s, which are involved in copper assimilation (25-fold increase for *CTR2*, eightfold increase for *CTR3*, and fourfold increase for *CTR1*), and ZIP family transporters (23-fold increase for *IRT2*, fourfold increase for *ZRT2*, and threefold increase for *ZIP7*) are especially notable. Previously, we had suggested that IRT2 (whose expression is also regulated by iron deficiency) may serve as a back-up of the FOX1/FTR1 pathway (41). The *CTR*s do show slightly increased expression in copper-replete cells, presumably because cell division depletes the medium of copper, but the induction is less pronounced than the induction observed under copper deficiency. Although copper export from the chloroplast should be catalyzed by a transporter, we were unable to identify a candidate gene for this function from the transcriptome analysis. It is certainly possible that export occurs by passive transport relying on a constitutively expressed transporter. Only one gene, *CTH1* (encoding an enzyme in chlorophyll biosynthesis), appeared to be down-regulated in copper deficiency. *CTH1* is also a target of CRR1; its expression is reciprocal to the expression of its homolog, *CRD1*, whose expression increases in copper deficiency (42).

When we expand this analysis to include all expressed genes (≥10 FPKMs) that increased eightfold or more over the course of the experiment, we identified an additional 12 genes that increase in expression as copper becomes depleted in the 20 nM cultures. Two of these genes, *HYDEF* (25-fold increase) and *AOX2* (26-fold increase), were identified previously as being regulated by copper but were not known to be CRR1-dependent because these genes were also regulated in *crr1* (25). The remaining 10 copper-regulated genes [Cre01.g004750, Cre01.g029700, Cre03.g203569, Cre05.g241647, Cre05.g244000, Cre07.g350976, Cre10.g426800, *CBD1* (listed only as Cre14.g609900 in Phytozome version 10), Cre16.g656750, and Cre17.g734644] were not identified in that earlier study. In support of the hypothesis that these genes are regulated by CRR1, all had one or more putative copper response elements (CuREs) in the 300 nt upstream of the transcription start site (25).

We were particularly interested in identifying the protease that degrades plastocyanin to liberate its copper. Copper is very tightly

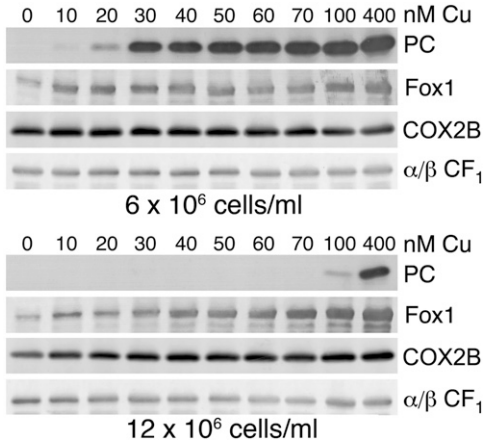

Fig. 3. Hierarchical accumulation of copper-containing proteins in copper-deficient cells. Cells of strain CC-4532 were grown in acetate-containing medium supplemented with the indicated concentrations of copper and 1 μM iron. Cells from the same culture were collected at the indicated densities, and the abundances of plastocyanin (PC), Fox1, COX2B, and the α/β subunit of CF_1 (α/β CF_1) in 1×10^6 cells (per lane) were monitored by immunoblotting.

Table 2. Copper is depleted from the medium during cell growth

Preinoculation, nM		Spent medium, nM	
Calculated	Measured	6×10^6	12×10^6
0	<0.2	<0.2	<0.2
10	9.3	<0.2	<0.2
20	19.5	<0.2	<0.2
30	29.8	<0.2	<0.2
40	40.3	<0.2	0.5
50	51.5	<0.2	1.7
60	63.2	0.4	2.4
70	69.8	1.8	2.6
100	101.8	7.9	6.0
400	408.5	202.3	72.9

WT strain CC-4532 was grown in TAP medium containing various amounts of copper. The copper content of the medium was measured by ICP-MS before inoculation and after growth of cells to the indicated densities. The detection limit in this experiment was 0.2 nM.

bound to plastocyanin and does not dissociate in a physiologically relevant time scale; furthermore, previous work invoked a process involving regulated degradation (40). Given the tight reciprocal accumulation of plastocyanin vs. Cyt c_6 (Fig. 4*E*), we expect the degradative activity to be expressed in parallel with the expression of *CYC6*. Therefore, we searched the dataset for genes coregulated with *CYC6* (Fig. S5). The most likely candidate, *RSEP1*, named for the relationship of the encoded protein with a bacterial membrane-localized zinc metalloprotease called RseP (43), clusters closely with *CYC6*. Its expression increases 54-fold as copper is depleted in the 20 nM cultures.

