Role of iron in gene expression and in the modulation of copper uptake in a freshwater alga: Insights on Cu and Fe assimilation pathways.

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Abstract

Metal uptake and toxicity can generally be related to its aqueous speciation and to the presence of competitive ions as described by the biotic ligand model. Beyond these simple chemical interactions at the surface of aquatic organisms, several internal biological feedback mechanisms can also modulate metal uptake. This is particularly important for essential elements for which specific transport systems were developed over the course of evolution. Based on the results of short-term Cu^{2+} uptake experiments and on the analysis of the expression of certain genes involved in Cu and Fe homeostasis, we studied the effects of Fe^{3+} on Cu^{2+} uptake by the freshwater green alga *Chlamydomonas reinhardtii*. We observed a significant increase in Cu^{2+} uptake rate in algal cells acclimated to a low Fe^{3+} medium up to 4.7 times greater compared to non-acclimated algal cells. The overexpression of the ferroxidase FOX1 and permease FTR1 genes suggests an activation of the high affinity Fe^{3+} assimilation system, which could constitute a plausible explanation for the increase in Cu^{2+} uptake rate in acclimatized algae. We show that Fe availability can have a significant impact on Cu uptake. Our observations reinforce the importance of considering physiological factors to better predict metal bioavailability.

Key words: metals, transport, effects, algae, biotic ligand model, transcriptomics

1. Introduction

Copper (Cu) and iron (Fe) are two of the most important essential elements because of the role they play in cells. Due to their chemical properties (especially their redox potential), they are able to act as enzymatic cofactors in several redox reactions in cell metabolism (Burkhead et al., 2009; La Fontaine et al., 2002b; Ravet and Pilon, 2013). However, Cu can become toxic to living organisms, especially at high concentrations, and cause irreversible damage that can lead to cell death (Balamurugan and Schaffner, 2006; Marchetti and Maldonado, 2016; Wang and Wang, 2021). Therefore, ingenious systems of regulation and control of the availability of these trace elements exist in organisms, allowing them to ensure homeostasis of these elements within cells (Beauvais-Flück et al., 2019; Blaby-Haas and Merchant, 2017; Burkhead et al., 2009). Homeostasis is a highly

modulated physiological equilibrium that results from a long evolution process in response to harsh environmental conditions encountered in the past (Hanikenne, 2003; Hennigar and McClung, 2015). Cu and Fe transporter proteins (biotic ligands) play a central role in these physiological processes that aim to maintain element homeostasis (Kropat et al., 2015; Maldonado et al., 2006). In particular, they act as a gate of entry and possibly of exit for trace metals in the cell (Blaby-Haas and Merchant, 2012; Hennigar and McClung, 2015). To carry out their role and allow the organism to survive in various environmental conditions (along the range of trace element availabilities in natural systems), ion transporters have been subjected, through evolution, to various inter and/or intra-specific specializations, varying from one element to another (Annett et al., 2008; Blaby-Haas and Merchant, 2012). In *Chlamydomonas reinhardtii*, Cu and Fe uptake has been widely studied and the involved mechanisms to modulate their assimilation are now well documented (Hanikenne, 2003; La Fontaine et al., 2002b; Merchant et al., 2006; Wang and Wang, 2021). Typically, assimilation of most trace elements is mainly ensured by two types of systems, responding according to the ambient element concentration (availability) and/or to the elemental nutritional status of the organisms. High affinity systems use a set of membrane proteins mobilized to meet the needs of the cell in an environment where the element of interest is limited or low. On the other hand, low affinity systems are used in an environment where this element is abundant (Glaesener et al., 2013; Hanikenne, 2003; Kropat et al., 2015; Lavoie et al., 2016).

Trace metal bioavailability and toxicity predictive models such as the biotic ligand model (BLM) notably use the affinity constants of metal-ligand interactions as input. Hence, understanding the metal transport mechanisms is important to adequately predict their uptake and toxicity as pointed out by Lavoie et al. (2016). Several decades of research have led to the modeling of metal bioavailability and toxicity as a function of its free ion, resulting first in the free ion activity model (FIAM) predictive model, then the BLM (Adams et al., 2020; Schlekat et al., 2020). Various improvements have been made over the years to improve these models, such as accounting for pH and concentrations of major cations (Mebane et al., 2020; Niyogi and Wood, 2004). Despite this progress, several other challenges still remain in order to further improve bioavailability models, in particular by considering the effects of other important factors such as temperature, concentrations of

essential elements and macro-nutrients, acclimatization, *etc.* (Bossuyt and Janssen, 2005; Mebane et al., 2020; Wang and Dei, 2006). Our work falls within this overall perspective to improve such models.

