Title

From economy to luxury: Copper homeostasis in Chlamydomonas and other algae

Journal

Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1867(11)

ISSN

0167-4889

Authors

Merchant, Sabeeha S
Schmollinger, Stefan
Strenkert, Daniela
et al.

Publication Date

2020-11-01

DOI

10.1016/j.bbamcr.2020.118822

Peer reviewed

HHS Public Access

Author manuscript

Biochim Biophys Acta Mol Cell Res. Author manuscript; available in PMC 2021 November 01.

Published in final edited form as:

Biochim Biophys Acta Mol Cell Res. 2020 November ; 1867(11): 118822. doi:10.1016/j.bbamcr.2020.118822.

From economy to luxury: copper homeostasis in Chlamydomonas and other algae

Sabeeha S. Merchant[1], **Stefan Schmollinger**[1], **Daniela Strenkert**[1], **Jeffrey L. Moseley**[1], **Crysten E. Blaby-Haas**[2]

[1]Departments of Molecular and Cell Biology and Plant and Microbial Biology, University of California – Berkeley, Berkeley, CA 94720

[2]Department of Biology, Brookhaven National Laboratory, Upton, NY

Abstract

Plastocyanin and cytochrome c_6, abundant proteins in photosynthesis, are readouts for cellular copper status in Chlamydomonas and other algae. Their accumulation is controlled by a transcription factor copper response regulator (CRR1). The replacement of copper-containing plastocyanin with heme-containing cytochrome c_6 spares copper and permits preferential copper (re)-allocation to cytochrome oxidase. Under copper-replete situations, the quota depends on abundance of various cuproproteins and is tightly regulated, except under zinc-deficiency where acidocalcisomes over-accumulate Cu(l). CRR1 has a transcriptional activation domain, a Zn-dependent DNA binding SBP-domain with a nuclear localization signal, and a C-terminal Cys-rich region that represses the zinc regulon. CRR1 activates >60 genes in Chlamydomonas through GTAC-containing CuREs; transcriptome differences are recapitulated in the proteome. The differentially-expressed genes encode assimilatory copper transporters of the CTR / SLC31 family including a novel soluble molecule, redox enzymes in the tetrapyrrole pathway that promote chlorophyll biosynthesis and photosystem I accumulation, and other oxygen-dependent enzymes, which may influence thylakoid membrane lipids, specifically polyunsaturated galactolipids and γ-tocopherol. CRR1 also down-regulates 2 proteins in Chlamydomonas: for plastocyanin, by activation of proteolysis, while for the di-iron subunit of the cyclase in chlorophyll biosynthesis, through activation of an upstream promoter that generates a poorly-translated 5’ extended transcript containing multiple short ORFs that inhibit translation. The functions of many CRR1-target genes are unknown, and the copper protein inventory in Chlamydomonas includes several

Credit Author Statement
First draft by SM
Editing and revision by SS, DS, JLM, CEB
Data, data analysis and visualization by SS, DS, JLM, CEB
Credit author statement
Sabeeha Merchant: Conceptualization, Writing – Original draft, Supervision, Project Administration, Funding acquisition Stefan Schmollinger: Conceptualization, Writing – Review and editing, Methodology, Formal analysis, Investigation, Visualization Daniela Strenkert: Investigation, Writing – Review and Editing, Visualization Jeffrey L. Moseley: Investigation, Writing – Review and Editing Crysten E. Blaby-Haas: Investigation, Writing – Review and Editing, Visualization, Funding acquisition

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

whose functions are unexplored. The comprehensive picture of cuproproteins and copper homeostasis in this system is well-suited for reverse genetic analyses of these under-investigated components in copper biology.

Copper proteins are recent innovations

The low mass elements are the most abundant elements on earth, and also the most abundant elements in life forms. Typically, about 12 of the first 20 elements in the periodic table contribute to biomass (Figure 1). Yet, more than 60 other naturally occurring elements are found in various organisms, albeit in trace amounts [1]. Elements like the first row transition metals enable redox reactions that are central to life, and that cannot readily be catalyzed by functional groups found on amino acid side chains. For instance, the oxidation of water in oxygenic photosynthesis relies on Mn, Fe and Cu chemistry, and the reduction of dinitrogen to ammonium, relies on Fe and Mo (or V) during nitrogen fixation. Because organisms depend on redox reactions for extraction of energy from inorganic compounds for autotrophic growth or organic compounds for heterotrophic growth, the trace elements are essential nutrients for life. Protein catalysts evolved over the course of a few billion years to use metals that were environmentally accessible, meaning abundant and easily extractable, and comparative genomics, especially of microorganisms, can offer a paleontological record of events that shaped today's metalloprotein repertoire [2]. We note that while iron was readily available at the origin of life, especially in an anaerobic environment, accounting for its wide use and abundance throughout biology, copper was essentially invisible to biology until the rise of O_2 (Figure 2), well after the iron-proteome was established. Accordingly, there are many fewer unique copper proteins in biology (compared to iron) and their substrates are often oxygen or oxygenated compounds, which were prevalent only after oxygenation of the planet. Typically, an organism's copper quota is an order of magnitude less than its iron quota (Figure 1). Still, copper-deficiency does occur in nature, either because of a sulfidic or anaerobic environment, dietary deficiency, competition with other metals in the nutrient milieu, or mutations in copper assimilation pathways.

Copper sparing in algae – the plastocyanin / cytochrome c_6 switch

We have developed *Chlamydomonas reinhardtii* (simply Chlamydomonas, hereafter) as a reference organism [3] for understanding acclimation to copper deficiency in the context of photosynthesis. *Chlamydomonas* spp. have been found in peat bogs and sewage lagoons, where copper availability may be reduced because of poor oxygenation [4, 5]. Chlamydomonas is a unicellular chlorophyte alga in the green plant lineage (Viridiplantae), and accordingly aspects of its biology, especially in the context of chloroplast metabolism, are similar to those in land plants. Yet, Chlamydomonas has retained ancestral eukaryotic features and functions that have been lost in present-day plants [6]. Our interest in nutritional copper signaling and homeostasis was motivated by the decades-old observations [7, 8] that many algae and cyanobacteria can use either the blue copper-protein plastocyanin or the heme protein cytochrome c_6 as soluble one-electron carriers between reduced cytochrome *f* of the cytochrome b_6f complex and oxidized $P700^+$ in photosystem I. Blue copper proteins are a more recent innovation in biology relative to the ancient *c*-type cytochromes [4]. In organisms that have genetic information for both proteins, the

occurrence of one or the other protein was determined by the copper content in the environment. This suggested that these algae had a mechanism for measuring / sensing copper and a signaling pathway for responding to copper levels. We used a Chlamydomonas plastocyanin-deficient mutant, strain *ac-208* (carrying an early stop-codon in the *PCY1* transcript encoding pre-apoplastocyanin [9]), to validate the existence of a copper-sensing signaling pathway and to document the functional equivalence of plastocyanin and Cyt c_6 [10]. Specifically, strain *ac*-208 showed a copper-nutrition-conditional defect in photosynthesis; the strain could not grow photoautotrophically in copper-replete medium but was rescued by growth in copper-deficient medium where Cyt c_6 is expressed and compensates for the loss of plastocyanin (Figure 3). Furthermore, suppressors of *ac*-208 grow photoautotrophically by restricting copper assimilation and hence activating expression of Cyt c_6.

In Chlamydomonas, the accumulation of plastocyanin vs. Cyt c_6 is controlled by different mechanisms for each protein. For plastocyanin, copper stabilizes the protein both in vitro and in vivo; the apoprotein is unfolded and more protease-susceptible, but degradation in vivo requires expression of a protease (whose identity is not yet known) [11]. For Cyt c_6, the *CYC6* gene is under tight transcriptional control [12, 13] by a transcriptional activator, copper response regulator 1 (CRR1), that works through copper response elements (CuREs) [14, 15]. The sequence GTAC within the CuRE is critical for its function. Mutation of any one of these nucleotides to any other nucleotide abolishes CuRE activity in vivo. *CYC6* transcription increases also in response to hypoxia and nickel ions, and these responses also occur through the CuREs [16, 17]. While the response to hypoxia may be physiologically meaningful (with low oxygenation of the medium promoting less soluble Cu(l) over more soluble Cu(ll) species [4]), the response to nickel ions is non-physiological and may relate to the mechanism of the coppersignaling pathway. Quantitative blot hybridization [18] and RNA-Seq analysis [19] indicate that *CYC6* transcripts are completely absent in copper-replete medium, making the CuREs+CRR1 useful for regulated expression of transgenes in Chlamydomonas [17, 20].

CRR1

CRR1 contains a DNA-binding domain, called SBP (for the prototype sequence in the *SQUAMOSA* promoter binding protein [21]) (Figure 4A). The 76-amino acid SBP domain has a nuclear localization signal and shows Zn(II)-dependent sequence-specific binding to the CuREs associated with the *CYC6* gene [22]. The zinc ion is coordinated to conserved Cys and His residues in a novel zinc-binding domain [23]. In in vitro experiments, Cu(I), which is the likely species in vivo, can compete with Zn(II) because of 10^5-fold higher affinity for the metal binding site, but the Cu(I) containing form does not bind DNA [22]. This property suggests a mechanism for copper-sensing and signaling by CRR1 in vivo. A conserved His residue within the SBP domain, distal to the zinc binding ligands, is required for Cu(I) binding in the in vitro system.

The SBP DNA-binding domain is unique to the plant lineage. Green algae have from 1 (in *Dunaliella salina)* to 24 (in Chlamydomonas) SBP-domain proteins, and the reference plant Arabidopsis has 17 (although SPL13A and SPL13B are identical at the nucleotide/protein

level). Green algal genomes encode diverse SBPs (Figure 4B) with the vast majority uncharacterized. Among the 17 Arabidopsis proteins (named SPLs), SPL1, SPL7 and SPL12 are most similar to CRR1 with respect to sequence similarity outside the highly conserved SBP domain. However, because the land plant SBP family expanded after the chlorophyte-streptophyte split, a one-to-one orthology relationship between CRR1 and land plant SBPs cannot be made (Figure 4B and C). Reverse-genetic analysis indicates that Arabidopsis SPL7 is a functional homologue of Chlamydomonas CRR1 [24], while SPL1 and SPL12 do not have a role in copper homeostasis [25]. The spl7 plants grow poorly in copper deficient conditions, and are not able to activate the expression of assimilatory copper transporters (see below) nor down-regulate cuproprotein abundance for copper sparing [24, 26]. The SBP domain of SPL7, like CRR1, also has affinity for the GTAC-core sequence of the CuRE [21, 26].

CRR1 is a large protein, containing a Med15 transcriptional activation domain in the N-terminal half and a conserved sequence, WLxxxPxxxExxIRPGC, just downstream of the SBP domain [15, 24, 25]. We refer to the region containing this sequence as the “extended SBP domain” [15]. The extended SBP domain is found in most algal SBPs in group A (the group of SBP-domain proteins more closely related to CRR1), plus Arabidopsis SPL1, SPL7, SPL12, SPL14 and SPL16, and closely related homologs in other land plants (Figure 4). Unique to CRR1 is a Cys-rich metallothionein-like domain at the C-terminus, with several ankyrin repeats adjacent [15] (Figure 4A). Neither of these domains is required for copper-responsive gene expression, but they are important for the nickel- and hypoxia-induced expression of the copper regulon [22]. The Cys-rich region is conserved in closely related algae from Chlorophyceae, speaking to its functional relevance (Figure 4A). We noted, unexpectedly, that Chlamydomonas strains in which the Cys-rich region is deleted (CRR1-ΔCys) are phenotypically normal for nutritional copper signaling, but are de-repressed for the nutritional zinc regulon [22]. The CRR1-ΔCys version of the protein is recessive to the wild-type form. The high-affinity ZRT transporters of the ZIP-family are expressed in CRR1-ΔCys Chlamydomonas strains in fully zinc-replete medium, and the strains accordingly hyper-accumulate Zn. This result suggests cross-talk between Cu and Zn signaling pathways, but the mechanistic connection has not yet been illuminated. A connection between Cu and Zn uptake was documented decades ago for humans where excess zinc supplementation resulted in copper-deficiency owing to an impact of zinc on intestinal copper absorption [27], but this connection is not seen in Arabidopsis [25].

