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Regulating cellular trace metal economy in algae

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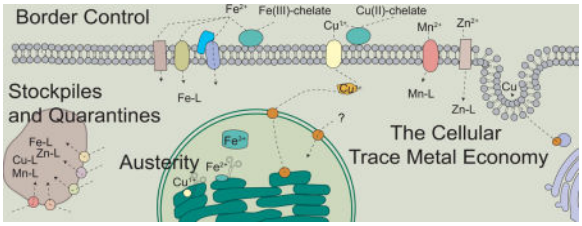
Abstract

As indispensable protein cofactors, Fe, Mn, Cu and Zn are at the center of multifaceted acclimation mechanisms that have evolved to ensure extracellular supply meets intracellular demand. Starting with selective transport at the plasma membrane and ending in protein metalation, metal homeostasis in algae involves regulated trafficking of metal ions across membranes, intracellular compartmentalization by proteins and organelles, and metal-sparing/recycling mechanisms to optimize metal-use efficiency. Overlaid on these processes are additional circuits that respond to the metabolic state as well as to the prior metal status of the cell. In this review, we focus on recent progress made toward understanding the pathways by which the single-celled, green alga *Chlamydomonas reinhardtii* controls its cellular trace metal economy. We also compare these mechanisms to characterized and putative processes in other algal lineages. Photosynthetic microbes continue to provide insight into cellular regulation and handling of Cu, Fe, Zn and Mn as a function of the nutritional supply and cellular demand for metal cofactors. New experimental tools such as RNA-Seq and subcellular metal imaging are bringing us closer to a molecular understanding of acclimation to supply dynamics in algae and beyond.

Graphical abstract



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Keywords

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Introduction

Economics of metal ion metabolism

Homeostasis is the property of a biological system to regulate its internal environment at or near a steady-state as the external environment varies. For the trace metal nutrients Zn, Cu, Mn and Fe, homeostasis is the process by which organisms maintain a steady-state level of metal ion availability inside the cell as the abundance and availability of metal ions varies outside the cell. It is important to note that metal ion availability inside the cell is not synonymous with a cell's metal quota, which is defined as the total metal content of a cell. Because of luxury consumption, the ability to compartmentalize excess ions, the presence of metal-sparing/recycling strategies during sustained metal limitation, and changing metabolic requirements for metal ions, the metal quota can vary [1–11]. Metal homeostasis embodies a multitude of mechanisms that each act to ensure the cellular demand for metal ions is met. Supply and demand is one of the most fundamental concepts of economics, and it forms the foundation of cellular metal economy.

Definitions and scenarios

Supply refers to the concentration/amount of a metal ion or metal-chelate that is nutritionally available for uptake. Demand refers to how many proteins in the cell require that metal ion to function – resulting in a baseline or minimal cellular quota for that metal (metal/cell). For microorganisms, the ideal situation of supply being equal to demand is rare. The availability of metal ions in the environment can increase or decrease as can the cellular demand for that metal. For instance, acclimation to a change in carbon or nitrogen source may involve de novo synthesis (or degradation) of abundant metalloproteins resulting in a change in cellular quota for that metal [12,13]. Therefore, metal economy involves consideration of the environmental variable (supply) and also the metabolic/developmental variable (demand). Maintaining homeostasis requires multiple regulatory loops involving selective uptake in response to changing nutrition (or in the real world, competition for nutrients), intracellular distribution of the metal nutrient, and expression of metalloproteins (demand). Overlaid on the regulatory scheme are additional circuits dependent on the prior history of the cell. For instance, if a prior period of selective storage occurred because supply exceeded demand, mobilization from such reserves might be the first level response. Without metal economy, organisms would lose the flexibility to occupy varied niches, respond to other organisms (such as host-pathogen or host-symbiont interactions), or develop in response to environmental or biotic cues (Figure 1A). Most organisms, but microbes in particular, have evolved mechanisms to thrive within the range of feast and famine scenarios (Figure 1B).

Border Control

Some of the most important players in intracellular metal economy are membranes and the transporters embedded within them. Together they create a selective barrier that separates a