In previous work (Kochoni and Fortin, 2019) we observed a hypertoxicity of Cu to the green alga *C. reinhardtii* following its abnormal accumulation in a low metal environment which faded when the medium was supplemented in Fe^{3+}, while other essential trace elements (Co, Mn, Zn) had no effect. We hypothesized that both Cu and Fe are using the same uptake systems and that the level of Fe in the antecedent growth medium impacts the level of Cu accumulation in a subsequent exposure. The objectives of the present study are therefore, to verify this hypothesis and to gain an overview of the mechanisms triggered from these low metal growth conditions by *C. reinhardtii*. Two methodological approaches were used to characterize membrane transporters involved in the assimilation process of these two elements. First, Cu uptake rates using short-term uptake experiments were determined to quantify the kinetic parameters characterizing the uptake of these metals by the membrane transporters. Second, we examined the transcriptomic response of the cells for known transport systems to gain insight on the cellular coping mechanisms typically when Fe deficiency occurs. The analysis and interpretation of our key findings allowed us to conclude that cells grown in low-Fe conditions increase the number of Fe transporters that use Cu as a cofactor, which could be used to internalize Cu.

2. Materials and methods

2.1. Algae culture conditions

The freshwater green microalga *C. reinhardtii* (wild type strain CPCC11, Waterloo University, ON, Canada), was used as a model organism in this work as described in a previous publication (Kochoni and Fortin, 2019). This species is easy to grow and is well suited for controlled studies. In order to better quantify the internalization fluxes of Cu during the exposure experiments, cells were grown in media supplemented with the stable isotope ^{63}Cu (Enrichment 99%, Trace Sciences International) and exposure experiments were carried out in media containing only ^{65}Cu (Enrichment 99%, Trace Sciences International). The resulting $^{65}Cu/^{63}Cu$ ratios inside algae prior to the experiments reached 0.22 ± 0.08 and 0.30 ± 0.02 for algae grown or not in low-Fe media, respectively, compared

to a natural abundance ratio of 0.45. This approach allowed us to better differentiate between "pre-existing" and "new" Cu inside the algae to facilitate the quantification of Cu uptake above base levels. In order to study how Fe influences Cu uptake and homeostasis, two types of algae were used in this work depending on their culture condition; (*i*) the algal cells (L-alga) that were grown in LM1 (Low metal) medium (Lavoie et al., 2012b), that is less concentrated in Fe^{3+} ($[Fe^{3+}]_{free} \sim 10^{-19}$ M) and (*ii*) the algal cells (M-alga) cultivated in the basic MHSM1 medium (Modified High Salt Medium) whose Fe^{3+} content is 10x higher ($[Fe^{3+}]_{free} \sim 10^{-18}$ M) than that in LM1 medium. All culture media were adjusted to pH 7 with concentrated HNO_3 (35%, Trace Metal Grade, Fisher Chemical) and stored at 4°C for later use. Table SI1 details the culture media chemical compositions.

Prior to the experiments, algal cells were maintained in a good physiological condition by renewing the axenic culture weekly and by inoculating a fresh culture with cells in mid-exponential growth. For L-alga cells, the acclimation consisted of a minimum of two transfers and eight days of culture in the targeted medium to achieve acclimation and to proceed with exposure experiments. All algal cells were cultured in polycarbonate or glass Erlenmeyer flasks (of 250 mL or 1 L, depending on the amount of algae needed) in a controlled environmental growth chamber (Conviron, CMP4030, Controlled Environments Ltd., Canada) under continuous light (80-100 $\mu mol \cdot m^{-2} \cdot s^{-1}$), using 110 ± 10 rpm of agitation at 20°C until they reached the mid-exponential growth phase.

All growth media were filtered (0.2 µm polycarbonate membrane filters, Isopore, Merck Millipore) prior to sterilization using an autoclave (121°C, 15 min). To keep cultures axenic, precautions were taken at each step of the experiment. In addition to the use of a sterile laminar flow hood, periodic monitoring of the absence of other living microbes (e.g. bacteria) was carried out by plating on agar with a 48-72 h incubation time at 37°C. To avoid metal cross contamination, all materials used were acid washed (for 24 h in 15%, v/v, HNO_3, ACS grade, Fisher Chemical), rinsed seven times with Milli-Q water (resistivity > 18.2 MΩ·cm; TOC < 2 $\mu g \cdot L^{-1}$) and sterilized in an autoclave.

2.2. Exposure and sampling conditions

All exposure solutions (50 mL) were left to equilibrate for 24 h prior to their use. Experiments were carried out in triplicate using both types of algae (L-/M-alga), in both

the presence (+) and the absence (–) of Fe^{3+}. At the end of the exposure period (≤ 60 min, a short exposure time to minimize physiological feedback reactions of algal cells) the cells were harvested on 2.0 µm polycarbonate filter membranes (Isopore TM, Merck Millipore). Two superimposed filters were used to control for adsorptive losses of Cu by using the bottom filter as background signal indicator. A volume of 10 mL of low metal rinse (LMR) solution supplemented with ethylenediaminetetraacetic acid (EDTA; 0.1 mM) were added on top of the filters and kept in contact with the cells for 10 min to remove adsorbed metals (Hassler et al., 2004). The remaining metal was operationally defined as intracellular.