RSEP1 belongs to the S2P family of membrane-bound metalloproteases, which includes At2g32480 in *Arabidopsis* that is localized to the inner chloroplast envelope membrane and required for chloroplast development (44). Orthologs of *Arabidopsis* At2g32480 are found in all plants, including *Chlamydomonas* (RSEP2) and other green algae. RSEP1 distribution, on the other hand, is restricted to *Chlamydomonas* and *Volvox*, consistent with a specialized function, perhaps in plastocyanin degradation. The protein is predicted to be targeted to an organelle: chloroplast according to ChloroP and TargetP mitochondria according to Predalgo. Expression of *RSEP1* in *Chlamydomonas* is directly responsive to copper nutrition through CRR1, and immunoblot analysis confirms that the protein accumulates in parallel with the RNA. Nevertheless, the protein appears to be unstable, which has precluded fractionation and purification. Conclusive demonstration of function awaits reverse genetic analysis; to date, knock-down experiments have not substantially reduced RSEP1 abundance. The founding member of this family of proteases is the *E. coli* RseP, which targets transmembrane domains of proteins subsequent to their cleavage by an S1P protease (DegS) (45). There are several Deg proteases in the chloroplast, including in the thylakoid lumen, and it is possible that RSEP1 works in concert with one of these Deg proteases (46). An active site near the membrane surface would allow it to act on a soluble protein like plastocyanin, which is known to interact with the membrane (43).

We took advantage of the *crr1* mutant to validate the role of the copper regulon in maintenance of the copper proteome of *Chlamydomonas*. The mutant is completely deficient in the activity of CRR1, which is a transcription factor that recognizes CuREs and is required for activating the copper regulon in copper deficiency (23). Although the mutant grows poorly in copper deficiency (Fig. S7), it is possible to collect sufficient material for biochemical analysis of protein abundance. The samples were analyzed by nondenaturing PAGE to separate holo-plastocyanin from apo-plastocyanin as well as denaturing gels to monitor COX2B (Fig. 6). The *crr1* mutants are unable to maintain Cyt oxidase levels at a low nutritional supply of copper relative to the complemented strain. Indeed, even with 400 nM