potentially capricious source of nutrients in the environment or growth milieu and the controlled chemical reactions within the cell. Genes encoding candidate metal transporters for Cu, Zn, Fe and Mn have been identified in the *C. reinhardtii* genome by several techniques, including sequence similarity to previously characterized transporters, differential expression (Figure 3) and reverse genetics (reviewed in [14]). These transporters belong to a mix of protein families with origins in various lineages and unique phylogenetic occurrences (Figure 2). For instance, Fe uptake at the plasma membrane occurs by a reduction-oxidation-transport mechanism, involving FRE1 (Fe reductase), FOX1 (Fe oxidase) and FTR1 (permease) [15–19], which is found in other algae, such as rhodophytes and diatoms [20], but absent from most land plants and prasinophytes [21,22] (Figure 2A). Additional components of high-affinity Fe uptake include the soluble secreted proteins FEA1 and FEA2, which are only found in the algal lineages [17,23,24]. The Cu-ATPases encoded in the *C. reinhardtii* genome reflect the origin of metalloproteins to whom they supply Cu. For instance, in the green lineage two Cu-ATPases (PAA1 and PAA2 in Arabidopsis) and a Cu chaperone (PCH1 in Arabidopsis) supply Cu to plastocyanin within the chloroplast [25]. This intracellular Cu transport pathway only exists in algae that contain plastocyanin; red algae constitutively express cytochrome c_6 (requires heme instead of Cu) and orthologs of PAA1, PAA2 and PCH1 are missing (Figure 4B). Interestingly, the diatoms *Thalassiosira oceanica* and *Fragilariopsis cylindrus* and the haptophyte *Emiliana huxleyi* have plastocyanin [26,27], but like red algae, which are the modern relatives of the engulfed alga that became their plastid, these algae are also missing orthologs of PAA1, PAA2 and PCH1, suggesting a different pathway exists to metalate plastocyanin.

Stockpiles and quarantines

It is advantageous to bank or hoard a limiting resource when supplies of the resource wax and wane, and the same is true for the essential trace metals. They are essential because each metal has unique chemical properties that render it irreplaceable as a catalyst (Figure 4): for example, Mn in photosystem II, Fe in the Fe-S centers of photosystem I, and Cu in plastocyanin. If a metal ion is unavailable for synthesis of metalloproteins, and at least one of those metal-dependent proteins is essential [28] (such as RNA polymerase (Zn) [29,30], Rli1p (Fe) [31,32], the linear photosynthetic electron transport chain (Fe, Mn and Cu) during photoautotrophic growth, or respiratory electron transport chain (Fe and Cu) during heterotrophic growth), cell viability is lost. The capacity for storage offers a distinct selective advantage.

The best understood storage mechanism in algae is ferritin for Fe. In *C. reinhardtii*, as in land plants, ferritin is located in the chloroplast, and this is likely true in other algae and diatoms as well [33,34]. Given the location of ferritin in the chloroplast and the abundance of Fe in the photosynthetic complexes, a physiological role for ferritin in mediating Fe availability to those complexes during various developmental stages and stress conditions occurs both on land and in the oceans (reviewed in [35]). However, the regulation and roles of ferritin in Fe homeostasis differs. In Arabidopsis, ferritin expression is induced by a supply of excess Fe, and a role in storage and protection has been prescribed [36]. In *C. reinhardtii*, expression of two ferritins Fer1 (recovered in soluble fraction) and Fer2 (recovered in particulate fraction) is increased during Fe limitation [34]. Instead of Fe-

storage, these ferritins are proposed to mediate an Fe-sparing/recycling process involving degradation of the Fe-rich photosystem I (PSI) complex [37]. The *Ostreococcus tauri* ferritin appears to play a similar role in buffering Fe in the chloroplast (rather than storage) as a function of the day/night cycle [22,38]. Although ferritin is central to iron homeostasis in both green algae and diatoms [39], it is not found in all algal genomes (Figure 4B).

The acidic vacuole, or acidocalcisome, is less understood but potentially of broader relevance to metal metabolism in algae (Figure 4B). The acidocalcisome, first characterized in trypanosomatids, is a storage organelle that contains polyphosphate, pyrophosphate, calcium and divalent metal ions, such as Fe and Zn, and appears as an electron-dense granule in transmission electron micrographs [40]. Acidocalcisomes share properties with lysosomes and lysosome-related organelles from mammalian cells and, like the plant vacuole, contain both V-type ATPases and V-type pyrophosphatases, which acidify the interior. In addition to storage, these compartments can quarantine potentially toxic metals such as cadmium [41] and are expected to play a significant role in balancing intracellular accessibility of essential and non-essential (even deleterious) metal ions in *C. reinhardtii* and other algae [42]. Indeed, acidic vacuoles appear to be waypoints for Fe in *Dunaliella salina* [43] and recent investigation of unexpected Cu hyper-accumulation during zinc starvation [44] has revealed a role of lysosome-like organelles in *C. reinhardtii* in Cu homeostasis. These compartments trap Cu in a bio-unavailable state concurrent with the induction of the well-characterized Cu-deficiency response [45]. The Cu does not stay ensnared forever. When Zn is resupplied, the Cu-response is turned off, the size and number of bodies decreases and Cu is redistributed to Cu-dependent proteins [45], demonstrating that the acidocalcisome is not a trash can, but rather a storage silo. The purpose of this Cu stockpiling during Zn starvation is not yet known, but one attractive hypothesis is that this phenomenon serves to protect Zn-dependent proteins from mismetallation by the highly competitive Cu ion [46].