2.2.1. Cu uptake kinetics

Cu uptake kinetic experiments were performed in exposure media containing 25.7 nM of free $^{65}Cu^{2+}$ ions (i.e. about ten times the equivalent of the total concentration of Cu in algae culture media) with (+Fe) and without Fe (–Fe). The exposure media (Table SI2) composition was simplified to improve the control of Cu^{2+} and Fe^{3+} speciation during exposure. Typically, exposure media contained only Cu and/or Fe as source of metal, were buffered with NTA and no phosphorus was added. Algae in culture were harvested during the exponential growth phase by filtration on a 2-µm polycarbonate filter membrane (Isopore, Merck Millipore, vacuum pressure ≤ 10 cm Hg), rinsed three times with LMR rinse solution (Table SI2) and suspended again in 20 mL of LMR. Cell population numbers as well as size distributions were then determined using an electronic particle counter (Multisizer TM 3 Coulter Counter®; Beckman) and the quantity of algal cells needed to reach 40 000 cell·mL^{-1} in the exposure media was calculated. Immediately after, cells were introduced in the exposure media, Cu uptake was determined for exposure times of 0, 5, 20, 40, and 60 min. For each time point, metal uptake was stopped, as described above (section 2.2), by rinsing the cells with 10 mL of LMR containing 0.1 mM EDTA for 10 min. For the experiment performed to quantify Cu^{2+} internalization as a function of concentration, algae were harvested as above and exposed for 60 min to a range of free ion concentrations of $^{65}Cu^{2+}$ (up to 43 pM Cu^{2+}; Table SI3). At the end of the exposure time, metal uptake was stopped as described above.

Following the 10 min EDTA contact period, filters were then rinsed three times with 10 mL of LMR rinse solution to remove any residual trace metal present on the filters so that

only metals accumulated inside the cells could be determined. The upper filter, containing all the collected algae, and the lower one were recovered separately in 50 mL polypropylene tubes. All samples were then digested by adding 5 mL of HNO_3 (65%, Trace Metal™ Grade, Fisher Chemical) and 1 mL of H_2O_2 (30-32%, Optima™ Grade, Fisher Chemical) on the filters and by heating them at 95°C for 4 h. At the end of digestion process, samples were diluted (with milli-Q water) to 50 mL (10% v/v HNO_3) prior to trace metal analysis.

2.2.2. Transcriptomic experiments

For gene expression experiments (performed in parallel to the bioaccumulation experiments), the green alga *C. reinhardtii* was exposed for 60 min to 25.7 nM of $^{65}Cu^{2+}$ (free concentration, Table SI2). As above each type of algae were exposed to ^{65}Cu in triplicate, for 60 min, and using 50 mL exposure media in the presence (+) and in the absence (–) of Fe^{3+}. At the end of the exposure time, Cu uptake was stopped by adding EDTA (final concentration 0.1 mM) for 10 min and algae were collected by centrifuging the exposure solutions (10 min, 4500 rpm). Pellets were transferred to an Eppendorf tube and were diluted in 600 µL of RNA later buffer (to avoid RNA degradation) and stored at -80 °C until required for mRNA extraction.

2.3. Metal quantification in algae and media

Total, dissolved, and cellular metal (Fe, ^{63}Cu and ^{65}Cu) concentrations were determined following mineralization by inductively coupled plasma mass spectrometry (ICP-MS; XSeries 2, Thermo Scientific) and atomic emission spectrometry (ICP-AES, Varian Vista AX). Cu content of the algae was corrected by accounting for the passive adsorptive losses of Cu (substraction of the Cu found on the lower filter). Mass balances were performed by dividing the initial Cu concentration (measured in triplicate) by the sum of the algal filtrate and algal digest. Each experimental point was performed in triplicate and the quality assurance and control was similar as in the work of Kochoni and Fortin (2019). Briefly, Fisher certified reference standards for ICP analyses and the measurement repeatability were ≥97%. The calibration curves, used to quantify metals of both ICP instruments, were prepared using single element standards (SCP Science). As for the quality control, multi-elemental certified standards (900-Q30-100, SCP Science) and a sample from a proficiency

testing study for trace elements in water (#TE105-05, Environment and Climate Change Canada) were used and the recovery levels of each control material were 99.6 ± 4.6% and 110.5 ± 5.1%, respectively. The average recovery for Cu mass balances after the exposure was 85.2 ± 0.1%. With regards to the acid digestions, the quality control was performed using IAEA-413 algae material (International Atomic Energy Agency) and the average recovery reached for Cu was 96.8 ± 0.1% (Table SI4).

2.4. Gene expression

2.4.1. Extraction and Reverse Transcription of RNA

RNA extraction and reverse transcription procedures were adapted based on the protocol of Kim Tiam et al. (2012). In total, thirty-one genes were studied. Table 1 provides a list of the studied genes and the cellular functions in which they would be involved.

2.4.2 Quantitative real-time polymerase chain reaction (q-RT-PCR)

Based on the molecular characterization of the target genes, specific q-RT-PCR primer pairs were determined for each gene using Primer3Plus. The detailed primers for each gene are provided in Table SI5. The q-RT-PCR were performed in a LightCycler (Roche LightCycler 480) following the manufacturer's instructions. Briefly, the pre-incubation lasted 3 min at 95 °C, then the amplification consisted of 45 cycles with 1 cycle at 95 °C for 5 sec and 60 °C for 10 sec. The melting curve continued at 95 °C for 5 sec and at 65 °C for 1 min. Finally, the cooling step was performed at 40 °C for 30 sec. Melting curves of every reaction were analyzed to assess reaction specificity. The data were normalized to the mean of the Ct values for three reference genes, β-act, elf1a and rpl13 and analyzed using the $2^{-\Delta Ct}$ method described by Livak and Schmittgen (2001) (Table SI6). The mean values of the level of expression of the genes studied were finally used to calculate the induction (>2) or repression (<0.5) factors (indicating at least a decrease or increase by a factor of 2) (Moisset et al., 2015; Santos et al., 2019) of the genes according to the treatments received by the algae (Table SI7). Results are shown as fold changes of exposed to control group.