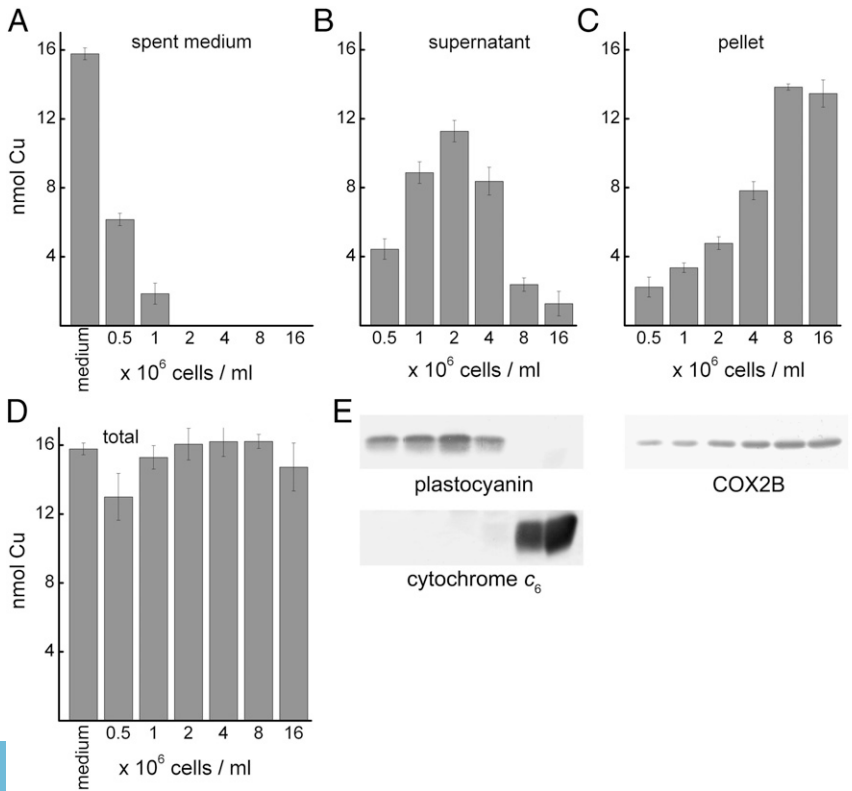

Fig. 4. Reallocation of copper from plastocyanin to Cyt oxidase. Copper-deficient cells of strain CC-4532 were inoculated into TAP medium containing 20 nM supplemental copper to a density of 1×10^4 cells per milliliter. The culture was sampled (volume equivalent to 2×10^8 cells) at 0.5×10^6, 1×10^6, 2×10^6, 4×10^6, 8×10^6, and 16×10^6 cells per milliliter. (*A*) Total copper content of 1 L of spent medium after removal of cells (the lane marked with medium refers to the medium before inoculation). Supernatant (containing plastocyanin) and pellet (containing Cyt oxidase) fractions of the cells were quantitatively recovered and analyzed for copper content (*B* and *C*) and abundance of plastocyanin, Cyt c_6, and Cyt oxidase (*E*). For *B* and *C*, the *y* axis shows the copper content of the supernatant and pellet fractions of cells in 1 L of culture. (*D*) Sum of the copper content from *A*–*C*. The experiment was conducted in biological triplicate, and the values show the average ± SD. For *E*, the sample was loaded on the basis of an equivalent volume of culture and only one representative blot is shown. The soluble fraction was also analyzed by HPLC, both by size-exclusion and anion-exchange HPLC (copper repletion is shown in Fig. S4) coupled to the ICP-MS. All copper-containing peaks could be attributed to either free copper or plastocyanin in various samples (copper-replete and copper-deficient soluble extract from a mutant lacking plastocyanin, purified plastocyanin, and an aliquot of copper solution used to supplement the medium).

Table 3. Photosynthesis and respiration capacity is maintained during a Cu-limited algal bloom

Cell density, $\times 10^6$ cells per milliliter	Respiration	Photosynthesis	Respiration	Photosynthesis
	nmol of O_2 $min^{-1} \cdot mL^{-1}$		nmol of O_2 per minute per 10^7 cells	
0.5	1.0 ± 0.1	1.3 ± 0.1	16.6 ± 1.7	22.2 ± 1.9
1	1.4 ± 0.2	3.4 ± 0.4	13.8 ± 1.9	34.0 ± 5.1
2	2.6 ± 0.3	6.7 ± 2.0	13.4 ± 1.2	34.7 ± 9.7
4	5.7 ± 1.4	12.9 ± 0.5	14.1 ± 3.2	32.0 ± 0.9
8	12.1 ± 3.0	29.5 ± 4.5	16.2 ± 1.7	34.9 ± 2.4
16	22.1 ± 1.2	47.9 ± 1.7	18.7 ± 1.5	36.8 ± 3.8

Copper-deficient cells of strain CC-4532 were inoculated into TAP medium containing 20 nM supplemental copper to a density of 1×10^4 cells per milliliter. When cells reached 0.5×10^6 cells per milliliter, and subsequently at 1×10^6, 2×10^6, 4×10^6, 8×10^6, and 16×10^6 cells per milliliter (corresponding to one doubling each time), 2 mL of culture was removed and analyzed with a ChloroLab2 electrode from Hansatech. Oxygen consumption in the dark was measured for a period of 10 min after the addition of 17 mM acetate. After the addition of 10 mM potassium bicarbonate, the cultures were illuminated with 300 μmol of photons per $m^{-2} \cdot s^{-1}$ and the rate of oxygen evolution was observed for an additional 10 min. The values shown represent the average and SD of three independent experiments.

copper supplementation, which is adequate in the WT (discussed above), *crr1* is unable to accumulate its full complement of cuproproteins. The loss of Cyt oxidase likely results both from reduced copper assimilation (because of an inability to activate the *CTR* genes) and the absence of a holo-plastocyanin pool from which to harvest copper (Fig. 6). The plastocyanin polypeptide