Austerity

When unable to import sufficient supply of a particular metal ion and if the stockpiles are depleted, *C. reinhardtii* and other algae initiate austerity measures. The best characterized austerity responses in algae are metal nutrition responsive accumulation of electron-transfer proteins in the chloroplast: plastocyanin (Cu-containing) replaced by cytochrome c_6 (Fe-containing) as a function of Cu availability and ferredoxin (Fd, Fe-containing) replaced by flavodoxin (Fld, flavin-containing, Fe-independent) as a function of Fe availability [49] (Figure 4B). Note that the specific mechanism by which Cu-responsive regulation is achieved can differ between algae [47,48]. While these metal-sparing mechanisms permit a reduction in the cellular Cu and Fe quota, respectively, recently plastocyanin was found to serve as an intracellular stockpile of Cu [8], and suggests that in those algae that contain Fd and Fld, Fd is a reservoir for Fe that can be recycled after the switch to Fld. When faced with Cu starvation, Cu can be mobilized from plastocyanin and transferred to the mitochondrion, where it is used to sustain the activity of cytochrome *c* oxidase, an irreplaceable component of the respiratory electron transfer chain. If plastocyanin were lost in the green algal lineage and constitutively replaced by cytochrome c_6, these organisms would also lose a copper

reservoir. Plastocyanin therefore provides an ingenious mechanism for algae to compete with other copper-utilizing organisms in their environment.

Sometimes austerity and reduced quota takes place without direct functional replacement. During mixotrophic growth (when the cells are supplied with air, light and acetate), *C. reinhardtii* down-regulates the abundance of Fe-containing complexes of the photosynthetic chain, in particular PSI, cytochrome b_6f and Fd [50–52]. The cell sacrifices photosynthetic capacity in favor of heterotrophic oxygen-dependent metabolism with a reduced Fe quota: Fe is recycled from the photosynthetic complexes to Fe-dependent superoxide dismutase within the chloroplast and to respiratory complexes in the mitochondria [37,52,53]. However, because Fd is involved in indispensable processes, a low level is maintained [51]. Transcriptome and proteome studies have provided evidence of similar metal-sparing events, whereby the demand for the limiting nutrient is decreased without necessarily providing a replacement [54].

Sensing and responding

At the center of a healthy metal economy is the ability to sense changes and respond quickly and appropriately to accommodate those changes. Understanding the mechanistic details of how *C. reinhardtii* (and other eukaryotic microbes) senses an imbalance between intracellular metal availability and the number of proteins requiring that metal ion is an active area of research with much to be discovered. Indeed, the mechanisms responsible for sensing metal status and adjusting gene expression or protein activity are largely unknown. A few exceptions do exist, which highlight the complexity of regulating a balance between supply and demand and underscore the regulatory mechanisms that remain to be discovered.

The Cu-response regulator CRR1

CRR1 is a transcription factor with a DNA binding domain (named SBP for the prototype identified as a *S*QUAMOSA promoter binding protein) that is unique to the plant lineage [55]. Its SBP domain recognizes the GTAC core of copper response elements (CuREs) found within the promoters of roughly 60 *C. reinhardtii* genes [56]. CRR1 regulates Cu-responsive processes such as the plastocyanin/cytochrome c_6 switch, Cu salvage from plastocyanin, and Cu assimilation (CTR genes) [8,56]. Other targets are involved in subtle modifications of the photosynthetic apparatus, such as desaturation of thylakoid membrane galactolipids and changes in minor Chl-protein abundances, which may be required to accommodate the switch to Cyt c_6 [56,57]. Remarkably, this transcription factor is also involved in the hypoxia-, Ni- and Zn-sensing pathways [58,59,60], but the mechanisms behind these regulatory pathways are yet to be discerned.

Identifying proteins involved in Fe-responsive regulation

A multitude of enzymes in *C. reinhardtii* require Fe as a catalytic cofactor [61] and, as discussed above, Fe-responsive gene regulation is different depending on trophic status. Therefore, whereas a single central transcription factor has been found at the heart of Cu-dependent gene regulation, Fe sensing and signaling is expected to be more complex. Some aspects are being uncovered, such as the identification of TAA1 (TRANSLATION OF

psaA1), which is proposed to be involved in down-regulation of PSI during Fe starvation [62]. How TAA1, in turn, is regulated by Fe status is unknown. Proteins potentially involved in *FOX1* expression have also been identified through a genetic screen assaying absence of *FOX1*-promoter-arylsulfatase fusion activity during Fe starvation. The corresponding loci encode a putative protein kinase [63] and a RING-type Zn finger protein that contains a Ran-binding domain (Ran regulates receptor-mediated transport between the nucleus and cytoplasm) [64]. However, it remains unknown whether these two proteins are directly involved in Fe-responsive signaling. Indeed, the experimentally determined consensus sequence for the RING-finger protein is not found in the promoter region of *FOX1*.