Chlamydomonas copper regulon

Transcriptome analysis of copper-deficient vs. copper-replete Chlamydomonas cells identified 149 differentially accumulating transcripts (using a cut-off of 2-fold change and at least 10 FPKM in either condition), of which 129 showed increased abundance in copper-deficiency, indicative of substantial metabolic changes beyond the replacement of plastocyanin by Cyt c_6 [19]. A comparison of copper-deficient wild-type cells vs. the *crr1* mutant indicates that about half (63) of these changes are dependent on the CRR1 locus; computational analysis showed enrichment of GTAC sequences upstream of the putative transcription start sites of those genes, consistent with their being direct CRR1 targets. Parallel proteomics experiments captured about 25% of the copper-deficiency target genes

and in each case re-capitulated the pattern of expression, which underscores the key contribution of transcription to nutritional copper signaling in Chamydomonas [28].

In addition to *CYC6*, comparative transcriptomics re-identified *CPX1* and *CRD1*, encoding enzymes in tetrapyrrole biosynthesis [29, 30] and *CTRs*, encoding copper assimilation proteins [31]. Both CPX1 and CRD1 function at oxygen-dependent steps that can be rate-limiting for flux through the tetrapyrrole pathway. CPX1 is coproporphyrinogen (coprogen) oxidase, and CRD1 is the di-iron subunit of Mg-protoporphyrin IX monomethylester cyclase in chlorophyll biosynthesis [32]. Other components required for function and assembly of the cyclase enzyme, CGL78/ycf54 and FDX6, were discovered subsequently [33, 34], and the corresponding genes are also co-expressed in the Chlamydomonas nutritional copper regulon with *CRD1* [28]. Increased coprogen oxidase activity may accommodate the need for more heme for synthesis of Cyt c_6, which accumulates to ~ 2×10^6 molecules per cell.

CRD1 is one of two paralogues; the other one is named CTH1 (for copper target homolog). The two proteins are reciprocally expressed with respect to copper nutrition [35]: CRD1 is more abundant in copper-deficient cells while CTH1 is more abundant in the copper-replete situation. Interestingly, the proteins are more diverged than are the corresponding genes, consistent with neo-functionalization. Duplication of this gene is prevalent in the green algal lineage, and may represent an adaptive strategy coincident with replacement of plastocyanin with Cyt c_6. The phenotype of *crd1* strains suggests that CRD1-dependent chlorophyll biosynthesis favors photosystem (PS) 1 accumulation [30]. Constitutive (i.e. copper-independent) expression of *CTH1* can only incompletely cover the loss of CRD1, speaking to a specialized role for CRD1 in maintaining PS1 [30].

The reciprocal accumulation of CTH1 (down in low copper) and CRD1 (up in low copper) is determined by CRR1. The CRR1- and CuRE-dependent decrease of CTH1 in copper-poor cells occurs through an interesting mechanism in which a) an alternative 5’ extended transcript containing multiple short upstream ORFs blocks synthesis of the corresponding downstream CTH1 polypeptide, and b) utilization of the alternative CuRE-associated promoter interferes with the downstream promoter that generates the well-translated shorter mRNA form [35]. The ZAP1 transcriptional activator of zinc-deficiency responses in yeast also works through transcriptional interference to down-regulate alcohol-dehydrogenase-encoding transcripts, *ADH1* and *ADH3* [36]. More recently, the use of promoter switching combined with translational repression through short upstream ORFs has been shown to operate for hundreds of genes in yeast [37].

Transcripts encoding enzymes involved in modification of fatty acids and lipids, including sterol hydroxylases / desaturases, lipid and acyl-ACP desaturases, a sterol methyl transferase, 4-hydroxyphenylpyruvate dioxygenase (HPPD1) and P450s acting on sterols and carotenoids, are also increased in copper-deficient cells, and about half of the corresponding genes are CRR1 targets [19]. Lipid and lipid cofactor analysis identified increased desaturation of chloroplast galactolipids in copper-deficient cells, attributed primarily to increased *FAB2* expression, and increased γ-tocopherol attributed to *HPPD1* expression [19, 38]. It is possible that these changes are needed to reorganize the

bioenergetic membrane for Cyt c_6 instead of plastocyanin in the photosynthetic electron transfer chain.

Besides plastocyanin, Cyt oxidase is another metabolically important copper protein in eukaryotic cells. The protein is prioritized to receive copper (see below), yet transcripts for key proteins involved in anaerobic pyruvate metabolism, PAT1, PFR1 and several hydrogenase assembly factors, as well as for a mitochondrial alternative oxidase isoform (AOX2) show increased abundance in copper-deficient cells [19]. Except for hydrogenase [39], the corresponding genes are not CRR1 targets, suggesting that they may be responding to compromised Cyt oxidase activity or metabolic signals calling for flux through fermentation or alternative bioenergetic pathways. Transcripts for some Cyt oxidase assembly factors, Cox17, which is relevant to assembly of the Cu_A site [40], and Cox15, required for heme a synthesis [41], are also increased in abundance [19], but again the genes do not appear to be CRR1-responsive; instead, they may be regulated by feedback response to Cyt oxidase levels or function.

CDO1, encoding cysteine dioxygenase, a non-heme iron enzyme, is another differentially-expressed gene that is also not a direct CRR1 target. Increased *CDO1* expression in copper-deficiency is intriguing, since the enzyme is important for maintaining intracellular Cys levels in animal cells [42, 43]. A mechanism for decreasing Cys in copper-deficiency may reduce competition for copper ions. A related cupin-fold enzyme is a key hypoxia regulator [44], making *CDO1* another connection between hypoxia- and Cu-deficiency-signaling.

Copper assimilation, distribution and sequestration

Chlamydomonas cells have a remarkable ability to assimilate copper, drawing down the copper content in the medium to undetectable levels (< 0.2 nM), yet once the cells assimilate enough copper to saturate the copper-binding sites of cuproproteins, they do not take up any more (see below), speaking to a high-affinity, copper-regulated system [31]. The increased sensitivity of copper-deficient vs. -replete cells to nM levels of Ag(I) is consistent with an inducible high-affinity Cu(I) transporter [45].

In eukaryotic cells, copper is assimilated as Cu(I) by transporters of the CTR family or SLC31 after reduction of Cu(ll) by cell-surface cupric reductases [46–48]. CTRs are permeases that transport Cu(I) down a concentration gradient, either from the external milieu or from intracellular storage compartments towards the cytoplasm [49, 50]. Structural studies indicate a trimeric organization of CTR polypeptides, each with three trans-membrane helices, that together form an ion-channel / transmembrane pore [51]. The extra-membrane regions of the prototype yeast and mammalian CTRs typically contain copper-binding residues, forming a ($Mx_{1\text{-}4}M$) Mets motif on the N-terminus of each polypeptide on the outside the cell and Cys- and His- containing regions towards the C-terminus on the inside of the cell. The extracellular Mets motifs are not required for Cu(I) transport but confer high-affinity properties. A trans-membrane Mets motif, $Mx_4Mx_{12}Gx_4G$, conserved across species, is a diagnostic signature of the CTR family and may be involved in Cu(I) transport across the membrane [47].

The Chlamydomonas genome encodes 4 proteins of the CTR family [52, 53]. Of these, COPT1 is related to Arabidopsis COPT proteins [54], but is not regulated by copper nutrition or CRR1, and does not appear to be quantitatively important in copper-deficiency based on relative mRNA abundance. CTR1, CTR2 and CTR3 form a distinct family [52]. Each gene is associated with CuREs and is a CRR1 target [19, 31]. CTR1 and CTR2 are membrane-associated with 3 predicted trans-membrane segments and large N-terminal extra-membrane domains with multiple distinct Cys-Met motifs, the most common of which is CxxMxxMxxC-$x_{5/6}$-C, and a juxtamembrane MxMxxH motif on the N-terminal side of TM1. These distinctive motifs could contribute to the high copper scavenging capacity of Chlamydomonas; they are found also in other algae and some amoebae [31]. CTR1 and CTR2 function as transporters in yeast, but their in vivo functions in Chlamydomonas are not yet articulated. The identity of the inducible cell surface cupric reductase in Chlamydomonas [55] is also not firmly established.

The *CTR3* gene is physically linked to *CTR2* (~31.5 kb away) and appears to have arisen from it by partial duplication. The N-terminal Cys-Met motifs are present, but it lacks the membrane-spanning α-helices and the juxta-membrane MxMxxH motif, and proteomic analysis identifies it as a soluble protein [28]. It is clearly not a canonical transporter, but its mRNA is relatively abundant (~ 10x compared to *CTR1* and *CTR2)*, and the gene is co-expressed with *CTR1* and *CTR2* through CuREs and CRR1, consistent with a function in copper homeostasis. Similar soluble versions of CTR are found in other algae, amoebae, slime molds and fungi (often, the gene is physically linked to a membrane CTR transporter gene). Reverse-genetic analysis or functional reconstitution of Cu(I) may illuminate the in vivo role of these derived soluble forms.

Once Cu(I) is taken up into the cell, it is handed over to a copper chaperone for transfer to distributive copper transporters of the P_{IB}-ATPase family [56–58]. None of these proteins is regulated by copper nutrition at the level of the transcriptome. An ATX1 orthologue was identified by sequence similarity, by increased expression in iron-deficiency and by functional complementation of a yeast *atx1* mutant, consistent with a role in copper delivery to the secretory pathway for biogenesis of the Chlamydomonas multi-copper oxidase, FOX1, involved in iron uptake [59, 60]. In Chlamydomonas, 5 members of the P_{IB}-ATPase family have been described; named CTP1 through CTP4 and HMA1 [52]. These pumps use ATP hydrolysis to concentrate ions in intracellular locations or to pump ions out of the cell. HMA1 is an orthologue of Arabidopsis HMA1 localized to the chloroplast envelope membrane whose function is still debated. CTP1 is an orthologue of yeast Ccc2 and mammalian ATP7s that deliver copper to the secretory pathway for assembly of secreted copper proteins like ferroxidases and PAM (peptidylglycyl α-amidating monooxygenase) [61–63]. CTP2 and CTP4 are orthologues of PAA1 and PAA2 from Arabidopsis, which are located in the envelope and thylakoid membranes, respectively and pump copper ions into the chloroplast stroma and then lumen for assembly of plastocyanin [64]. CTP3 has not been characterized in Chlamydomonas, but the prevailing view is that it may pump copper into endosomes or vacuoles, ultimately for detoxification of copper.

In addition to ATX1, the Chlamydomonas genome encodes 3 putative copper chaperones. Based on the presence of a signal peptide and co-expression with copper-dependent amine

oxidases, Cre03.g207650 may function as a copper chaperone in the secretory pathway. Candidate copper chaperones in the chloroplast are PCC1 and PCH1. PCC1 is one of very few proteins whose expression is decreased in copper-deficiency [19]. Orthologues co-occur in algae with plastocyanin, consistent with a function in copper delivery to plastocyanin. PCH1 is derived from PAA1/CTP2 by an alternative splicing event that has been conserved broadly throughout the green plant lineage [65].

The copper quota in Chlamydomonas is tightly regulated, presumably by regulation and balance between assimilation and efflux, and varies between 1-3 × 10^7 Cu atoms / cell depending on the expression of cuproproteins (see below). The only situation in which we noted over-accumulation of copper is under zinc-deficiency where Cu(I) is sequestered with an unknown S-containing ligand in acidic compartments, called acidocalcisomes , that also contain Ca^{2+} and polyphosphate [66–68]. Sequestered copper may be visualized directly with high spatial resolution by nanoscale secondary ion mass spectrometry (nanoSIMS) relying on the mass of the element or by X-ray fluorescence relying on the absorption of hard X-rays by Cu, or indirectly, by using a Cu(I)-sensitive fluorescence probe with confocal microscopy [69]. The mechanism underlying copper over-accumulation is not understood, but it involves mis-expression of the copper regulon in zinc-deficiency. The sequestered copper can be made bio-available by zinc supplementation to restore copper homeostasis, indicating that the acidocalcisomes are dynamic copper reservoirs rather than terminal storage or detoxification sites.