2.5. Data treatment and analysis

Unless otherwise indicated, all means are expressed with standard deviation (±SD) and the experiments were carried out in triplicate (n=3). The effects of Fe on Cu uptake on the

acclimated and non-acclimated algae were tested by a one-way ANOVA analysis using JMP Pro 13.0.0 (SAS Institute, Riverside, CA, U.S.A.) followed by a t-test to determine if significant differences among means were observed ($p<0.05$). A nonlinear regression based on the Michaelis-Menten model were applied to the internalization fluxes of Cu^{2+} to determine J_{max} (saturation flux) and K_M (stability constant) parameters which describe the interactions of Cu^{2+} with the biotic ligands (membrane transporters) as described by Slaveykova and Wilkinson (2005).

For gene expression, a two-way ANOVA test was used to evaluate simultaneously the effect of the Fe content in both culture and exposure media on the expression of genes involved in Cu metabolism (Table SI8). These statistical analyses were performed with R software (version 3.5.0) using the packages tidyverse and vegan. The normality and homogeneity were verified prior to ANOVA analysis by the Shapiro-Wilk ($p<0.01$) and the Levene ($p<0.05$) test respectively. If the ANOVA results were significant, a Tukey HSD test was computed to perform multiple pairwise comparisons between the means of groups. If data were not normal, then the non-parametric Kruskal-Wallis test ($p<0.05$) was performed followed by the Wilcoxon test for performing the multiple pairwise comparisons between groups.

All speciation data, free element concentrations, and activities in the media were determined by thermodynamic calculations using MINEQL 5.0. Graphs, curves and regression models were realized by using mainly Sigma Plot software (version 12.5). Error propagation calculations were performed each time measured values were pooled.

3. Results and discussion

To design the culture and exposure media used in this work, the concentrations of the two metals of interest (Cu and Fe) were set by taking into account their typical environmental levels and to remain coherent with the values used in our previous work (Kochoni and Fortin, 2019). Indeed, Fe concentration is often high in freshwater, but its expected free ion concentration is much lower and varies between 10^{-20} and 10^{-14} M depending on pH (Lofts et al., 2008). As for Cu, its total dissolved concentration is also relatively high in natural waters while its corresponding free ions can vary from 10^{-12} to 10^{-8} M (Mueller et al., 2012). Concentrations of Fe^{3+} and Cu^{2+} in our different study media were within these

ranges. Finally, in our experimental conditions, the concentrations of the other essential trace elements such as Co, Mn and Zn in our study media were shown not to have an effect on Cu^{2+} uptake and toxicity to *C. reinhardtii* (Kochoni and Fortin, 2019).

3.1. Fe effect on Cu uptake rate by *C. reinhardtii*

Short-term (60 min) Cu uptake experiments were performed to characterize the transporter system(s) involved in "Cu-biotic ligand" interactions. This allowed for the probing of initial cellular responses with different Fe nutritional status and minimal interference by biological feedback mechanisms that could modulate these rates. The accumulation of the stable isotope ^{65}Cu ion ($^{65}Cu^{2+}$) in the algal cells grown in –Fe media (L-algae) increased steadily with time in both the low-Fe (–Fe, $[Fe^{3+}]_{free} << 10^{-19}$ M) and the Fe-replete (+Fe, $[Fe^{3+}]_{free} \approx 10^{-18}$ M) exposure media (Figure 1 and Table SI9). This trend is observed with *C. reinhardtii* cells, when exposed to $^{65}Cu^{2+}$ ($[^{65}Cu^{2+}]_{free}$ = 27.5 nM) in both exposure media. Internalization fluxes were determined from the slopes of $^{65}Cu^{2+}$ uptake as a function of time and were 1.17 ± 0.34 and 1.15 ± 0.14 amol·cell^{-1}·min^{-1} in –Fe and +Fe exposure media, respectively. There is no significant difference in $^{65}Cu^{2+}$ uptake rates, whether the ^{65}Cu exposure medium contains Fe or not ($p > 0.05$) (see Figure 1). Thus, the absence of an effect on Cu uptake whether in the presence or absence of Fe suggests that our previously observed change in Cu uptake and toxicity as a function of Fe concentration (Kochoni and Fortin, 2019) could likely be due to physiological effects (e.g. non-competitive inhibition). In addition, the use of the stable isotope ^{63}Cu in the growth media prior to exposure to ^{65}Cu allowed us to track Cu export. Since the base level of ^{63}Cu (already present in the algal cells) remained constant over the course of the exposure to ^{65}Cu (Figure SI1), this suggests that no significant Cu efflux occurred over the 1-h exposure time frame.