Identifying proteins involved in Zn- and Mn-responsive regulation

Transcriptome sequencing of *C. reinhardtii* cells grown with or without Zn suggests a specific transcriptional Zn response involving ZIP-family transporters and members of the COG0523 family of putative Zn chaperones [44]. While CRR1 is clearly involved at some level based on analysis of *CRR1* mutants [58], how CRR1 regulates Zn-responsive gene expression and the identity of other transcription factors is currently unknown. In contrast, transcriptome sequencing of Mn-limited cells has not revealed a specific Mn-response bringing to question whether a Mn-specific transcription factor exists in *C. reinhardtii* (Castruita and Merchant, unpublished).

Conclusions

While significant progress has been made in identifying the means by which *C. reinhardtii* and other algae have evolved to regulate their cellular trace metal economy, many questions remain. Transporters encoded in the genome are routinely identified by homology-based techniques and the response of those transporters' expression to metal status is easily quantified, but the intracellular location of most metal transporters is unknown. This information is central to understanding function. For instance, an importer localized to the plasma membrane has a different function (assimilate metal ions) from a homologous transporter in the vacuolar membrane (liberate metal store). Ongoing development of a genome-wide library of *C. reinhardtii* gene disruptants [65] and the characterization of mutants defective in metal homeostasis are expected to yield considerable insight, especially for those proteins, such as membrane-bound transporters, that are notoriously difficult to characterize *in vitro*. Reverse genetics by inducible artificial miRNAs [66] and fluorescent protein tags [67,68] combined with subcellular metal imaging by live cell imaging and imaging mass spectrometry also promises to generate insight into the molecular mechanisms underlying subcellular metal speciation and compartmentalization. For instance, given the disparate observations of vacuole-like compartmentalization for both essential and non-essential metal ions, how is selectivity determined? Do different compartments have different compositions in terms of transporter profile, ligands and element content? In addition, recent studies with *Ostreococcus* have revealed diel rhythms for Fe and Zn quotas [38,69], further highlighting our lack of understanding concerning how these metals are sensed and regulated. In general, (with the exception of CRR1) the proteins responsible for linking metal status to gene/protein expression are unknown. In some cases, these networks

are expected to be complex as is the case for regulatory circuits that enable Fe-responsive gene expression as a function of carbon metabolism in *C. reinhardtii*.

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Highlights

- Trace metal economy: border control, stockpiles, quarantines, and austerity
- Compartmentalized metal sequestration and storage is dynamic
- The molecular details of metal-status sensing in algae remain to be detailed