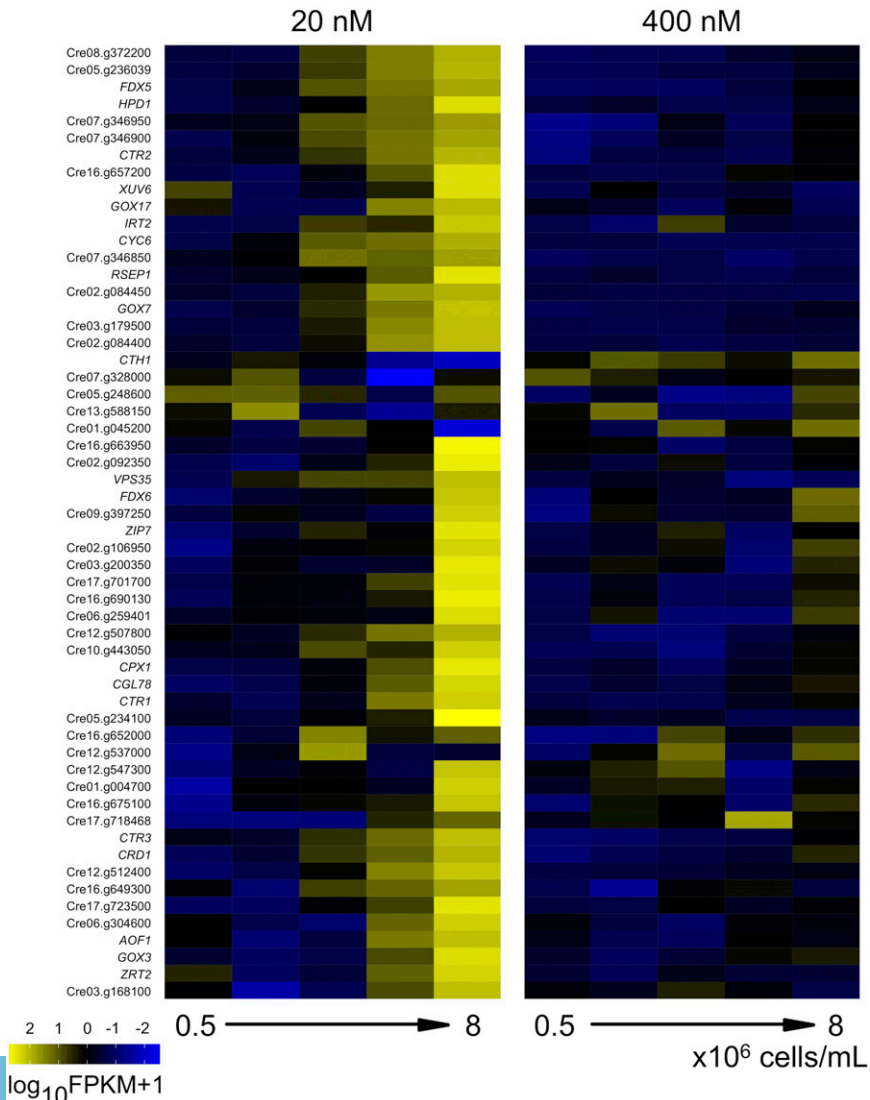

Fig. 5. CRR1 targets are differentially expressed during the transition from copper repletion to copper depletion. WT strain CC-4532 was inoculated at 1×10^4 cells per milliliter into TAP medium supplemented with either 20 or 400 nM copper. RNA was purified from cells of each culture once the cell density reached 0.5×10^6, 1×10^6, 2×10^6, 4×10^6, and 8×10^6 cells per milliliter. RNA was quantified in terms of FPKMs by RNA-seq. Expression estimates from 56 genes that had previously been identified as targets of the CRR1 transcription factor are plotted as a clustered heat map. Log_{10} transformations of FPKMs are presented on a scale ranging from blue (lowest expression) to yellow (highest expression). *AOF1*, amine oxidase, flavin-containing 1; *CGL78*, conserved in green lineage 78; *CPX1*, coproporphyrinogen III oxidase 1; *CRD1*, copper response defect 1; *CTH1*, copper target homolog 1; *CTR1*, copper transporter 1; *FDX5*, ferredoxin 5; *GOX3*, glyoxal or galactose oxidase 3; *HPD1*, 4-hydroxyphenylpyruvate dioxygenase 1; *IRT2*, iron responsive transporter 2; *VPS35*, vacuolar protein sorting-associated protein 35; *XUV6*, xanthine/uracil/vitamin C permease-like protein 6; *ZIP7*, zinc/iron transporter 7; *ZRT2*, zinc responsive transporter 2.

accumulates to substantial levels, although largely in the nonfunctional apoform (presumably because of reduced copper assimilation capacity of the *crr1* strain). Any holoprotein that is made appears not to be degraded in copper-deficient cells of *crr1*. Accordingly, copper cannot be salvaged for maintenance of Cyt oxidase. Thus, *crr1* mutants fail to grow either phototrophically or heterotrophically in copper-poor medium (Fig. S7), which reinforces the importance of CRR1 in nutritional copper homeostasis in the plant lineage. Note that *RSEP1* is not induced in *crr1*, which is not inconsistent with the hypothesis that *RSEP1* might encode a plastocyanin-degrading protease (25).