Short-term $^{65}Cu^{2+}$ uptake experiments performed with the algae grown in +Fe media (M-algae) showed significant accumulation of $^{65}Cu^{2+}$, whether in –Fe or +Fe exposure media. But it is only after 60 min of exposure that $^{65}Cu^{2+}$ uptake by M-alga became significantly different from the base level (background noise) (Figure SI2, A and B). This could be due to fewer Cu transporters under these experimental conditions for sufficient Cu accumulation to be significantly detectable in the first 60 min, suggesting that the up- and

down-regulation of Cu transport is likely taking longer than 60 min to come into play. This assertion regarding the low number of Cu transporters is consistent with the negative feedback regulation mechanism described in the literature (Knauer et al., 1997; Lavoie et al., 2016; Sunda and Huntsman, 1998). In fact, the Cu^{2+} uptake rate is known to be under an inductive and a negative feedback regulation by cellular Cu nutritional state (Burkhead et al., 2009; Merchant et al., 2006) i.e. in the case of Cu-sufficient cells, Cu^{2+} uptake rates will be reduced to a strict minimum, no matter the content of exposure media of Cu^{2+}, mainly in response to the negative feedback control system lowering the synthesis of membrane transporters (Sunda, 2012; Sunda and Huntsman, 1995). The accumulation of $^{65}Cu^{2+}$ increased linearly over a 300 min (5-h) exposure time frame. As previously observed in the case of L-alga, there is no significant difference in the $^{65}Cu^{2+}$ uptake rates for the M-alga, whether the exposure medium is Fe^{3+}-depleted or not ($p > 0.05$) (Figure SI2, C). According to the slopes of the linear uptake curves, the $^{65}Cu^{2+}$ uptake rates in –Fe and +Fe media were 0.23 ± 0.05 and 0.28 ± 0.02 amol·cell^{-1}·min^{-1}, respectively. This also indicates that there is no competitive inhibition of Cu uptake by Fe in our exposure conditions. In addition, the level of $^{63}Cu^{2+}$ in the M-alga remained unchanged over time (Figure SI2, A and B), as previously noticed in the case of L-alga, suggesting that Cu^{2+} export is negligible in our experimental conditions. Furthermore, it should be noted that the observed linearity of Cu^{2+} accumulation (Cu_{acc}^{2+}) as a function of time is also an indication that the metal was not significantly excreted by algae during the duration of the exposures (Crémazy et al., 2013). Our results suggest the absence of a substantial Cu^{2+} export process in *C. reinhardtii*, no matter the level of Fe in the medium. We can therefore deduce, by using the means of the Cu^{2+} uptake rates obtained from L-algae (1.159 amol·cell^{-1}·min^{-1}) and M-algae (0.246 amol·cell^{-1}·min^{-1}), that the Cu uptake rate by L-algae is 4.7-fold greater than those of M-algae (Figure 1 and Figure SI2).

An increase in the uptake rate of an element (Fe, Cu, Zn, *etc.*) when it is present at a low concentration is well documented in eukaryote cells (Annett et al., 2008; Hsieh et al., 2013; Merchant et al., 2006). However, low concentrations of one element (e.g. Fe^{3+}) in the medium or organism inducing an increased accumulation of another element (e.g. Cu^{2+}) has rarely been observed (Chen et al., 2004; Lavoie et al., 2012a; Malasarn et al., 2013) and suggests a link with their homeostasis. The fact that L-algae (grown in –Fe media)

were able to accumulate more Cu^{2+} than M-algae (grown in +Fe) strongly supports the idea of a common metabolic pathway in Cu and Fe assimilation in *C. reinhardtii* (Herbik et al., 2002; La Fontaine et al., 2002b; Maldonado et al., 2006). Recently, Wang and Wang (2021) also established the existence of a strong metabolic link between Cu and Fe in *C. reinhardtii*. Therefore, we hypothesize that the activity and functioning of Cu transporters are strongly modulated by Fe content in the growth medium.

3.2. Cu uptake kinetic parameters under depleted and replete Fe conditions in *C. reinhardtii*

A series of short-term (60 min) Cu uptake experiments were carried out with algae cells either grown in low or high metal concentrations (L-algae and M-algae). These were exposed to a range of low $^{65}Cu^{2+}$ concentrations (as described in section 2.2.1) to characterize the uptake parameters of membrane transporter(s) involved in the surface binding of Cu and its subsequent transmembrane transport. Following these uptake experiments, the internalization fluxes of $^{65}Cu^{2+}$ ions were calculated and plotted as a function of $[^{65}Cu^{2+}]_{free}$ from the exposure medium.