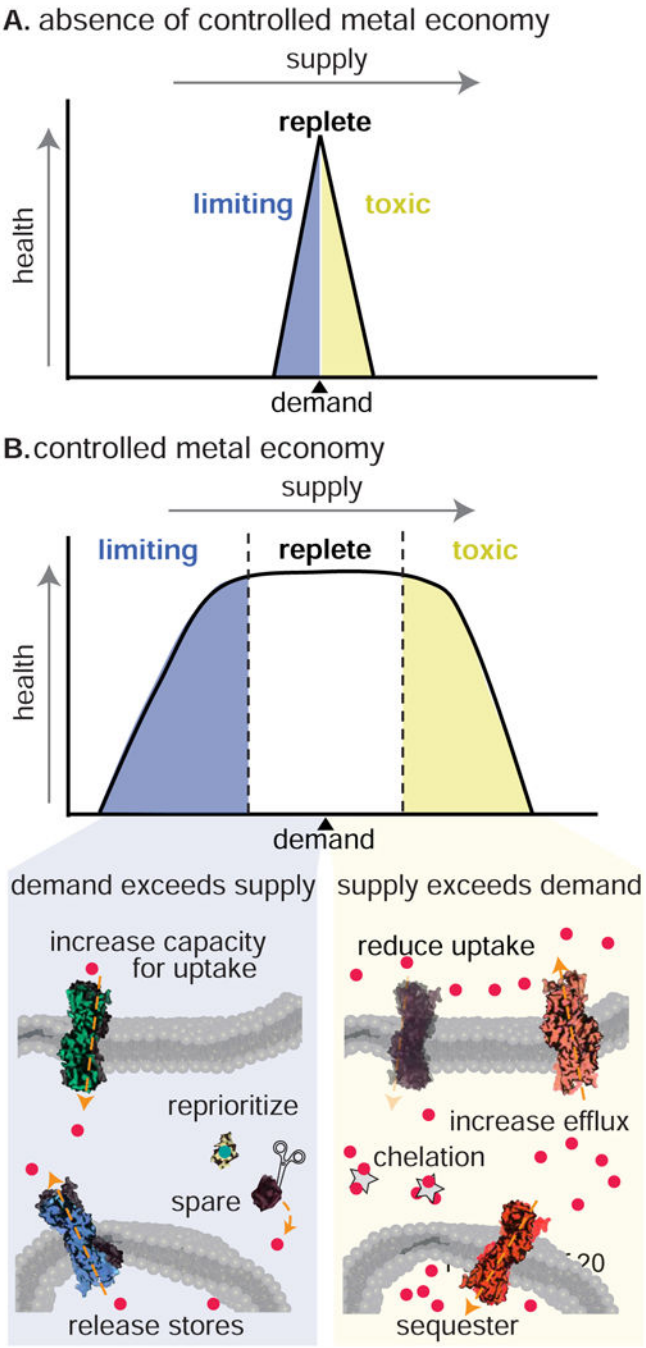

Figure 1. Controlled metal economy

Organisms have evolved to respond to and control their interaction with nutrients supplied by the environment. Metal ions such as Cu, Fe, Zn and Mn provide a particular challenge in that they are often required as essential cofactors for indispensable functions within the cell. Therefore, each organism has to ensure that enough of these metals are concentrated within the cell in the face of supply fluctuations and possible starvation. Nevertheless, if allowed to accumulate in excess within the cell, these ions can cause damage through deleterious reactions with macromolecules, particularly in the presence of oxygen. Each cell must strike

a balance between the essential and potentially toxic nature of these elements. In the absence of controlled metal economy (A), organisms would be restricted to life in a static utopian environment where supply of these nutrients perfectly matches the cellular requirement. Instead, organisms have evolved to control their metal economy (B) through a collection of processes that serves to tolerate nutrient fluctuations and quickly acclimate to cellular demands.