Discussion

Metabolism Defines the Copper Quota. Three cuproproteins, plastocyanin, ferroxidase, and Cyt oxidase, each with a function in fundamental metabolic pathways (photosynthesis, iron assimilation, and respiration, respectively) are largely responsible for the copper quota in *Chlamydomonas*. Their contribution is evident from the change in copper quota in response to metabolic changes that affect the expression of the three cuproproteins. When acetate is provided in the medium, which allows respiratory growth, mitochondrial biogenesis is stimulated and Cyt oxidase stoichiometry and the associated copper content per cell increase two- to threefold, with an increase in the copper quota of the cell (Figs. 1 and 2 and Table 1). Although these experiments do not identify the cellular location of the copper, measurements of respiratory activity confirm an increase, indicating that at least some of the additional copper must be attributable to Cyt oxidase. Although the photosynthetic apparatus of *Chlamydomonas* is present in cells grown without light, the biogenesis is enhanced with illumination, resulting in higher plastocyanin content, and therefore a corresponding increase in the quota. In iron-deficient medium, the copper quota is increased relative to iron-replete medium, coincident with a 40- to 50-fold increase in the abundance of the ferroxidase (35, 47). The impact of these physiological changes on the copper quota is visible because of the high abundance of the proteins; plastocyanin and ferroxidase are certainly visible as heavily stained bands in cell extracts (17, 48). The nutritional requirement for copper for any organism (or, by extension, for any cell within a multicellular organism) is therefore not a fixed, absolute number but rather dependent on the external conditions, physiological state, or metabolic demands on that cell.

Biomarkers for Copper Status are Organism-Specific, Dictated by Copper-Dependent Metabolic Pathways. When there is insufficient copper nutrition to support the accumulation of all cuproproteins to the maximum stoichiometry demanded by their physiology or metabolic status, a copper distribution mechanism comes into play. This mechanism relies, in part, on expression of the distributive copper transporters and chaperones. For instance, CTP1 (ortholog of *S. cerevisiae* Ccc2p and *Arabidopsis* RAN1) and ATX1 expression is increased in iron-deficient cells, which may drive copper toward the secretory pathway (35). Likewise, the expression of COX17 is increased in acetate-grown cells, where mitochondrial biogenesis is promoted. Mitochondrial biogenesis has a heavy iron requirement, which probably accounts for the up-regulation of FOX1 in acetate-grown vs. CO_2-grown cells (Fig. S2). These copper distribution mechanisms may contribute to the observed hierarchical allocation of copper to Cyt oxidase over ferroxidase over plastocyanin. Cyt oxidase is also prioritized in fungi (over CuZn-superoxide dismutase and Gal oxidase) but not in some multicellular organisms, where loss of Cyt oxidase is used as a biomarker for the copper status of the organism (11, 49, 50). Indeed, Cyt oxidase is a poor marker for the copper status of *Chlamydomonas* or *Dactylium dendroides*. Rather, plastocyanin or Gal oxidase provides a better proxy for copper status in these species, and we recommend that plastocyanin be used as a biomarker for environmental copper status in marine and freshwater phytoplankton that have a genetic capacity for plastocyanin synthesis (18, 51–53). In *Chlamydomonas*, plastocyanin, whose abundance in a copper-replete situation is estimated at 8×10^6 molecules per cell, accumulates only after the copper demand for maintenance of Cyt oxidase is satisfied. Copper is retained in the medium only when it is provided in excess of the cellular copper quota, in this case, the amount required for synthesis of both plastocyanin and Cyt oxidase (17).

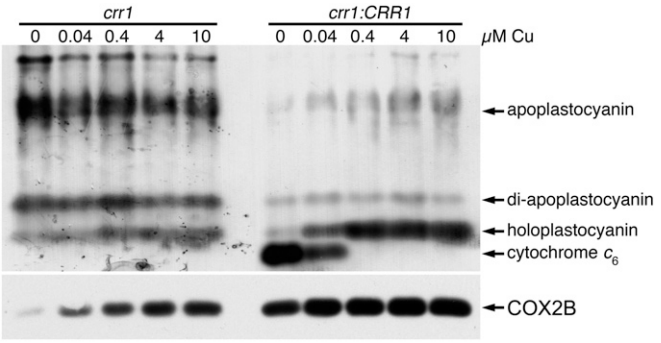

Fig. 6. CRR1 function is required for degradation of plastocyanin and maintenance of Cyt oxidase in limiting Cu. The *crr1* and the corresponding complemented (*crr1:CRR1*) strains were grown in TAP medium with copper supplementation as indicated to a density of 5×10^6 cells per milliliter. Proteins from soluble fractions (8 μg) were separated by native gel electrophoresis (*Upper*), proteins from membrane fractions (8 μg) were separated on SDS-containing gels (*Lower*), and both were analyzed by immunoblotting. Native gel electrophoresis separates holoplastocyanin from apoplastocyanin (40). The band marked di-apoplastocyanin represents a disulfide-linked apo-form. Cyt c_6 is detected because of its heme-dependent peroxidase activity.