Copper quota and copper sparing

A survey of the Chlamydomonas genome revealed dozens of cuproproteins in Chlamydomonas [19]. Among these are plastocyanin, encoded by *PCY1*, located in the chloroplast (see above) for photosynthesis, Cyt oxidase copper binding subunits COX2A and COX2B, located in the mitochondrion for respiration, a multi-copper oxidase encoded by *FOX1*, located on the plasma membrane for iron-assimilation [70]. PAM [71], 3 amine oxidases, a putative dopamine β-monooxygenase, other multi-copper oxidases of unknown function, several copper-binding matrix metalloproteinases [72], multiple galactose/glyoxal oxidases of unknown physiological function, urate oxidase, and other proteins with predicted copper-binding domains. Orthologues of copper chaperones and assembly factors for Cyt oxidase, ATX1, COX17, COX19, COX11, COX 23, SC01, are also found in the Chlamydomonas genome, indicating that copper trafficking in this organism is not different from most other eukaryotic cells.

Transcripts for *PCY1, COX2* and *FOX1* represent > 95% of cuproprotein-encoding RNA [19] and likely also represent the most abundant cuproproteins in Chlamydomas [73, 74] (Strenkert, unpublished data). Indeed, estimations of absolute abundance of these 3 cuproproteins by quantitative proteomics correlates very well with the copper quota of laboratory grown Chlamydomonas cultures (Figure 5), which is typically in the range of 1 × 10^7 Cu/cell (Figure 1). The quota can be manipulated by growth physiology [75]. For instance, when Chlamydomonas is grown on acetate as a source of carbon, mitochondria will proliferate relative to photoautotrophically grown cells so that the Cu quota is increased to match the ~ 2-fold increase in Cyt oxidase per cell. And, under iron-deficiency,

Chlamydomonas increases the expression of the *FOX1* gene with a concomitant increase in the Cu quota, corresponding to ~ 20-fold increase in accumulation of the ferroxidase [59, 75]. In the copper-deficient situation, copper is allocated to these abundant proteins in a hierarchical manner so that Cyt oxidase, whose function cannot be replaced by another protein, receives copper with the highest priority, whereas plastocyanin whose function is readily covered by the heme-containing Cyt c_6 is reduced drastically in abundance [75] (Figure 5). The copper-sparing mechanism has the greatest impact when it operates on abundant cuproproteins like plastocyanin [76]. While replacement of plastocyanin by Cyt c_6 is firmly established in cyanobacteria [77–80] and green algae (see above) by ecological, biochemical and genetic evidence, other sparing mechanisms remain untested. For instance, *IRT2*, encoding a member of the ZIP family / SLC39 and a candidate iron transporter, may partially compensate for the loss of FOX1 function in copper-deficient Chlamydomonas. Nevertheless, this has not yet been established. The *AOF1* gene, which encodes a flavin-containing amine oxidase may represent another example of copper sparing in a situation where organic amines are the sole N source for the organism [81].

Other algae

Algae are a phylogenetically diverse group of organisms [82]. They are distributed throughout the tree of life and are related through endosymbiotic events involving the plastid. Chlorophyte algae and land plants are the descendants of the primary endosymbiotic event involving a eukaryotic host and a cyanobacterium, giving rise to the Archaeplastida clade, but plastids have been transferred to various hosts by secondary and tertiary endosymbiosis giving rise to distinct other algal lineages [83]. Plastid and photosynthesis-related processes can therefore be ancestrally related among the different clades, but processes derived from the host eukaryotes can be rather diverged or distinct. A number of algal genomes were sequenced in the last few years [82], allowing us to survey the genomes for the co-occurrence of both plastocyanin and Cyt c_6, which gives an indication of the prevalence of the copper sparing phenomenon in nature [84].

We identified plastocyanin- and Cyt c_6-encoding algae in several stramenopiles as well as in green algae, which is consistent with the broad occurrence of the copper-sparing switch from plastocyanin to Cyt c_6 in extant cyanobacteria [85–87]. Among the green algae, it is more typical to identify both proteins in organisms closely related to Chlamydomonas, but not in the early diverging prasinophytes where plastocyanin prevails. CRR1 orthologs can be identified in closely related chlorophyte algae, including Chlamydomonales and Sphaeropleales consistent with the occurrence of a nutritional copper signaling pathway in this group of organisms. Besides the SBP domain itself, these CRR1 orthologs contain the extended SBP domain and the ankyrin repeats followed by the Cys-rich region (Figure 4). In Volvox and Gonium where a duplication has occurred, one paralog resembles the CRR1 prototype, and the other is truncated. In Volvox, the derived protein lacks the C-terminal Cys-rich region while in Gonium there is a deletion in the N-terminal region. Perhaps the regulatory circuit is more complex in these species.

Interestingly, a nearly-ubiquitous copper enzyme in eukaryotes, CuZn-superoxide dismutase (SOD), is absent in most of the green algae [88] except prasinophytes, although it is present

in plants [82]. In plants and also fungi, the CuZn-SOD can be replaced by iron- or manganeseutilizing enzymes [89–91]. In Arabidopsis, the miRNA-dependent down-regulation of *CSD* genes encoding CuZn-SOD is mediated by SPL7 activation of miR398 transcription [26], which validates CuZn-SOD as a target of copper sparing mechanisms. By relying solely on iron- or manganese-containing superoxide dismutases, the green algae have reached a permanent solution to a reduced copper quota. Interestingly, the branches within the green lineage that retain the CuZnSODs (prasinophytes and land plants) are also the ones where plastocyanin prevails.

Duplication events involving regulatory and target genes in various algae suggest tailoring of the nutritional copper regulon (Figure 6). Multiple paralogs of *PCY1* are found in *Haematococcus lacustris* and *Volvox carteri*, both of which lack the *CYC6* gene. They must have a mechanism for copper sparing other than plastocyanin degradation. Conversely, *Caulerpa lentillifera* has *CYC6* but not *PCY1.* An interesting mechanism for down-regulation of plastocyanin operates in *Pediastrium boryanum* where a 5' truncated *PCY1* mRNA that is incompetent for translation is generated in copper-deficient cells [92, 93]. The switch between two alternative promoters in copper-replete vs. copper-deficient cells is reminiscent of *CTH1* gene expression in Chlamydomonas (see above), but the mechanism for translational repression is unique. In *Gonium pectorale* on the other hand, two copies of *CYC6* (96.8% nucleotide identity, 95.5% amino acid identity) are found side-by-side, consistent with recent duplication. In *Chromochloris zofingiensis* the *CYC6* gene is immediately upstream of the *PCY1* gene, suggesting of a regulatory mechanism involving transcriptional interference for either / or expression in response to copper nutrition. Transcriptional regulation of both *PCY1* and *CYC6* has been suggested also for *Scenedesmus obliquus* [94].

For chlorophyll biosynthesis, most species among the Chlorophyceae have orthologues of CTH1/CHL27A and CRD1/CHL27B, but in *Scenedesmus obliquus* the CHL27B is more diverged from Chlamydomonas CRD1 than from Chlamydomonas CTH1, indicating an independent duplication event. *Monoraphidium neglectum* and *Raphidocelis subcapitata* each have a CTH/CHL27A orthologue, and gene sequences that may represent a second copy of *CHL27*, which is now an inactive pseudogene. *Monoraphidium* also contains duplicate, physically linked copies of *CGL78*, which are substantially diverged from each other (86.8% nucleotide identity, 80.8% amino acid identity). The Trebouxiophytes *Chlore. variabilis* and *Micractinium conductrix* encode, respectively, 3 and 4 CHL27 homologues, with one potential pseudogene in each set. Another component of the Mg-protoporphyrin IX monomethylester cyclase, CGL78, is present in all the species analyzed except *Chloropicon primus.* The absence in *Chloro. primus* may be a consequence of an incomplete genome assembly because all other enzymes in chlorophyll biosynthesis are found. Two ferredoxins, FDX5 and FDX6, which are controlled by CRR1 and up-regulated during Cu-deficiency, may provide electrons for the cyclase reaction [19, 34, 95]. The *FDX5* gene is present only in the Chlamydomonadales family (Chlamydomonas, Gonium, Tetrabaena and Volvox), but *FDX6* homologs are found in all species except Chloropicon. Taken together, these duplications and gene losses speak to a dynamic process of optimization of the genetic network controlling acclimation to Cu-deficiency, perhaps offering us an evolutionary record of the mineral environment of the niche occupied by each algal species.

Future directions

Despite our holistic understanding of nutritional copper signaling in Chlamydomonas at multiple levels, many details need to be illuminated and many questions remain unanswered, making the system attractive for further inquiry.

1. CuREs and the CRR1 regulon: What are sequences beyond the GTAC core of the CuRE that confer function? These have not yet been revealed by in vivo mutational analysis. Perhaps an in vitro SELEX experiment would be informative. Comparative transcriptomics (copper-replete vs-deficient or wild-type vs. *crr1* mutats) identified > 60 candidate genes in the CRR1-dependent nutritional Cu regulon. Although computational methods suggest that these genes are associate with CuREs, this remains to be documented by ChIP-Seq or DAP-Seq [96] with CRR1.

2. Copper sensing by CRR1: In vitro studies of the SBP domain document Zn-dependent binding to the GTAC-core of the CuRE. Cu(I) competes effectively in vitro with 10^5 higher affinity relative to Zn(II) for binding to the metal binding site in the SBP domain. The Cu(I)-bound form does not bind the CuRE, but whether this is the mechanism for inactivating CRR1 in vivo remains unanswered. How is copper delivered to CRR1 in vivo? How is the effect of Ni(II), Co(II) or hypoxia, each of which recapitulates copper-deficiency in vivo, explained? The analogy with mammalian hypoxic signaling should be considered. Reverse genetic functional analysis of the Med15 domain and the extended SBP domain should be more straightforward in Chlamydomonas relative to Arabidopsis, and may illuminate mechanistic aspects of nutritional copper sensing and signaling.

3. CRR1 in other algae: Orthologs of CRR1 occur in various algae. How does copper signaling operate in other algae? What genes are turned on in copper-deficiency in other algae? Which of these is conserved, and hence central to copper economy? What is the mechanism in algae where a CRR1 orthologue is not evident?

4. CRR1 targets: The biochemical functions of many CRR1 target genes in Chlamydomonas are not known, suggesting that there remains much to be discovered for understanding copper biology in the chlorophyte algae. Functional studies of the multiple copper-deficiency induced prolyl hydroxylases may inform on post-translational aspect of nutritional copper signaling in this organism.

In a ChIP experiment, the *CYC6* and *CPX1* genes were noted to be associated with acetylated histones H3 and H4, nucleosome displacement and an open chromatin conformation [97]. Genome-wide approaches can now be used to probe chromatin marks associated with each of the target genes and assess whether they are responsive to copper nutrition.

5. Promoter switching: Are there other examples of genes whose expression is decreased in copper-deficiency by the mechanism used for CTH1? Ongoing Iso-seq experiments should identify alternate transcript forms.

6. Chaperones and transporters: Only a few of these have been rigorously assigned function by reverse genetics, heterologous complementation and / or expression patterns. How are CTR1 and CTR2 distinguished? What is the function of the soluble CTR3 in algae? Which pumps function in copper detoxification? What is the mechanism of PCC1 and PCH1?

Acknowledgments

The research from the Merchant group, summarized in this article, was funded by the National Institutes of Health (GM 042143) since 1989. CB-H is supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, as part of the Quantitative Plant Science Initiative at Brookhaven National Laboratory.