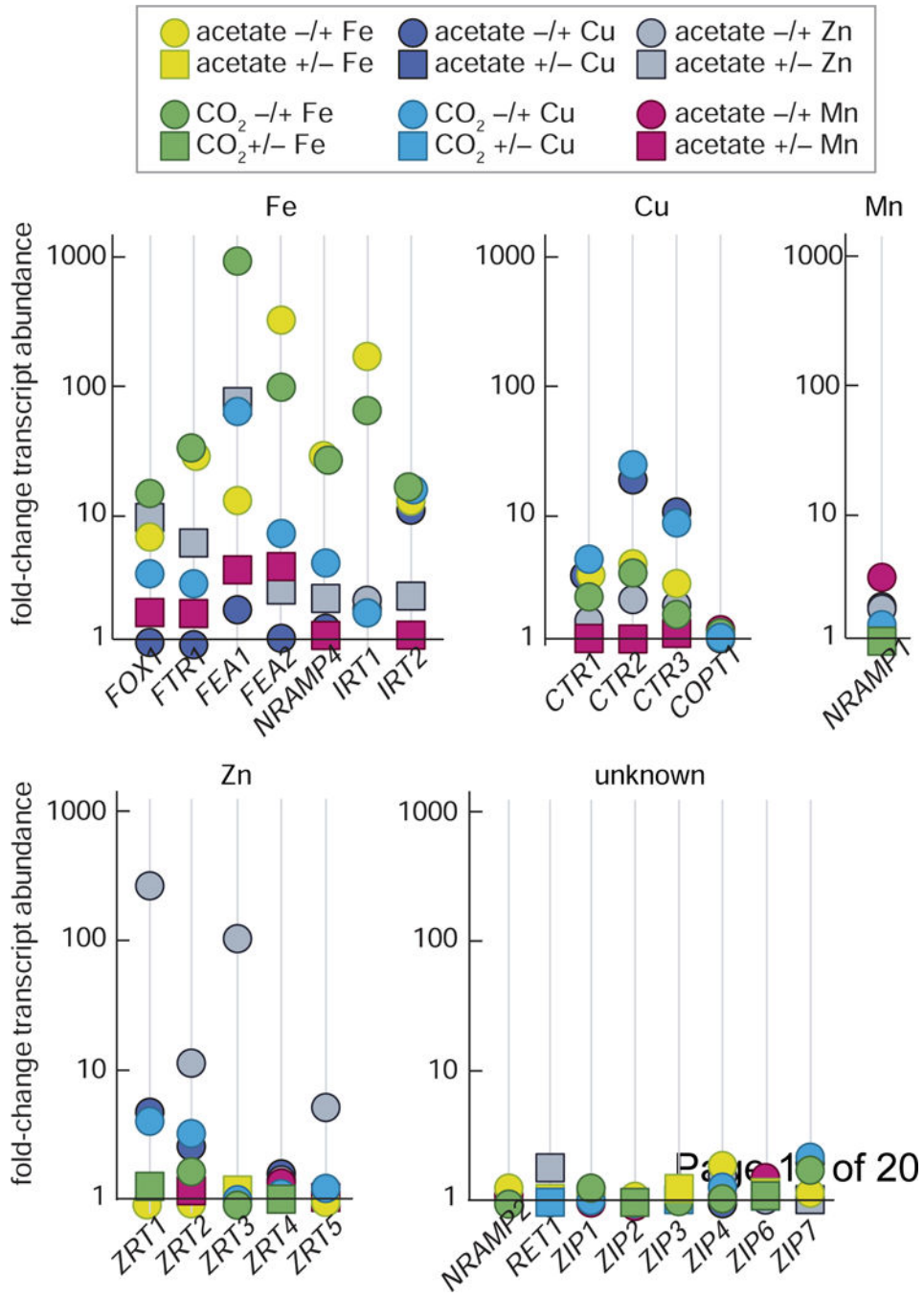

Figure 2. Metal-nutrition-specific transcript abundance of transporters involved in border control

Fold-change in transcript abundance for known and candidate transition metal transporters encoded in the *C. reinhardtii* genome. Data compiled from Castruita, et al. [56], Urzica, et al. [70], Malasarn, et al. [44] and Castruita and Merchant, unpublished. Each data point represents the fold-change in transcript abundance as measured by RNA-Seq. Circles indicate that the corresponding transcript increased in abundance under the metal-limitation condition, while a square indicates that transcript abundance decreased under the metal-

limitation condition. Where available, metal-responsive fold-changes in the presence of a fixed carbon source (acetate) or in the absence (CO_2) is given.