Although the particular proteins that are prioritized are specific to the metabolic needs of individual organisms, the concept of hierarchical distribution of a limiting cofactor is broadly applicable. For instance, in zebrafish, in a situation of copper limitation, a cuproprotein like tyrosinase involved in melanin synthesis is deprioritized over cuproproteins required for notochord development, presumably because the loss of tyrosinase is less consequential to survival of the organism (12). Likewise, patients with weak alleles of Menkes disease (and hence less copper-deficient) show connective tissue abnormality because of lysyl oxidase deficiency but are less affected in neurological processes (54). In land plants, plastocyanin is prioritized over CuZn-superoxide dismutase because land plants do not have genetic information for Cyt c_6 as a backup for plastocyanin and photosynthesis is essential for survival, whereas Fe-superoxide dismutase can cover the function of the cuproenzyme (37). The mechanisms for down-regulating the deprioritized protein vary, ranging from proteolysis of plastocyanin in *Chlamydomonas* to inhibition of cuproprotein mRNA function in *Arabidopsis* (37), but the outcome is the same.

Elemental Sparing Mechanisms May Offer a Selective Advantage. The reason that plastocyanin is dispensable under conditions of copper deficiency is because Cyt c_6 is a perfect substitute with respect to bioenergetic function. Growth rates of *Chlamydomonas* cells in copper-replete vs. copper-deficient conditions are indistinguishable, as also are the rates of photosynthetic electron transfer (Fig. S7 and Table 3). The absence of an obvious advantage of plastocyanin raises the question of why genetic information for plastocyanin is maintained in algae and cyanobacteria. We suggest that plastocyanin offers a selective advantage as an intracellular copper store. In a competitive natural environment, where copper can become depleted during an algal bloom (exacerbated by high respiratory activity generating an oxygen-poor environment that is not conducive to copper solubility), the possibility of recycling copper from plastocyanin to Cyt oxidase would be beneficial. Cyt oxidase is an

essential enzyme because its proton-pumping function cannot be substituted by a copper-independent version. The lack of a copper-independent version is evident from the poor growth of copper-deficient *Chlamydomonas* cells in the dark, where they are dependent on respiration and Cyt oxidase function (Fig. S7). Despite the operation of copper sparing and salvage, Cyt oxidase-dependent (cyanide-sensitive) respiration is greatly impaired in copper-deficient *Chlamydomonas* [25% of Cyt oxidase-dependent respiration in copper-replete cultures (Table S1)].

In a previous work on iron allocation in cyanobacteria, the idea of "hot bunking" was promoted as a mechanism of sparing iron during a diurnal cycle (55). Saito et al. (55) suggested that intracellular iron could be shared between photosynthesis and nitrogen fixation, which are uncoupled temporally because the former is active during the day and the latter at night. In so doing, the cyanobacterium reduces its iron quota, which gives it a competitive advantage in an iron-limited ocean. However, in the study of Saito et al. (55), it was not possible to monitor recycling quantitatively, which was possible in our study because of the very small number of abundant copper proteins. It is possible that *Chlamydomonas* cells grown on limiting copper may also recycle the cofactor between photosynthesis and respiration on a diurnal basis, but we have not yet tested this possibility. Although plastocyanin is degraded to support Cyt oxidase accumulation, Cyt oxidase occurrence and function are not required for plastocyanin degradation. For instance, plastocyanin is degraded in the *dum19* mutant (which does not accumulate Cyt oxidase) (Fig. S8).