References

[1]. Cvetkovic A, Menon AL, Thorgersen MP, Scott JW, Poole FL 2nd, Jenney FE Jr., Lancaster WA, Praissman JL, Shanmukh S, Vaccaro BJ, Trauger SA, Kalisiak E, Apon JV, Siuzdak G, Yannone SM, Tainer JA, Adams MW, Microbial metalloproteomes are largely uncharacterized, Nature., 466 (2010) 779–782 Epub 2010 Jul 2018. [PubMed: 20639861]

[2]. Dupont CL, Butcher A, Valas RE, Bourne PE, Caetano-Anollés G, History of biological metal utilization inferred through phylogenomic analysis of protein structures, Proc Natl Acad Sci U S A, 107 (2010) 10567–10572 Epub 12010 May 10524. [PubMed: 20498051]

[3]. Salomé PA, Merchant SS, A Series of Fortunate Events: Introducing Chlamydomonas as a Reference Organism, Plant Cell, 31 (2019) 1682–1707. [PubMed: 31189738]

[4]. Crichton RR, Pierre JL, Old iron, young copper: from Mars to Venus, Biometals, 14 (2001) 99–112. [PubMed: 11508852]

[5]. Harris EH, The *Chlamydomonas* Sourcebook: A comprehensive guide to biology and laboratory use, Academic Press, Place Published, 1989.

[6]. Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-Laylin LK, Marechal-Drouard L, Marshall WF, Qu LH, Nelson DR, Sanderfoot AA, Spalding MH, Kapitonov VV, Ren Q, Ferris P, Lindquist E, Shapiro H, Lucas SM, Grimwood J, Schmutz J, Cardol P, Cerutti H, Chanfreau G, Chen CL, Cognat V, Croft MT, Dent R, Dutcher S, Fernandez E, Fukuzawa H, Gonzalez-Ballester D, Gonzalez-Halphen D, Hallmann A, Hanikenne M, Hippler M, Inwood W, Jabbari K, Kalanon M, Kuras R, Lefebvre PA, Lemaire SD, Lobanov AV, Lohr M, Manuell A, Meier I, Mets L, Mittag M, Mittelmeier T, Moroney JV, Moseley J, Napoli C, Nedelcu AM, Niyogi K, Novoselov SV, Paulsen IT, Pazour G, Purton S, Ral JP, Riano-Pachon DM, Riekhof W, Rymarquis L, Schroda M, Stern D, Umen J, Willows R, Wilson N, Zimmer SL, Allmer J, Balk J, Bisova K, Chen CJ, Elias M, Gendler K, Hauser C, Lamb MR, Ledford H, Long JC, Minagawa J, Page MD, Pan J, Pootakham W, Roje S, Rose A, Stahlberg E, Terauchi AM, Yang P, Ball S, Bowler C, Dieckmann CL, Gladyshev VN, Green P, Jorgensen R, Mayfield S, Mueller-Roeber B, Rajamani S, Sayre RT, Brokstein P, Dubchak I, Goodstein D, Hornick L, Huang YW, Jhaveri J, Luo Y, Martinez D, Ngau WC, Otillar B, Poliakov A, Porter A, Szajkowski L, Werner G, Zhou K, Grigoriev IV, Rokhsar DS, Grossman AR, The Chlamydomonas genome reveals the evolution of key animal and plant functions, Science., 318 (2007) 245–250 PMC2875087. [PubMed: 17932292]

[7]. Wood PM, Interchangeable copper and iron proteins in algal photosynthesis. Studies on plastocyanin and cytochrome *c*-552 in *Chlamydomonas*, Eur J Biochem, 87 (1978) 9–19. [PubMed: 208838]

[8]. Sandmann G, Reck H, Kessler E, Boger P, Distribution of plastocyanin and soluble plastidic cytochrome *c* in various classes of algae, Arch Microbiol, 134 (1983) 23–27.

[9]. Quinn JM, Li HH, Singer J, Morimoto B, Mets L, Kindle KL, Merchant S, The plastocyanin-deficient phenotype of *Chlamydomonas reinhardtii* ac-208 results from a frame-shift mutation in the nuclear gene encoding pre-apoplastocyanin, J Biol Chem, 268 (1993) 7832–7841. [PubMed: 8463310]

[10]. Merchant S, Bogorad L, Metal ion regulated gene expression: use of a plastocyanin-less mutant of *Chlamydomonas reinhardtii* to study the Cu(II)-dependent expression of cytochrome *c*-552, EMBO J., 6 (1987) 2531–2535. [PubMed: 2824187]

[11]. Li HH, Merchant S, Degradation of plastocyanin in copper-deficient *Chlamydomonas reinhardtii*, J Biol Chem, 270 (1995) 23504–23510. [PubMed: 7559514]

[12]. Merchant S, Hill K, Howe G, Dynamic interplay between two copper-titrating components in the transcriptional regulation of cyt *c6*, EMBO J., 10 (1991) 1383–1389. [PubMed: 1863287]

[13]. Hill KL, Li HH, Singer J, Merchant S, Isolation and structural characterization of the *Chlamydomonas reinhardtii* gene for cytochrome c_6. Analysis of the kinetics and metal specificity of its copper-responsive expression, J. Biol. Chem, 266 (1991) 15060–15067. [PubMed: 1714451]

[14]. Quinn JM, Merchant S, Two copper-responsive elements associated with the Chlamydomonas Cyc6 gene function as targets for transcriptional activators, Plant Cell, 7 (1995) 623–638. [PubMed: 7780310]

[15]. Kropat J, Tottey S, Birkenbihl RP, Depège N, Huijser P, Merchant S, 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 U S A., 102 (2005) 18730–18735 Epub 12005 Dec 18713 PMC1311908. [PubMed: 16352720]

[16]. Quinn JM, Barraco P, Eriksson M, Merchant S, Coordinate copper-and oxygenresponsive *Cyc6* and *Cpx1* expression in *Chlamydomonas* is mediated by the same element., J Biol Chem, 275 (2000) 6080–6089. [PubMed: 10692397]

[17]. Quinn JM, Kropat J, Merchant S, Copper response element and Crr1-dependent Ni^{2+}-responsive promoter for induced, reversible gene expression in *Chlamydomonas reinhardtii*, Eukaryot Cell, 2 (2003) 995–1002. [PubMed: 14555481]

[18]. Hill KL, Merchant S, In vivo competition between plastocyanin and a copper-dependent regulator of the *Chlamydomonas reinhardtii* cytochrome c_6 gene, Plant Physiol, 100 (1992) 319–326. [PubMed: 16652963]

[19]. Castruita M, Casero D, Karpowicz SJ, Kropat J, Vieler A, Hsieh SI, Yan W, Cokus S, Loo JA, Benning C, Pellegrini M, Merchant SS, Systems biology approach in Chlamydomonas reveals connections between copper nutrition and multiple metabolic steps, Plant Cell., 23 (2011) 1273–1292 Epub 2011 Apr 1215. [PubMed: 21498682]

[20]. Ferrante P, Catalanotti C, Bonente G, Giuliano G, An optimized, chemically regulated gene expression system for *Chlamydomonas*, PloS one, 3 (2008) e3200. [PubMed: 18787710]

[21]. Birkenbihl RP, Jach G, Saedler H, Huijser P, Functional Dissection of the Plant-specific SBP-Domain: Overlap of the DNA-binding and Nuclear Localization Domains, J Mol Biol, 352 (2005) 585–596. [PubMed: 16095614]

[22]. Sommer F, Kropat J, Malasarn D, Grossoehme NE, Chen X, Giedroc DP, Merchant SS, The CRR1 nutritional copper sensor in *Chlamydomonas* contains two distinct metalresponsive domains, Plant Cell., 22 (2010) 4098–4113 Epub 2010 Dec 4093. [PubMed: 21131558]

[23]. Yamasaki K, Kigawa T, Inoue M, Tateno M, Yamasaki T, Yabuki T, Aoki M, Seki E, Matsuda T, Nunokawa E, Ishizuka Y, Terada T, Shirouzu M, Osanai T, Tanaka A, Seki M, Shinozaki K, Yokoyama S, A novel zinc-binding motif revealed by solution structures of DNA-binding domains of *Arabidopsis* SBP-family transcription factors, J Mol Biol, 337 (2004) 49–63. [PubMed: 15001351]

[24]. Bernal M, Casero D, Singh V, Wilson GT, Grande A, Yang H, Dodani SC, Pellegrini M, Huijser P, Connolly EL, Merchant SS, Krämer U, Transcriptome Sequencing Identifies SPL7-Regulated Copper Acquisition Genes *FRO4/FRO5* and the Copper Dependence of Iron Homeostasis in *Arabidopsis*, Plant Cell, 28 (2012) 28.

[25]. Schulten A, Bytomski L, Quintana J, Bernal M, Krämer U, Do Arabidopsis *Squamosa promoter binding Protein-Like* genes act together in plant acclimation to copper or zinc deficiency?, Plant direct, 3 (2019) e00150. [PubMed: 31276083]

[26]. Yamasaki H, Hayashi M, Fukazawa M, Kobayashi Y, Shikanai T, *SQUAMOSA* Promoter Binding Protein-Like7 Is a Central Regulator for Copper Homeostasis in *Arabidopsis*, Plant Cell., 21 (2009) 347–361 Epub 2009 Jan 2002. [PubMed: 19122104]

[27]. Hoffman HN 2nd, Phyliky RL, Fleming CR, Zinc-induced copper deficiency, Gastroenterology, 94 (1988) 508–512. [PubMed: 3335323]

[28]. Hsieh SI, Castruita M, Malasarn D, Urzica E, Erde J, Page MD, Yamasaki H, Casero D, Pellegrini M, Merchant SS, Loo JA, The proteome of copper, iron, zinc, and manganese micronutrient deficiency in *Chlamydomonas reinhardtii*, Mol Cell Proteomics., 12 (2013) 65–86 doi: 10 1074/mcp M1112 021840 Epub 022012 Oct 021813. [PubMed: 23065468]

[29]. Hill KL, Merchant S, Coordinate expression of coproporphyrinogen oxidase and cytochrome c6 in the green alga *Chlamydomonas reinhardtii* in response to changes in copper availability, The EMBO journal, 14 (1995) 857–865. [PubMed: 7889936]

[30]. Moseley J, Quinn J, Eriksson M, Merchant S, The *Crd1* gene encodes a putative di-iron enzyme required for photosystem I accumulation in copper deficiency and hypoxia in *Chlamydomonas reinhardtii*, Embo J., 19 (2000) 2139–2151. [PubMed: 10811605]

[31]. Page MD, Kropat J, Hamel PP, Merchant SS, Two *Chlamydomonas* CTR copper transporters with a novel cys-met motif are localized to the plasma membrane and function in copper assimilation, Plant Cell., 21 (2009) 928–943 Epub 2009 Mar 2024 PMC2671701. [PubMed: 19318609]

[32]. Tottey S, Block MA, Allen M, Westergren T, Albrieux C, Scheller HV, Merchant S, Jensen PE, Arabidopsis CHL27, located in both envelope and thylakoid membranes, is required for the synthesis of protochlorophyllide, Proc Natl Acad Sci U S A, 100 (2003) 16119–16124. [PubMed: 14673103]

[33]. Hollingshead S, Kopecná J, Jackson PJ, Canniffe DP, Davison PA, Dickman MJ, Sobotka R, Hunter CN, Conserved chloroplast open-reading frame ycf54 is required for activity of the magnesium protoporphyrin monomethylester oxidative cyclase in Synechocystis PCC 6803, J Biol Chem, 287 (2012) 27823–27833. [PubMed: 22711541]

[34]. Stuart D, Hunting a gene that makes a plant green., Lund University, Sweden, 2016.