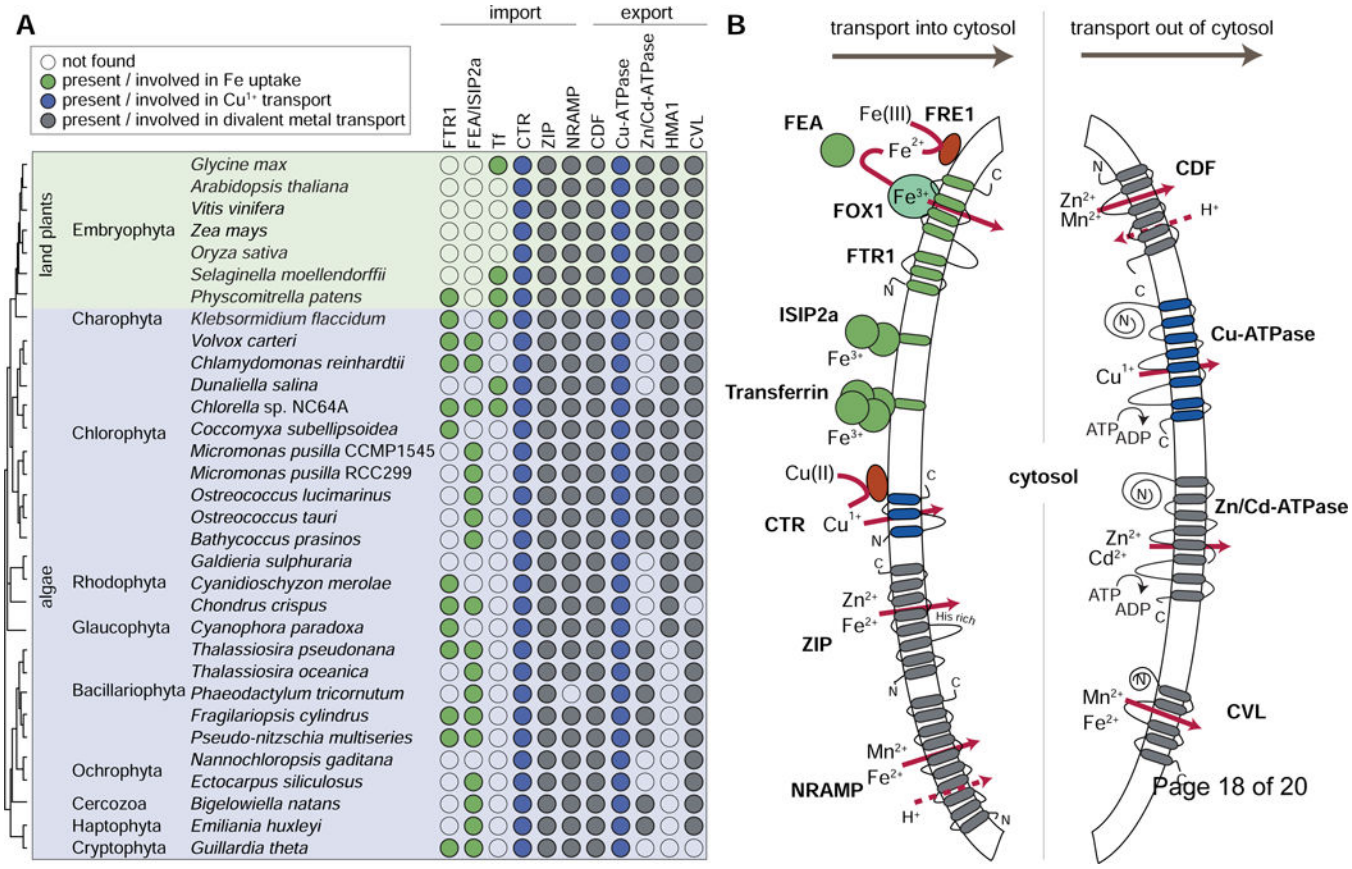

Figure 3. Transporters involved in border control

A, a filled circle signifies the presence of at least one homolog of the indicated protein family (FTR1, high-affinity iron permease; FEA/ISIP2a, Fe-assimilation proteins; Tf, transferrin; CTR, Cu assimilatory transport; ZIP, divalent metal transporter; NRAMP, divalent metal transporter; CDF, divalent metal transporter; Cu-ATPase, Cu-transporting subfamily of the P_{1B}-type ATPases; Zn/Cd-ATPase, Zn/Cd-transporting subfamily of the P_{1B}-type ATPases; HMA1, similar to Zn/Cd-subfamily of P_{1B}-type ATPases but with unknown function; CVL, Ccc1/VIT1-like. A schematic species tree is shown at the left. B, predicted membrane topologies and common substrates for each transporter family are shown. The *C. reinhardtii* protein names are given for the high-affinity Fe-transport complex involving FRE1, FOX1 and FTR1. Solid arrows represent the direction of metal ion transport either into the cytosol across the plasma or organelle membrane (left hand side of figure) or out of the cytosol across the intracellular membranes of organelles (right hand side of figure). Metal reductases (FRE1 for Fe reduction in *C. reinhardtii*) and an as yet unidentified reductase for Cu^{2+} reduction before transport by CTR are represented as dark red ovals Abbreviations for importers: FRE1, ferric reductase 1 ; FOX1, ferroxidase 1, MCO (multi-copper oxidase) family; FTR1, Fe transporter 1; FEA, Fe-assimilation protein; ISIP2a, iron starvation inducible protein 2a, first identified in *Phaeodactylum tricornutum*; ZIP, Zrt-, Irt-like proteins; CTR, Cu transporter; NRAMP (natural resistance-associated macrophage proteins). Abbreviations for exporters: CDF, cation diffusion facilitator, named

MTP in plants; CVL, Ccc1/VIT1, Ca(II)-sensitive cross-complementer 1/vacuolar iron transporter 1.