The internalization flux (J_{int}) data were fitted to a Michaelis-Menten model by nonlinear regression. In the case of L-algae, good fits were obtained with coefficients of determination of 0.90 and 0.88 for –Fe and +Fe exposure media, respectively (Figure 2 and Table SI10). The stability constants (K_M) and the saturation fluxes (J_{max}) obtained are presented in Table 2. There is no significant difference between the $^{65}Cu^{2+}$ fluxes no matter if the algae were exposed to Cu^{2+} in +Fe or –Fe medium ($p > 0.05$). As for M-algae, the ^{65}Cu content remained generally constant over the whole range of $^{65}Cu^{2+}$exposure concentrations. The resulting internalization fluxes of Cu in M-algae were at least 10 times lower than the maximum flux (J_{max}) reached with L-algae. This suggests that, for M-algae, Cu uptake was too low to be quantified with respect to the background levels of Cu present in the cells. Here again, the internalization fluxes of $^{65}Cu^{2+}$ obtained in the presence (+Fe) or absence (–Fe) of Fe were not significantly different ($p > 0.05$; Figure 2). The results obtained with L-algae show that (*i*) in both exposure media (±Fe), Cu was likely taken up by the same type of membrane transporters; and (*ii*) the algae grown in –Fe media (L-algae) were predisposed to accumulate more Cu^{2+} ions than algae grown in +Fe media(M-algae).

According to our data, the stability constants for the interaction of Cu^{2+} with membrane transporters of the L-algae ($10^{11.9}$ and $10^{11.6}$ M^{-1}) are five to six orders of magnitude greater than the values reported in the literature for *C. reinhardtii* at namely $10^{5.8}$ (Chen et al., 2010) and $10^{6.7}$ (Hill et al., 1996). This difference in affinity shows that transporters mobilized by *C. reinhardtii* under our experimental conditions would be of different nature or feature from those reported by Chen et al. (2010) and Hill et al. (1996). Such change in the affinity of an element of interest (i.e. Cu) to ligands following an acclimation to a low concentration of another element (i.e. Fe) is not well documented in freshwater green algae. However, a similar observation was reported in marine diatoms that remained for a long time in a low Fe medium, resulting in a high Cu demand and suggesting a change in its uptake system (Annett et al., 2008; Maldonado et al., 2006; Schoffman et al., 2016). It has been reported in the literature documented cases of element-ligands affinity change; but usually occurring after a pre-incubation of cells into media low in the same element (Hassler and Wilkinson, 2003; Lavoie et al., 2016; Lavoie et al., 2012a; Sunda, 2012). The mechanism responsible for this change in affinity remains to be identified. It could be (*i*) an induction, at low concentrations of metal ions, of a high affinity absorption system, undetectable in cells grown or pre-incubated in media containing relatively high concentrations of ions of the element of interest; or (*ii*) an affinity change of the same transport system resulting, for example, from isoforms having different affinities for the elements or from allosteric changes in molecular configuration at the binding sites; or simply (*iii*) localized changes in charges on the membrane surface (Lavoie et al., 2016; Sunda and Huntsman, 1992).

There are many feedback mechanisms that can allow cells to regulate the uptake of Cu. However, it remains unclear how Fe can modulate the uptake of Cu. It seems like Cu^{2+} could functionally replace Fe^{3+} as suggested in previous publications (Díaz-Quintana et al., 2003; Marchand et al., 2018; Wood, 1978). Our results on this point seem to support the existence of a very strong link in Cu and Fe homeostasis in *C. reinhardtii*. Several studies have indeed shown that in *C. reinhardtii*, like other algae, Cu-dependent plastocyanin can be substituted by Fe-dependent cytochrome c6 and vice versa depending on Cu and Fe medium nutritional status (Hanikenne et al., 2009; Kropat et al., 2015; Malasarn et al.,

2013; Merchant and Bogorad, 1987). Further investigation, for instance at the molecular level, is therefore needed to get insight on the mechanisms involved.

3.3. Gene expression profiles in response to the Fe nutritive status of the medium

Transcriptomic analyses were carried out to get insight on the metabolic pathway potentially involved in Cu homeostasis in algae. Genes known to play an important role in Cu and Fe metabolism, (Blaby-Haas and Merchant, 2012; Hanikenne et al., 2009; Merchant et al., 2006; Tripathi and Poluri, 2021), were thus targeted. Results summarized in Figure 3 show that the main significant changes in the gene expressions occurred in cells grown in low Fe media (L-algae), indicating that acclimation of algal cells to low Fe^{3+} culture media is the main factor that could best explain the differences in gene expression. Acclimation effects were indeed significantly different in at least 20 genes (~65%) versus 11 genes (~36%) for which only the exposure conditions effect significantly differentiate gene expression. As for the heatmap, it clearly distinguishes between L-algae and M-algae (Figure 3), based also on the primacy of the acclimation effect on the genes expression level. In addition, a global analysis of the heatmap shows that the expression level of at least 50% of the genes studied could also discriminate between L-algae (for which these genes have been repressed) and M-algae (for which they have been overexpressed). These include genes involved in the handling of oxidative stress (*cat*, *sodMn* and *sodFe*), mitochondrial metabolism (*nad5*, *cox1*, 12S), and photosystems (*psaA*, *d1*), part of Fe-transport system (*fer1, irt1, fea1, fea2*), and part of Cu^{2+} transporter system genes (*ctr3*, *ctp1* and *ctp3*). According to the gene induction and repression factors (Tables SI7 and SI13), there are more repressed genes (22/31, i.e. ~71%) than overexpressed genes (11 out of 31, i.e. ~36%). The gene expression of *fea1, fer2* and *fre1* (3 of the 9 Fe transporter genes), *sodMn* and *sodFe* (2 of the 4 oxidative stress gene markers), and *ctp3* (one of the 3 HMA transporter genes, a P-Type ATPase Cu transporters subfamily) were significantly different in all of the experimental conditions compared to the control cells (M-algae). The Fe transporter gene expressions were equivocal. The *fea1* gene was inhibited while the *fer2* gene was up-regulated regardless of experimental conditions. The *fre1* gene expression was inhibited in L-algae no matter the Fe exposure condition (–Fe/+Fe) but up-regulated in M-algae in the +Fe exposure condition.