[35]. Moseley JL, Page MD, Alder NP, Eriksson M, Quinn J, Soto F, Theg SM, Hippler M, Merchant S, Reciprocal expression of two candidate di-iron enzymes affecting photosystem I and light-harvesting complex accumulation, Plant Cell, 14 (2002) 673–688. [PubMed: 11910013]

[36]. Bird AJ, Gordon M, Eide DJ, Winge DR, Repression of *ADH1* and *ADH3* during zinc deficiency by Zap1-induced intergenic RNA transcripts, The EMBO journal, 25 (2006) 5726–5734. [PubMed: 17139254]

[37]. Cheng Z, Otto GM, Powers EN, Keskin A, Mertins P, Carr SA, Jovanovic M, Brar GA, Pervasive, Coordinated Protein-Level Changes Driven by Transcript Isoform Switching during Meiosis, Cell, 172 (2018) 910–923.e916. [PubMed: 29474919]

[38]. Strenkert D, Limso CA, Fatihi A, Schmollinger S, Basset GJ, Merchant SS, Genetically Programmed Changes in Photosynthetic Cofactor Metabolism in Copper-deficient *Chlamydomonas*, J Biol Chem, 291 (2016) 19118–19131. [PubMed: 27440043]

[39]. Pape M, Lambertz C, Happe T, Hemschemeier A, Differential expression of the *Chlamydomonas* [FeFe]-hydrogenase-encoding *HYDA1* gene is regulated by the copper response regulator1, Plant Physiol, 159 (2012) 1700–1712. [PubMed: 22669892]

[40]. Glerum DM, Shtanko A, Tzagoloff A, Characterization of *COX17*, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase, J Biol Chem, 271 (1996) 14504–14509. [PubMed: 8662933]

[41]. Glerum DM, Muroff I, Jin C, Tzagoloff A, *COX15* codes for a mitochondrial protein essential for the assembly of yeast cytochrome oxidase, J Biol Chem, 272 (1997) 19088–19094. [PubMed: 9228094]

[42]. Stipanuk MH, Ueki I, Dominy JE Jr., Simmons CR, Hirschberger LL, Cysteine dioxygenase: a robust system for regulation of cellular cysteine levels, Amino acids, 37 (2009) 55–63. [PubMed: 19011731]

[43]. Stipanuk MH, Simmons CR, Karplus PA, Dominy JE Jr., Thiol dioxygenases: unique families of cupin proteins, Amino acids, 41 (2011) 91–102. [PubMed: 20195658]

[44]. Masson N, Keeley TP, Giuntoli B, White MD, Puerta ML, Perata P, Hopkinson RJ, Flashman E, Licausi F, Ratcliffe PJ, Conserved N-terminal cysteine dioxygenases transduce responses to hypoxia in animals and plants, Science (New York, N.Y.), 365 (2019) 65–69.

[45]. Howe G, Merchant S, Heavy Metal-Activated Synthesis of Peptides in *Chlamydomonas reinhardtii*, Plant Physiol, 98 (1992) 127–136. [PubMed: 16668603]

[46]. Banci L, Bertini I, Cantini F, Ciofi-Baffoni S, Cellular copper distribution: a mechanistic systems biology approach, Cellular and molecular life sciences : CMLS, 67 (2010) 2563–2589. [PubMed: 20333435]

[47]. Pope CR, Flores AG, Kaplan JH, Unger VM, Structure and function of copper uptake transporters, Current topics in membranes, 69 (2012) 97–112. [PubMed: 23046648]

[48]. Georgatsou E, Mavrogiannis LA, Fragiadakis GS, Alexandraki D, The yeast Fre1p/Fre2p cupric reductases facilitate copper uptake and are regulated by the coppermodulated Mac1p activator, J Biol Chem, 272 (1997) 13786–13792. [PubMed: 9153234]

[49]. Dancis A, Haile D, Yuan DS, Klausner RD, The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake, J Biol Chem, 269 (1994) 25660–25667. [PubMed: 7929270]

[50]. Rees EM, Lee J, Thiele DJ, Mobilization of intracellular copper stores by the CTR2 vacuolar copper transporter, J Biol Chem., 279 (2004) 54221–54229 Epub 52004 Oct 54219. [PubMed: 15494390]

[51]. De Feo CJ, Aller SG, Unger VM, A structural perspective on copper uptake in eukaryotes, Biometals., 20 (2007) 705–716 Epub 2007 Jan 2009. [PubMed: 17211682]

[52]. Blaby-Haas CE, Merchant SS, The ins and outs of algal metal transport, Biochim Biophys Acta., 1823 (2012) 1531–1552 Epub 2012 May 1531. [PubMed: 22569643]

[53]. Hanikenne M, Krämer U, Demoulin V, Baurain D, A comparative inventory of metal transporters in the green alga *Chlamydomonas reinhardtii* and the red alga *Cyanidioschizon merolae*, Plant Physiol, 137 (2005) 428–446. [PubMed: 15710683]

[54]. Sancenón V, Puig S, Mira H, Thiele DJ, Peñarrubia L, Identification of a copper transporter family in *Arabidopsis thaliana*, Plant Mol Biol, 51 (2003) 577–587. [PubMed: 12650623]

[55]. Hill KL, Hassett R, Kosman D, Merchant S, Regulated copper uptake in *Chlamydomonas reinhardtii* in response to copper availability, Plant Physiol, 112 (1996) 697–704. [PubMed: 8883382]

[56]. González-Guerrero M, Argüello JM, Mechanism of Cu+-transporting ATPases: soluble Cu+ chaperones directly transfer Cu+ to transmembrane transport sites, Proc Natl Acad Sci U S A., 105 (2008) 5992–5997 Epub 2008 Apr 5915. [PubMed: 18417453]

[57]. Williams LE, Mills RF, P_{1B}-ATPases--an ancient family of transition metal pumps with diverse functions in plants, Trends Plant Sci., 10 (2005) 491–502. [PubMed: 16154798]

[58]. Argüello JM, Eren E, González-Guerrero M, The structure and function of heavy metal transport P_{1B}-ATPases, Biometals., 20 (2007) 233–248 Epub 2007 Jan 2012. [PubMed: 17219055]

[59]. La Fontaine S, Quinn JM, Nakamoto SS, Page MD, Göhre V, Moseley JL, Kropat J, Merchant S, Copper-dependent iron assimilation pathway in the model photosynthetic eukaryote *Chlamydomonas reinhardtii*, Eukaryot Cell, 1 (2002) 736–757. [PubMed: 12455693]

[60]. Chen JC, Hsieh SI, Kropat J, Merchant SS, A ferroxidase encoded by *FOX1* contributes to iron assimilation under conditions of poor iron nutrition in *Chlamydomonas*, Eukaryot Cell, 7 (2008) 541–545 PMC2268516. [PubMed: 18245275]

[61]. Yuan DS, Stearman R, Dancis A, Dunn T, Beeler T, Klausner RD, The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake, Proc Natl Acad Sci U S A, 92 (1995) 2632–2636. [PubMed: 7708696]

[62]. El Meskini R, Culotta VC, Mains RE, Eipper BA, Supplying copper to the cuproenzyme peptidylglycine α-amidating monooxygenase, J Biol Chem, 278 (2003) 12278–12284. [PubMed: 12529325]

[63]. Steveson TC, Ciccotosto GD, Ma XM, Mueller GP, Mains RE, Eipper BA, Menkes protein contributes to the function of peptidylglycine α-amidating monooxygenase, Endocrinology, 144 (2003) 188–200. [PubMed: 12488345]

[64]. Abdel-Ghany SE, Müller-Moulé P, Niyogi KK, Pilon M, Shikanai T, Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts, Plant Cell., 17 (2005) 1233–1251 Epub 2005 Mar 1216. [PubMed: 15772282]

[65]. Blaby-Haas CE, Padilla-Benavides T, Stübe R, Argüello JM, Merchant SS, Evolution of a plant-specific copper chaperone family for chloroplast copper homeostasis, Proc Natl Acad Sci U S A, 111 (2014) E5480–5487. [PubMed: 25468978]

[66]. Hong-Hermesdorf A, Miethke M, Gallaher SD, Kropat J, Dodani SC, Barupala D, Chan J, Domaille DW, Shirasaki DI, Loo JA, Weber PK, Pett-Ridge J, Stemmler TL, Chang CJ, Merchant SS, Selective sub-cellular visualization of trace metals identifies dynamic sites of Cu accumulation in Chlamydomonas, Nat. Chem. Biol, 10 (2014) 1034–1042. [PubMed: 25344811]

[67]. Docampo R, de Souza W, Miranda K, Rohloff P, Moreno SN, Acidocalcisomes - conserved from bacteria to man, Nat Rev Microbiol., 3 (2005) 251–261. [PubMed: 15738951]

[68]. Goodenough U, Heiss AA, Roth R, Rusch J, Lee JH, Acidocalcisomes: Ultrastructure, Biogenesis, and Distribution in Microbial Eukaryotes, Protist, 170 (2019) 287–313. [PubMed: 31154072]

[69]. Ackerman CM, Lee S, Chang CJ, Analytical Methods for Imaging Metals in Biology: From Transition Metal Metabolism to Transition Metal Signaling, Analytical chemistry, 89 (2017) 22–41. [PubMed: 27976855]

[70]. Herbik A, Bölling C, Buckhout TJ, The involvement of a multicopper oxidase in iron uptake by the green algae *Chlamydomonas reinhardtii*, Plant Physiol, 130 (2002) 2039–2048. [PubMed: 12481087]

[71]. Kumar D, Blaby-Haas CE, Merchant SS, Mains RE, King SM, Eipper BA, Early eukaryotic origins for cilia-associated bioactive peptide-amidating activity, J Cell Sci, 129 (2016) 943–956. [PubMed: 26787743]

[72]. Heitzer M, Hallmann A, An extracellular matrix-localized metalloproteinase with an exceptional QEXXH metal binding site prefers copper for catalytic activity, J Biol Chem, 277 (2002) 28280–28286. [PubMed: 12034745]

[73]. Mergner J, Frejno M, List M, Papacek M, Chen X, Chaudhary A, Samaras P, Richter S, Shikata H, Messerer M, Lang D, Altmann S, Cyprys P, Zolg DP, Mathieson T, Bantscheff M, Hazarika RR, Schmidt T, Dawid C, Dunkel A, Hofmann T, Sprunck S, Falter-Braun P, Johannes F, Mayer KFX, Jürgens G, Wilhelm M, Baumbach J, Grill E, Schneitz K, Schwechheimer C, Kuster B, Mass-spectrometry-based draft of the Arabidopsis proteome, Nature, 579 (2020) 409–414. [PubMed: 32188942]

[74]. Vogel C, Marcotte EM, Insights into the regulation of protein abundance from proteomic and transcriptomic analyses, Nature reviews. Genetics, 13 (2012) 227–232.

[75]. Kropat J, Gallaher SD, Urzica EI, Nakamoto SS, Tottey S, Mason AZ, Merchant SS, Copper economy in Chlamydomonas: prioritized allocation and re-allocation of Cu from respiration to photosynthesis, Proc Natl Acad Sci U S A., 112 (2015) 2644–2651. [PubMed: 25646490]

[76]. Merchant SS, Helmann JD, Elemental economy: microbial strategies for optimizing growth in the face of nutrient limitation, Adv Microbiol Physiol, 60 (2012) 91–210. [PubMed: 22633059]

[77]. Zhang L, McSpadden B, Pakrasi HB, Whitmarsh J, Copper-mediated regulation of cytochrome c_{553} and plastocyanin in the cyanobacterium *Synechocystis* 6803, J Biol Chem, 267 (1992) 19054–19059. [PubMed: 1326543]

[78]. Ho KK, Ulrich EL, Krogmann DW, Gomez-Lojero C, Isolation of photosynthetic catalysts from cyanobacteria, Biochim Biophys Acta, 545 (1979) 236–248. [PubMed: 216399]

[79]. Ho KK, Krogmann DW, Electron-Donors to P700 in Cyanobacteria and Algae – an Instance of Unusual Genetic-Variability, Biochim, Biophys. Acta, 766 (1984) 310–316.