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1. Kaim W, Schwederski B (1994) *Bioinorganic Chemistry: Inorganic Elements in the Chemistry of Life—An Introduction and Guide* (Wiley, Chichester, UK).
2. Crichton RR, Pierre JL (2001) Old iron, young copper: From Mars to Venus. *Biometals* 14(2):99–112.
3. Ochiai EI (1983) Copper and the biological evolution. *Biosystems* 16(2):81–86.
4. Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM (2008) Metal ions in biological catalysis: From enzyme databases to general principles. *J Biol Inorg Chem* 13(8): 1205–1218.
5. Danks DM (1988) Copper deficiency in humans. *Annu Rev Nutr* 8:235–257.
6. Broun ER, Greist A, Tricot G, Hoffman R (1990) Excessive zinc ingestion. A reversible cause of sideroblastic anemia and bone marrow depression. *JAMA* 264(11):1441–1443.
7. Fosmire GJ (1990) Zinc toxicity. *Am J Clin Nutr* 51(2):225–227.
8. Kadrabová J, Madaric A, Ginter E (1993) Zinc and copper in the tissues and serum of cadmium intoxicated guinea-pigs: Influence of vitamin C. *Physiol Res* 42(4):261–266.
9. Suttle NF (2012) Copper imbalances in ruminants and humans: Unexpected common ground. *Adv Nutr* 3(5):666–674.
10. Madsen E, Gitlin JD (2007) Copper deficiency. *Curr Opin Gastroenterol* 23(2):187–192.
11. Broderius MA, Prohaska JR (2009) Differential impact of copper deficiency in rats on blood cuproproteins. *Nutr Res* 29(7):494–502.
12. Mendelsohn BA, et al. (2006) Atp7a determines a hierarchy of copper metabolism essential for notochord development. *Cell Metab* 4(2):155–162.
13. Marschner H (1995) *Mineral Nutrition of Higher Plants* (Academic, London).
14. Merchant SS, Helmann JD (2012) Elemental economy: Microbial strategies for optimizing growth in the face of nutrient limitation. *Adv Microb Physiol* 60:91–210.
15. Sandmann G, Reck H, Kessler E, Boger P (1983) Distribution of plastocyanin and soluble plastidic cytochrome c in various classes of algae. *Arch Microbiol* 134:23–27.
16. Blaby-Haas CE, Merchant SS (2013) *Metals in Cells*, eds Culotta V, Scott RS (Wiley, Chichester, UK), pp 51–63.
17. Merchant S, Hill K, Howe G (1991) Dynamic interplay between two copper-titrating components in the transcriptional regulation of cyt *c6*. *EMBO J* 10(6):1383–1389.
18. Peers G, Price NM (2006) Copper-containing plastocyanin used for electron transport by an oceanic diatom. *Nature* 441(7091):341–344.
19. Merchant S, Bogorad L (1986) Regulation by copper of the expression of plastocyanin and cytochrome c552 in Chlamydomonas reinhardi. *Mol Cell Biol* 6(2):462–469.
20. Merchant SS, et al. (2006) Between a rock and a hard place: trace element nutrition in Chlamydomonas. *Biochim Biophys Acta* 1763(7):578–594.
21. Merchant S, Bogorad L (1986) Rapid degradation of apoplastocyanin in Cu(II)-deficient cells of *Chlamydomonas reinhardtii*. *J Biol Chem* 261(34):15850–15853.
22. Quinn JM, Merchant S (1995) Two copper-responsive elements associated with the Chlamydomonas Cyc6 gene function as targets for transcriptional activators. *Plant Cell* 7(5):623–628.
23. Kropat J, et al. (2005) A regulator of nutritional copper signaling in Chlamydomonas is an SBP domain protein that recognizes the GTAC core of copper response element. *Proc Natl Acad Sci USA* 102(51):18730–18735.
24. Sommer F, et al. (2010) The CRR1 nutritional copper sensor in Chlamydomonas contains two distinct metal-responsive domains. *Plant Cell* 22(12):4098–4113.
25. Castruita M, et al. (2011) Systems biology approach in Chlamydomonas reveals connections between copper nutrition and multiple metabolic steps. *Plant Cell* 23(4): 1273–1292.
26. Hsieh SI, et al. (2013) The proteome of copper, iron, zinc, and manganese micronutrient deficiency in Chlamydomonas reinhardtii. *Mol Cell Proteomics* 12(1):65–86.
27. Quinn JM, Merchant S (1998) Copper-responsive gene expression during adaptation to copper deficiency. *Methods Enzymol* 297:263–279.
28. Moseley JL, et al. (2002) Adaptation to Fe-deficiency requires remodeling of the photosynthetic apparatus. *EMBO J* 21(24):6709–6720.
29. Howe G, Merchant S (1992) The biosynthesis of membrane and soluble plastidic c-type cytochromes of Chlamydomonas reinhardtii is dependent on multiple common gene products. *EMBO J* 11(8):2789–2801.