3.3.1. Gene expression dynamics in L- vs M-algae

Figure 4 shows the gene expression correlation heatmap performed using the Pearson method (see Table SI11). This approach allows for the investigation of co-expression patterns in gene expression data and the highlighting of genes displaying similarity in their expression profiles which would suggest a co-regulation. Then, by analysing the dendrogram, genes can be classified in nine groups depending on their expression in the different conditions (Table SI12). The correlation heatmap revealed that the upregulation of the gene groups 1, 2, 3 and 4 were strongly correlated, as were the groups 7, 8 and 9.

As pointed out above, acclimation of algae is the factor which best explains differences observed in gene expression. This is a regulating mechanism describing the molecular changes that occur in an organism in response to variations in environmental conditions over the short to medium term (Raven and Geider, 2003). It is thus understandable that, abilities acquired by algal cells during this time frame have governed the expression of the genes studied. This points to the resilience of algae in facing inevitable environmental changes or fluctuations (Bird, 2015; La Fontaine et al., 2002a). Therefore, understanding our results requires studying how the acclimation of algae to low Fe environments is conducted. Acclimation is a well-documented process that requires an adapted reaction from the organism to be able to cope with variations in environmental conditions (La Fontaine et al., 2002a; Niyogi and Wood, 2003). Iron assimilation by *C. reinhardtii* is mediated by two main mechanisms specific to Fe^{2+} and Fe^{3+} (Eckhardt and Buckhout, 1998; Weger, 1999). Ferric Fe is taken up by a system of high affinity membrane transporters, the activity of which is subjected to negative feedback in response to the nutrient status of Fe^{3+} in the medium (Blaby-Haas and Merchant, 2012; La Fontaine et al., 2002a; La Fontaine et al., 2002b). This pathway is therefore supposed to become more active when the Fe^{3+} concentration in the medium is low (Eckhardt and Buckhout, 1998; Merchant et al., 2006). We therefore expected to see, at the molecular level, gene expression profiles clearly indicating an activation of the high affinity Fe^{3+} assimilation pathway through the expression or overexpression of genes encoding FRE, FOX1, FEA1, FEA2, FTR1 and possibly FER (Hanikenne et al., 2009; La Fontaine et al., 2002a; La Fontaine et al., 2002b; Merchant et al., 2006). According to our data, the gene expression profile in L-algae shows a pattern mainly dominated by significantly repressed genes

(Table SI13), four of which (*fea1, fea2, fer1* and *irt1*) play a role in iron assimilation in *C. reinhardtii* (Hanikenne et al., 2009; Merchant et al., 2006). However, the overexpression of other genes (*fox1, ftr1* and *fer2*) also involved in the same iron assimilation pathway (Allen et al., 2007a; Hanikenne et al., 2009; Merchant et al., 2006) leads us to bring some nuance to our initial conclusions. Gene expression dynamics is indeed a very finely regulated compromise between expression, repression and overexpression (Ritter et al., 2014), the profile of which only reflects an instant and/or early responses to environmental conditions (Beauvais-Flück et al., 2019; La Fontaine et al., 2002a; Raven and Geider, 2003). In addition, gene transcription does not always imply its effective translation into protein, and therefore not in a physiological response; the cell having possibly solicit other physiological approaches to address the issue (Halbeisen et al., 2008; Mohler and Ibba, 2017). Our results would therefore reflect an image of a transient physiological effect, probably in favor of Cu^{2+} assimilation, given that algae are exposed to Cu (Beauvais-Flück et al., 2019). This hypothesis is consistent with our data regarding the expression of genes encoding proteins involved in Cu^{2+} assimilation pathway, that were mostly overexpressed genes (see Table SI13; *ctr1, ctr2, copt1*).

3.3.2. Gene expression and the putative link of Fe and Cu assimilation pathways

This eventual physiological transition mentioned above is most likely in favor of the assimilation or homeostasis of Cu^{2+} via that of Fe^{3+}. This sheds some light on the probable link between Fe and Cu through their assimilation pathways. Several studies indeed underline metabolic interactions of the two pathways in several species, in particular in *C. reinhardtii* (Annett et al., 2008; La Fontaine et al., 2002b; Maldonado et al., 2006). It is well known that in some Cu and Fe assimilation pathways there is a redox step that could be catalyzed by the same enzymes or enzyme systems (Eckhardt and Buckhout, 1998; La Fontaine et al., 2002b), and that the multi-copper ferroxidase (FOX1) and its associated permease (FTR1) are involved in this uptake system (Castruita et al., 2011; Herbik et al., 2002; Page et al., 2009). This is supported by the classification of genes studied into nine different groups (Table SI12; Figure 4). For example, group 9 contains genes coding *ctr1, ctr2, fox1* and *mdr1*, the first three of which are known to play a very active role in Cu^{2+} assimilation or homeostasis (Blaby-Haas and Merchant, 2017; Merchant et al., 2006; Page et al., 2009). These correlated genes suggest they were co-regulated (meaning genes