[80]. Durán RV, Hervás M, De La Rosa MA, Navarro JA, The efficient functioning of photosynthesis and respiration in *Synechocystis* sp. PCC 6803 strictly requires the presence of either cytochrome c_6 or plastocyanin, J Biol Chem, 279 (2004) 7229–7233. [PubMed: 14660567]

[81]. Palenik B, Morel FM, Amine oxidases of marine phytoplankton, Appl Environ Microbiol., 57 (1991) 2440–2443. [PubMed: 16348545]

[82]. Blaby-Haas CE, Merchant SS, Comparative and Functional Algal Genomics, Annual Review of Plant Biology, 70 (2019) 605–638.

[83]. Keeling PJ, The number, speed, and impact of plastid endosymbioses in eukaryotic evolution, Annu Rev Plant Biol, 64 (2013) 583–607. [PubMed: 23451781]

[84]. Blaby-Haas CE, Merchant SS, Regulating cellular trace metal economy in algae, Current opinion in plant biology, 39 (2017) 88–96. [PubMed: 28672168]

[85]. Sandmann G, Böger P, Copper-induced exchange of plastocyanin and cytochrome c-*533* in cultures of *Anabaena variabilis* and *Plectonema boryanum*, Plant Sci Lett, 17 (1980) 417–424.

[86]. Navarro JA, Hervás M, De la Rosa MA, Purification of plastocyanin and cytochrome c6 from plants, green algae, and cyanobacteria, Methods in molecular biology (Clifton, N.J.), 684 (2011) 79–94.

[87]. Sandmann G, Formation of plastocyanin and cytochrome c-553 in different species of bluegreen algae, Arch Microbiol, 145 (1986) 76–79.

[88]. Asada K, Kanematsu S, Uchida K, Superoxide dismutases in photosynthetic organisms: absence of the cuprozinc enzyme in eukaryotic algae, Arch Biochem Biophys., 179 (1977) 243–256. [PubMed: 402888]

[89]. Broxton CN, Culotta VC, An Adaptation to Low Copper in Candida albicans Involving SOD Enzymes and the Alternative Oxidase, PloS one, 11 (2016) e0168400. [PubMed: 28033429]

[90]. Shatzman AR, Kosman DJ, The utilization of copper and its role in the biosynthesis of copper-containing proteins in the fungus, Dactylium dendroides, Biochim Biophys Acta, 544 (1978) 163–179. [PubMed: 568946]

[91]. Abdel-Ghany SE, Pilon M, MicroRNA-mediated systemic down-regulation of copper protein expression in response to low copper availability in *Arabidopsis*, J Biol Chem., 283 (2008) 15932–15945 Epub 12008 Apr 15911. [PubMed: 18408011]

[92]. Nakamura M, Yamagishi M, Yoshizaki F, Sugimura Y, The syntheses of plastocyanin and cytochrome c-553 are regulated by copper at the pre-translational level in a green alga, *Pediastrum boryanum*, J Biochem., 111 (1992) 219–224. [PubMed: 1314811]

[93]. Nakamura M, Yoshizaki F, Sugimura Y, Accumulation of plastocyanin mRNA lacking 5' region in the green alga *Pediastrum boryanum* grown under copper-deficient conditions, Plant Cell Physiol., 41 (2000) 33–41. [PubMed: 10750706]

[94]. Li HH, Merchant S, Two metal-dependent steps in the biosynthesis of *Scenedesmus obliquus* plastocyanin. Differential mRNA accumulation and holoprotein formation, J. Biol. Chem., 267 (1992) 9368–9375. [PubMed: 1577764]

[95]. Terauchi AM, Lu SF, Zaffagnini M, Tappa S, Hirasawa M, Tripathy JN, Knaff DB, Farmer PJ, Lemaire SD, Hase T, Merchant SS, Pattern of expression and substrate specificity of chloroplast ferredoxins from *Chlamydomonas reinhardtii*, J Biol Chem., 284 (2009) 25867–25878 Epub 22009 Jul 25867. [PubMed: 19586916]

[96]. Bartlett A, O'Malley RC, Huang SC, Galli M, Nery JR, Gallavotti A, Ecker JR, Mapping genome-wide transcription-factor binding sites using DAP-seq, Nature protocols, 12 (2017) 1659–1672. [PubMed: 28726847]

[97]. Strenkert D, Schmollinger S, Sommer F, Schulz-Raffelt M, Schroda M, Transcription factor-dependent chromatin remodeling at heat shock and copper-responsive promoters in *Chlamydomonas reinhardtii*, Plant Cell, 23 (2011) 2285–2301. [PubMed: 21705643]

[98]. Anbar AD, Oceans. Elements and evolution, Science (New York, N.Y.), 322 (2008) 1481–1483.

[99]. Papadopoulos JS, Agarwala R, COBALT: constraint-based alignment tool for multiple protein sequences, Bioinformatics, 23 (2007) 1073–1079. [PubMed: 17332019]

[100]. Price MN, Dehal PS, Arkin AP, FastTree: computing large minimum evolution trees with profiles instead of a distance matrix, Molecular biology and evolution, 26 (2009) 1641–1650. [PubMed: 19377059]

[101]. Miller MA, Schwartz T, Pickett BE, He S, Klem EB, Scheuermann RH, Passarotti M, Kaufman S, O'Leary MA, A RESTful API for Access to Phylogenetic Tools via the CIPRES Science Gateway, Evolutionary bioinformatics online, 11 (2015) 43–48. [PubMed: 25861210]

[102]. Letunic I, Bork P, Interactive Tree Of Life (iTOL) v4: recent updates and new developments, Nucleic acids research, 47 (2019) W256–w259. [PubMed: 30931475]

[103]. Boyle NR, Page MD, Liu B, Blaby IK, Casero D, Kropat J, Cokus SJ, Hong-Hermesdorf A, Shaw J, Karpowicz SJ, Gallaher SD, Johnson S, Benning C, Pellegrini M, Grossman A, Merchant SS, Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen

starvation-induced triacylglycerol accumulation in Chlamydomonas, J Biol Chem, 287 (2012) 15811–15825 Epub 12012 Mar 15818. [PubMed: 22403401]

[104]. Katoh K, Toh H, Parallelization of the MAFFT multiple sequence alignment program, Bioinformatics, 26 (2010) 1899–1900. [PubMed: 20427515]

[105]. Wiśniewski JR, Hein MY, Cox J, Mann M, A "proteomic ruler" for protein copy number and concentration estimation without spike-in standards, Molecular & cellular proteomics : MCP, 13 (2014) 3497–3506. [PubMed: 25225357]

Highlights

1. Plastocyanin replacement by a cytochrome is a key copper sparing response in algae.
2. 60+ genes are up-regulated in copper-deficiency by a transcriptional activator, CRR1.
3. Up-regulated genes include transporters, redox proteins, and chloroplast enzymes.
4. CRR1 down-regulation works through 5’ extended UTRs that are poorly translated.
5. CYC6, CRR1, and multiple forms of CRD1/CHL27 are found in many algae.

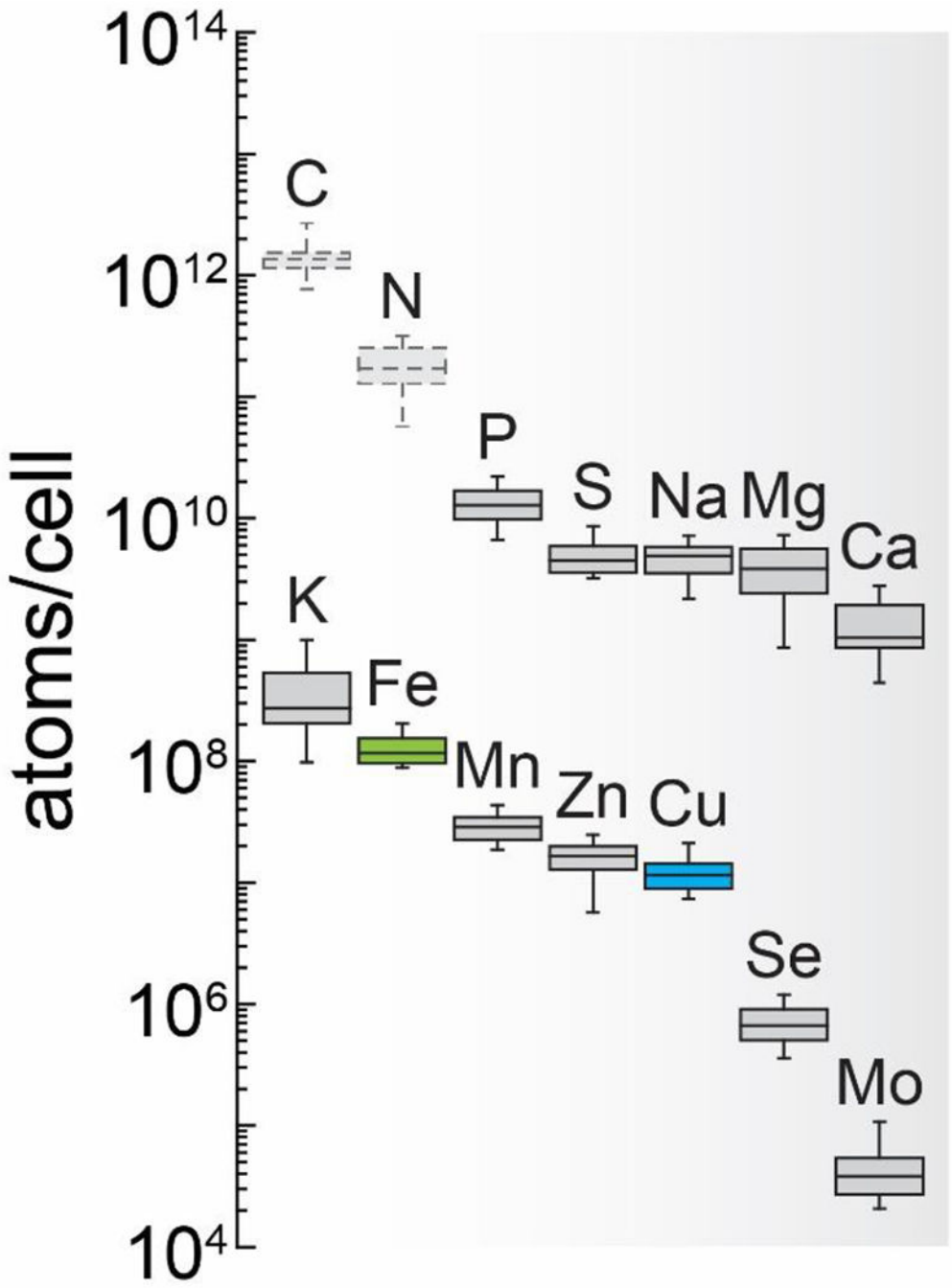

Figure 1: The elemental composition of Chlamydomonas.

Laboratory-grown wild-type cells were grown in nutrient-replete conditions. C and N were determined on a Shimadzu TOC/TN analyzer (dashed outline), and other elements by ICP-MS/MS on an Agilent 8800 (solid outline). A total of 12 samples was analyzed from wild-type Chlamydomonas laboratory strain CC-4532. The distribution is summarized in boxplots, where each horizontal line represents the 10, 25, 50, 75 and 90% quantile (from bottom to top).