30. Allen MD, Kropat J, Tottey S, Del Campo JA, Merchant SS (2007) Manganese deficiency in Chlamydomonas results in loss of photosystem II and MnSOD function, sensitivity to peroxides, and secondary phosphorus and iron deficiency. *Plant Physiol* 143(1):263–277.
31. Kim D, et al. (2013) TopHat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* 14(4):R36.
32. Thorvaldsdóttir H, Robinson JT, Mesirov JP (2013) Integrative Genomics Viewer (IGV): High-performance genomics data visualization and exploration. *Brief Bioinform* 14(2):178–192.
33. Trapnell C, et al. (2013) Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol* 31(1):46–53.
34. Gentleman RC, et al. (2004) Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biol* 5(10):R80.
35. La Fontaine S, et al. (2002) Copper-dependent iron assimilation pathway in the model photosynthetic eukaryote *Chlamydomonas reinhardtii*. *Eukaryot Cell* 1(5):736–757.
36. Blaby-Haas CE, Merchant SS (2012) The ins and outs of algal metal transport. *Biochim Biophys Acta* 1823(9):1531–1552.
37. Pilon M, Abdel-Ghany SE, Cohu CM, Gogolin KA, Ye H (2006) Copper cofactor delivery in plant cells. *Curr Opin Plant Biol* 9(3):256–263.
38. Pérez-Martínez X, et al. (2001) Subunit II of cytochrome c oxidase in Chlamydomonad algae is a heterodimer encoded by two independent nuclear genes. *J Biol Chem* 276(14):11302–11309.
39. Banci L, et al. (2010) Affinity gradients drive copper to cellular destinations. *Nature* 465(7298):645–648.
40. Li HH, Merchant S (1995) Degradation of plastocyanin in copper-deficient *Chlamydomonas reinhardtii*. Evidence for a protease-susceptible conformation of the apoprotein and regulated proteolysis. *J Biol Chem* 270(40):23504–23510.
41. Allen MD, del Campo JA, Kropat J, Merchant SS (2007) FEA1, FEA2, and FRE1, encoding two homologous secreted proteins and a candidate ferrireductase, are expressed coordinately with FOX1 and FTR1 in iron-deficient Chlamydomonas reinhardtii. *Eukaryot Cell* 6(10):1841–1852.
42. Moseley JL, et al. (2002) Reciprocal expression of two candidate di-iron enzymes affecting photosystem I and light-harvesting complex accumulation. *Plant Cell* 14(3): 673–688.
43. Kroos L, Akiyama Y (2013) Biochemical and structural insights into intramembrane metalloprotease mechanisms. *Biochim Biophys Acta* 1828(12):2873–2885.
44. Bölter B, Nada A, Fulgosi H, Soll J (2006) A chloroplastic inner envelope membrane protease is essential for plant development. *FEBS Lett* 580(3):789–794.
45. Li X, et al. (2009) Cleavage of RseA by RseP requires a carboxyl-terminal hydrophobic amino acid following DegS cleavage. *Proc Natl Acad Sci USA* 106(35):14837–14842.
46. Schuhmann H, Adamska I (2012) Deg proteases and their role in protein quality control and processing in different subcellular compartments of the plant cell. *Physiol Plant* 145(1):224–234.
47. Herbik A, Bölling C, Buckhout TJ (2002) The involvement of a multicopper oxidase in iron uptake by the green algae *Chlamydomonas reinhardtii*. *Plant Physiol* 130(4): 2039–2048.
48. Herbik A, Haebel S, Buckhout TJ (2002) Is a ferroxidase involved in the high-affinity iron uptake in *Chlamydomonas reinhardtii*. *Plant Soil* 241:1–9.
49. Shatzman AR, Kosman DJ (1978) The utilization of copper and its role in the biosynthesis of copper-containing proteins in the fungus, *Dactylium dendroides*. *Biochim Biophys Acta* 544(1):163–179.
50. Johnson WT, Anderson CM (2008) Cardiac cytochrome C oxidase activity and contents of subunits 1 and 4 are altered in offspring by low prenatal copper intake by rat dams. *J Nutr* 138(7):1269–1273.
51. Bohner H, Merkle H, Kroneck P, Böger P (1980) High variability of the electron carrier plastocyanin in microalgae. *Eur J Biochem* 105(3):603–609.
52. Nakamura M, Yoshizaki F, Sugimura Y (2000) Accumulation of plastocyanin mRNA lacking 5′ region in the green alga *Pediastrum boryanum* grown under copper-deficient conditions. *Plant Cell Physiol* 41(1):33–41.
53. Miramar MD, Inda LA, Saraiva LM, Peleato ML (2003) Plastocyanin/cytochrome c6 interchange in *Scenedesmus vacuolatus*. *J Plant Physiol* 160(12):1483–1486.
54. Tang J, Robertson S, Lem KE, Godwin SC, Kaler SG (2006) Functional copper transport explains neurologic sparing in occipital horn syndrome. *Genet Med* 8(11):711–718.
55. Saito MA, et al. (2011) Iron conservation by reduction of metalloenzyme inventories in the marine diazotroph *Crocosphaera watsonii*. *Proc Natl Acad Sci USA* 108(6): 2184–2189.