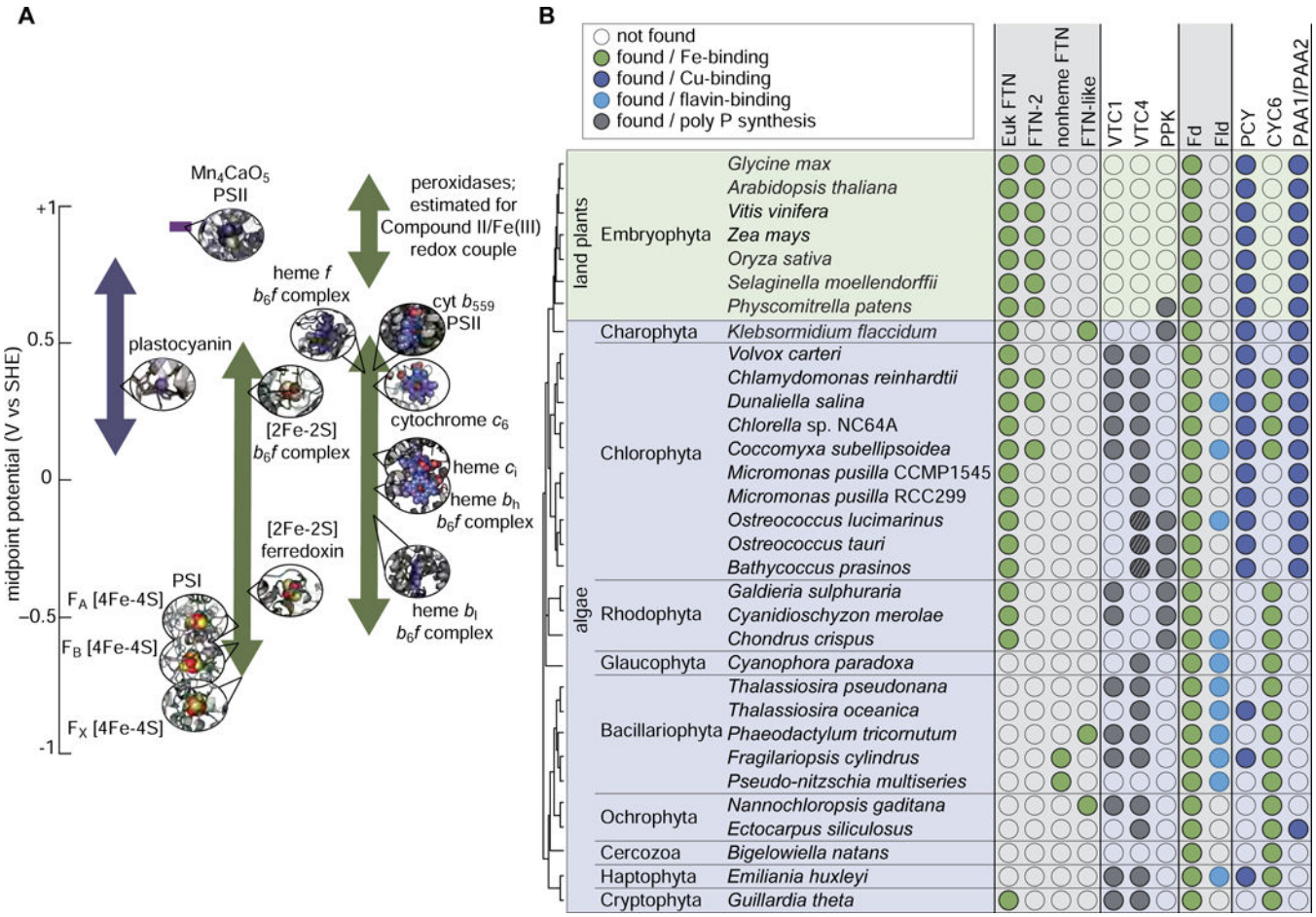

Figure 4. Austerity measures in algae

A, the same metal ion can catalyze different types of reactions, because the cognate apo-protein is able to tune the inherent chemistry of the metal ion. The range of midpoint potentials (measured in volts (V) vs. the Standard Hydrogen Electrode (SHE)) achieved by various combinations of metal ions, prosthetic groups and protein backbones illustrates both the functional versatility of metal ions and the control applied by the protein environment. The approximate midpoint potentials for specific metal-dependent redox centers in the oxygen-evolving electron transfer chain are given. The placement of the Mn cluster of photosystem II in this scheme is estimated. Illustrations and midpoint potentials for the photosynthetic redox centers are based on the following structures and references: plastocyanin (PDB entry 2PLT) [71], PSI (PDB entry 3LW5) [72,73], PSII (PDB entry 3ARC) [74], cytochrome c_6 (PDB entry 1CYI) [75], cytochrome b_6f (PDB entry 1Q90) [76,77]. B, identification of homologs and co-occurrence of selected protein families involved in metal storage or austerity measures. The identified ferritins are categorized based on the top domain hit in NCBI CDD: Euk FTN, cd01056; FTN-2 pfam13668; nonheme FTN, cd01055; FTN-like, cl00264. VTC1/VTC4 (vacuolar transport chaperone complex) and PPK (polyphosphate kinase) synthesize polyphosphate and are used as markers for acidocalcisome-like compartments. For the two *Ostreococcus* species and *Bathycoccus prasinos*, a protein similar to VTC4 is identifiable in their genomes, but these proteins lack

conservation within the N-terminal portion of the SPX domain. The *Thalassiosira pseudonana* VTC4 homolog also appears to lack the full-length N-terminal domain, but the apparent truncation is likely the result of an incorrect gene model (specifically, the full-length SPX domain is recovered if an alternative upstream methionine is used). Other abbreviations: Fd, ferredoxin; Fld, flavodoxin; PCY, plastocyanin; CYC6, cytochrome c_6; PAA1/PAA2, chloroplast-localized Cu-ATPases.