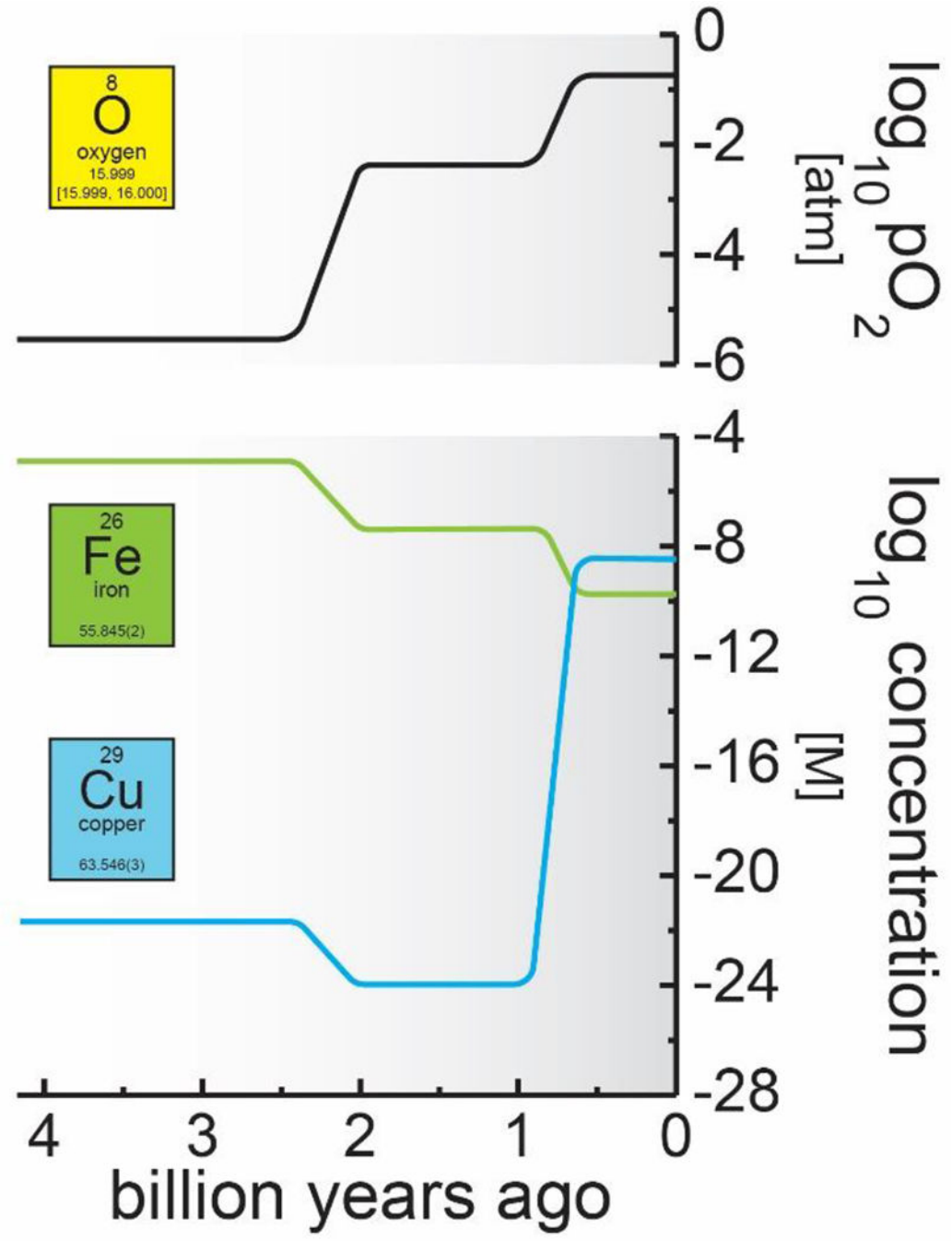

Figure 2: Increased bio-availability of copper in an oxygenated biosphere.
Elemental estimates are based on the chemical characteristics of ancient sediments [98].

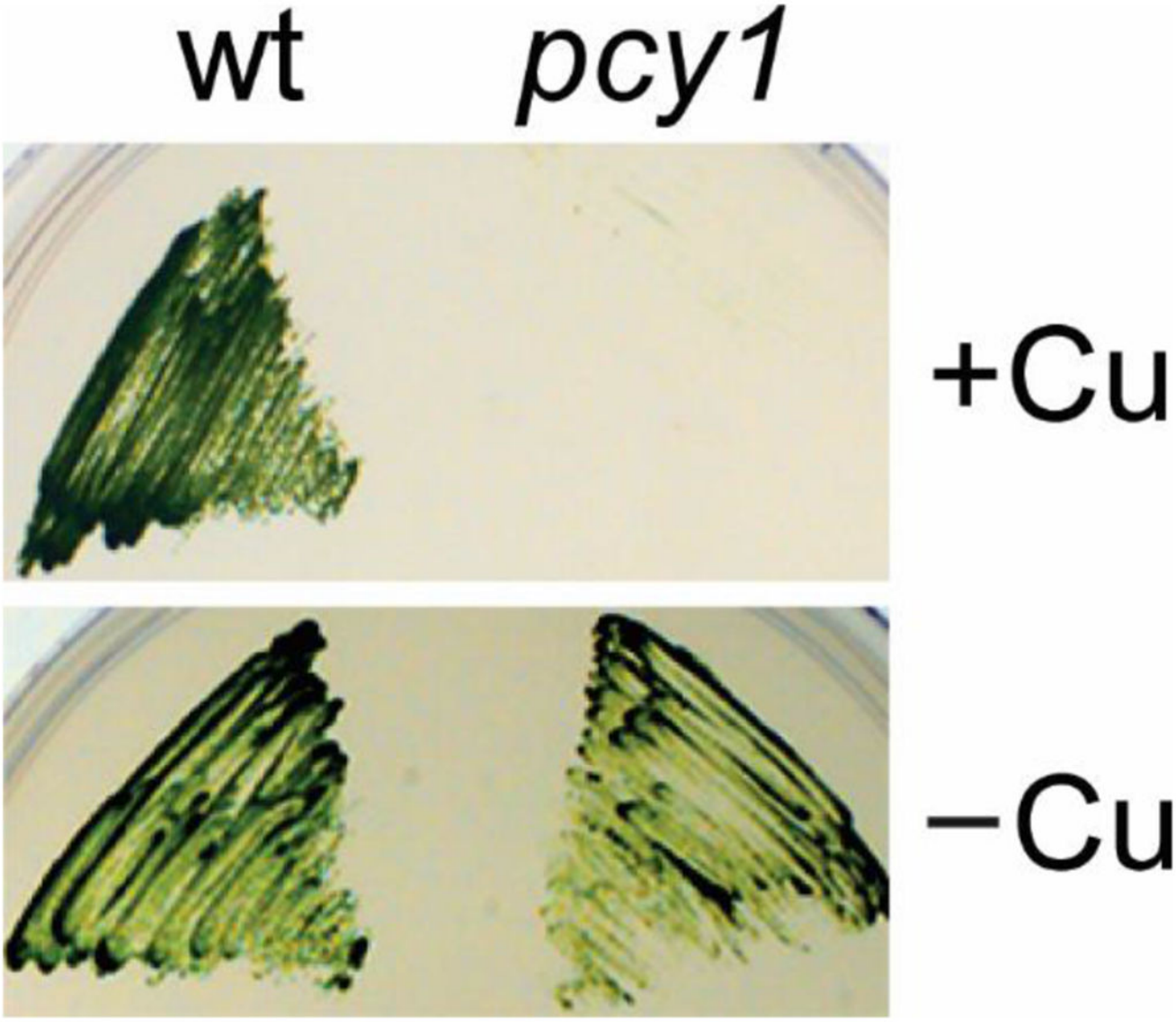

Figure 3: Copper conditional phenotype of a plastocyanin-deficient mutant.

Algal cells were grown photoautotrophically on plates supplemented with copper (+Cu) or not (−Cu). wt = wild-type for plastocyanin synthesis, *pcy1* = no synthesis of plastocyanin because of a frame-shift mutation [9].

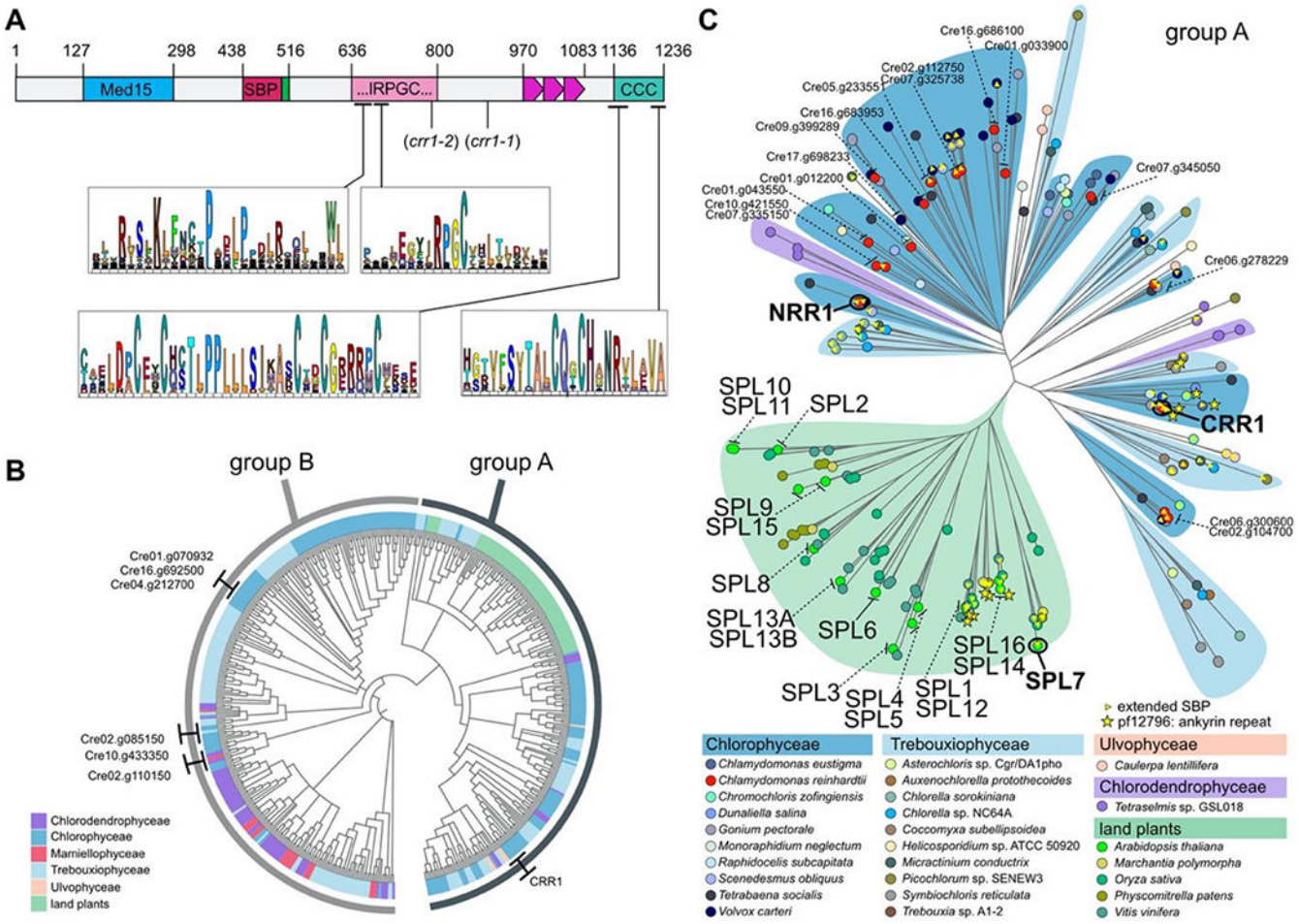

Figure 4: CRR1 schematic and family analysis.

A, Putative functional domains discussed in the text are indicated. Med15 = blue, SBP = dark pink, extended SBP = light pink, ankyrin repeats = purple, C-terminal Cys-rich region = turquoise, green= nuclear localization signal. The numbers on the top indicate amino acid positions in CRR1. The position of mutations in *crr1* mutants are indicated. Sequence logos for conserved motifs identified within the extended SBP region and their locations are shown. Homologs containing these motifs are highlighted in panel C with a triangle. The Cys-rich region resembles metallothionein and is found also in CRR1 from *Chlamydomonas eustigma, Volvox carteri, Gonium pectorale, Tetrabaena socialis, Raphidocelis subcapitata, Tetradesmus obliquus* and *Chromochloris zofingiensis.* Two conserved motifs identified from this region are shown as sequence logos. B, Phylogenetic tree of the SPB domain from green algae and land plants. Protein sequences were aligned using NCBI's Cobalt aligner [99]. An edited alignment encompassing just the SBP domain was used to build a phylogenetic tree with FastTree [100], using 1000 bootstrap replicates, on the CIPRES Science Gateway [101]. The tree was visualized with iTol [102] and branches with a bootstrap value less than 0.5 were deleted. Branch lengths are ignored. Leaves are colored by taxonomy according to legend. Based on this analysis, the family can be divided into two groups. Group B only contains algal sequences, while group A contains algal homologs (with the exception of prasinophytes) and land plant homologs. Chlamydomonas homologs

in group B and CRR1 are labeled. Chlamydomonas homologs from group A can be found in panel C. C, Unrooted phylogenetic tree of sequences from group A, which contains CRR1 from Chlamydomonas and SPL7 from Arabidopsis, as well as the previously-characterized nitrogen response regulator, NRR1 [103], from Chlamydomonas. Full-length sequences from group A were realigned using MAFFT [104] then the full-length alignment was used to build a phylogenetic tree as in panel B. Each circle represents an organism as indicated in the legend and the background is colored by taxonomy.

protein	proteins (10^6/cell) +Cu	-Cu	Cu (n)	Cu/protein (10^6) +Cu	-Cu
plastocyanin	5.4	0.20	1	5.4	0.20
Cyt oxidase	0.27	0.08	3	0.8	0.24
ferroxidase	0.07	0.01	6	0.4	0.07

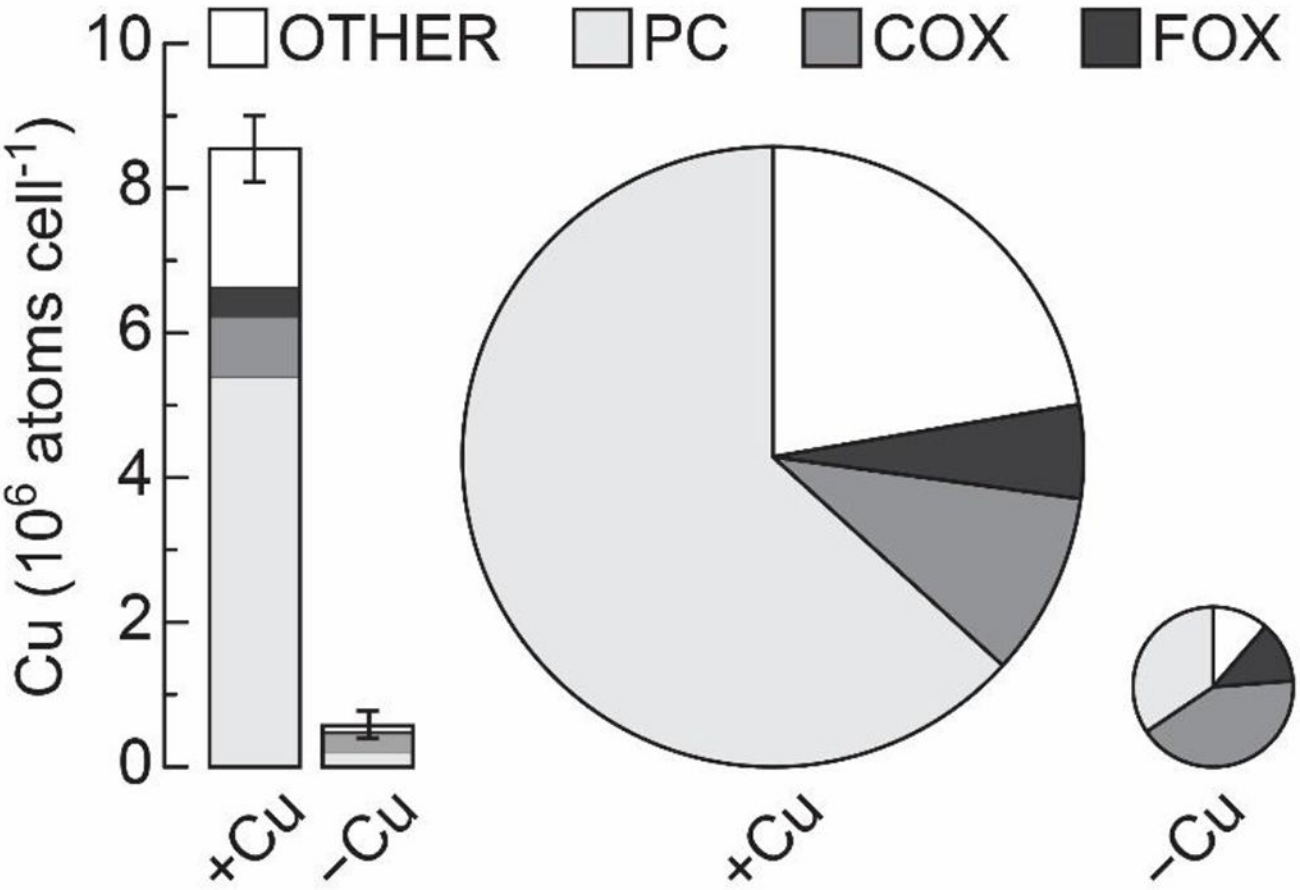

Figure 5 –. Altered distribution of copper depending on copper nutrition status.

The copper content of Chlamydomonas cells in replete vs. deficient medium was measured by ICP-MS. The abundances of copper proteins (in units of millions of molecules per cell) were estimated (left hand side of table) by quantitative proteomics using the proteomic ruler method [105]. The contribution of each protein to the copper quota was derived based on the number of copper binding sites (middle column) in each protein to yield the amount of copper (in millions of atoms) associated with that protein in each cell (right hand side of table). The distribution of total cellular copper (from ICP-MS) amongst the 3 most abundant cuproproteins (light grey = plastocyanin (PC), grey = Cyt oxidase (COX) and dark grey = ferroxidase (FOX)) in Chlamydomonas is presented within the bar and also as a pie chart

where the area reflects the amount of copper associated with each protein, and white represents the balance. Note that the cells were grown in iron-replete conditions, where the ferroxidase abundance is low.

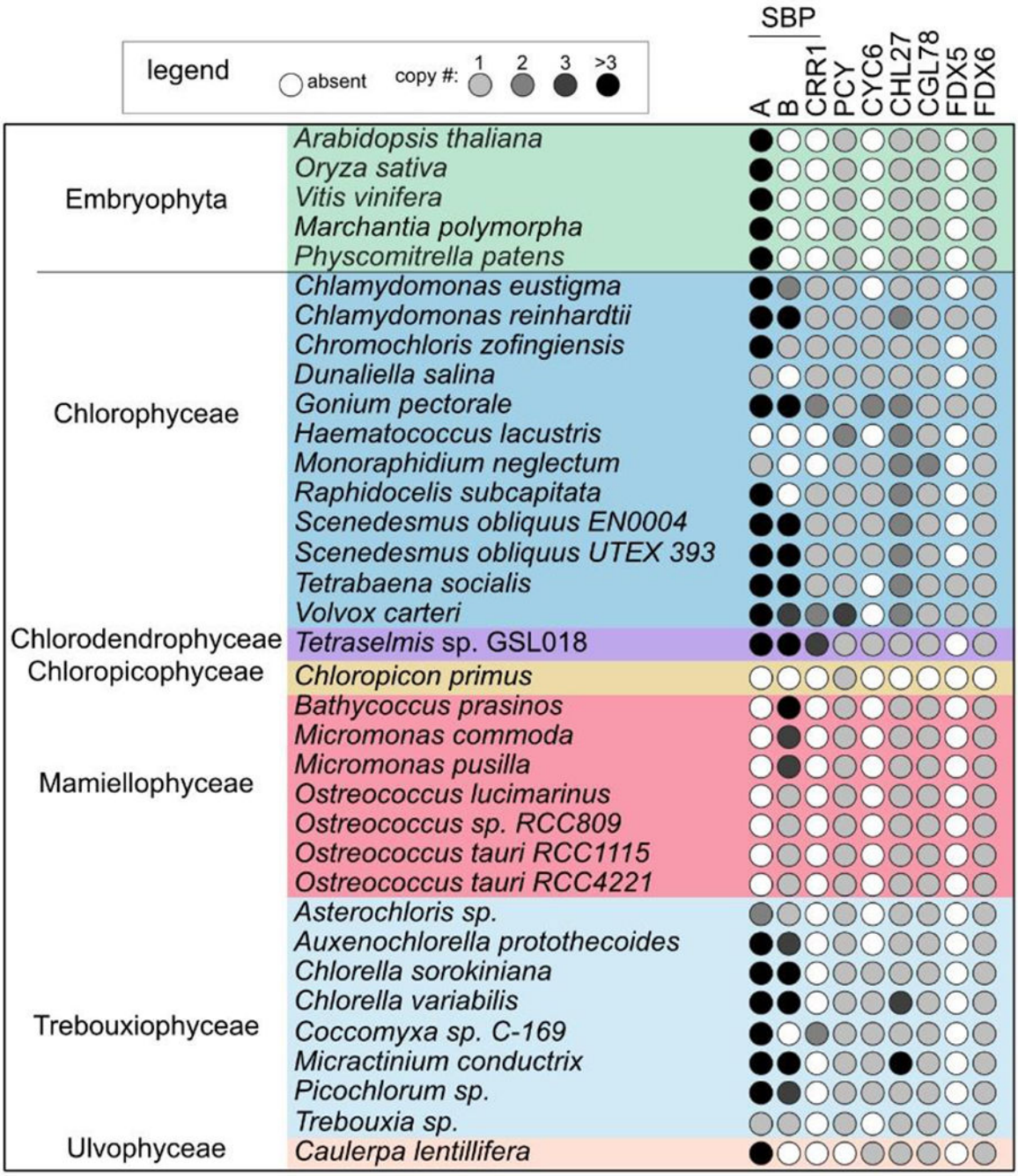

Figure 6. Co-occurrence of SBP-domains, CRR1 and Cu-deficiency targets in plant and green algal proteomes.

Proteins were predicted from public whole genome sequencing projects (as of June 2020). Assignments were based on reciprocal best hits with the *Chlamydomonas reinhardtii* homologs. In many cases faulty or absent gene models were corrected by searching the genome with tblastn and using comparisons with homologs from closely related species. Open circles signify the absence of a gene/protein. Absence of a protein may mean that it was never there or never acquired, or that it was lost, or that the genome assembly is incomplete. Copy # of paralogs within a species is indicated within the filled circles using a

grey scale. The “CRR1” designation in the figure was based on prediction of CRR1 orthologs using the tree in Figure 4C. However, we note that not all orthologs share the same motifs with CRR1, such as the extended SBP domain or the ankyrin motifs. These absence could be due to incorrect gene models or functional divergence. SBP Group A and Group B proteins may be functional homologs of CRR1 in other algae, as is the case for SPL7 in Arabidopsis. Cu-deficiency targets include *PCY1*, encoding a blue copper protein that transfers electrons from cytochrome b_6f to oxidized PSI. Heme-containing cyt c_6 (encoded by *CYC6)* functionally substitutes for PCY1 in Cu-deficient cells. In Chlamydomonas, the genes for Mg-protoporphyrin IX monomethylester cyclase components CHL27 (CTH1 & CRD1 parologs) and CGL78 are all regulated by CRR1 in response to Cu-availability. FDX5 and FDX6, both of whose genes are regulated by CRR1, may provide electrons for the cyclase reaction. Potential pseudogenes resulting from gene fusions, deletions, inversions and frame-shifts are included in the CHL27 copy # for *Monoraphidium neglectum, Raphidocelis subcapitata, Chlorella variabilis* and *Micractinium conductrix.* Abbreviations and gene product names: A, SBP Group A proteins; B, SBP Group B proteins; CRR1, copper response regulator; PCY1, plastocyanin; CYC6, cytochrome c_6; CHL27, Mg-protoporphyrin IX monomethylester cyclase; CGL78, conserved in green lineage 78 (component of the Mg-protoporphyrin IX monomethylester cyclase); FDX5, ferredoxin 5; FDX6, ferredoxin 6.