regulated by common transcription factors), thus supporting our previous conclusions (section 3.2) on the significant increase of Cu^{2+} uptake rate in L- vs M-algae. The mechanism that governs this whole process remains to be fully elucidated. Nevertheless, the overexpression of the gene encoding the FOX1 and FTR1 enzymes, classified in groups 9 and 6 respectively, strongly suggests that the redox step could likely be the most common step in Cu and Fe assimilation pathways. Knowing that Cu is a cofactor of ferroxidase (FOX1) (Terzulli and Kosman, 2010; Terzulli and Kosman, 2009) and that the overexpression of its gene (*fox1*) is an indicator of stress caused by low Fe conditions (Glaesener et al., 2013; Terauchi et al., 2010), the increase in Cu^{2+} uptake rate we noticed could be explained by the requirement in Cu^{2+} for FOX1 activity as mentioned by La Fontaine et al. (2002b) and Chen et al. (2008). This assertion is also supported by Terzulli and Kosman (2010) who have shown that high-affinity Fe uptake is dependent on both the FOX1 and Cu status of the cell. Such a process, also well known in yeast (*Saccharomyces cerevisiae*) and marine diatoms (*Thalassiosira spp*) would also be present in eukaryotic cells and transcriptionally induced in response to low-Fe (Chen et al., 2004; Rutherford and Bird, 2004). Accordingly, regulation of trace metal uptake by metal-responsive transcription factors is then an important aspect of their homeostasis (Bird, 2015; Rutherford and Bird, 2004). In *C. reinhardtii, crr1* (Cu response regulator) is a well-known transcription factor that is required as a sensor of Cu homeostasis and is also possibly involved (if only indirectly) in Fe homeostasis (Eriksson et al., 2004; Merchant, 2007; Merchant et al., 2020).

3.3.3. Gene expressions and homeostasis imbalance handling by microalgae

The overexpression of the *mdr1* gene, also classified in group 9 and known to play a role in the cell detoxification systems, could be to prevent plausible Cu stress following the accumulation of Cu^{2+}. Copper stress (physiological damage induce by Cu homeostatic imbalances) is known to activate a set of genes coding for, among other things, detoxification systems (Knauert and Knauer, 2008; Ritter et al., 2014; Tripathi and Poluri, 2021). The fact that the other gene (*pcs1*) also involved in the detoxification function was repressed could also be considered as consistent with this Cu stress (excess Cu) prevention, in particular if one considers that Cu^{2+} accumulation has not exceeded the regulation capacities of *mdr1*. Similar phenomena are also observed in other microalgae species. For

example, Tripathi and Poluri (2021) reported that in *Coccomyxa*, the mere presence of naturally high content of GSH is sufficient to induce a reduction in the expression of other antioxidants. The profile of oxidative stress genes also finds an explanation in the acclimation of algae to a low Fe concentration medium. Indeed, in *Chlamydomonas*, Fe-limited algal cells generally operate metabolic readjustments to avoid photo-redox damage that may result from FeS residues (Merchant et al., 2006). These readjustments could lead to losses of SODFe activity that could be compensated by an overexpression of the *sodMn* genes, as is the case in marine diatoms and cyanobacteria (Allen et al., 2007b; Hsieh et al., 2013; Merchant et al., 2006). However, with a simple expression of *sodMn*, our results are partially consistent with the profile described above, which nevertheless does not seem sufficient to rule out the idea of a repression of *sodFe* in favor of *sodMn*. The repression of most of the photosystem genes indeed pleads in favor of this readjustment. In fact, to cope with a low Fe environment, *C. reinhardtii* tends to reduce its functions to the most essential ones, such as reducing photosynthesis activity for the benefit of respiration (Blaby-Haas and Merchant, 2017; Kropat et al., 2015). This well-regulated strategy aims to allocate all the available resources to the mobilization of Fe to ensure the transfer of electrons in the mitochondria and thus ensure this vital function that is respiration (Blaby-Haas and Merchant, 2017; Glaesener et al., 2013; Kropat et al., 2015).

Linking gene expressions by comparing the correlation of their transcription factors with ion transport characterization is a promising investigative approach and allowed us to formulate new hypotheses. Many significant correlations were observed, some of them were expected, but most remain to be understood and explained (Beauvais-Flück et al., 2019). Thus, they constitute a basis for future research. Our results showed that Cu^{2+} and Fe^{3+} could share the same transporter systems so that when algal cells, grown in low-Fe conditions, increase the number of Fe transporters, the latter are in turn used by Cu to enter *C. reinhardtii* cells. Inversely, a protective effect of Fe against Cuaccumulation appears at relatively high Fe concentrations that result in a decrease in Fe transporters that could be used by Cu. But our results have also and above all pointed to a probable physiological link between Cu and Fe within their respective assimilation pathway in the freshwater green alga *C. reinhardtii*. The overexpression of the gene encoding the enzyme FOX1

strongly suggests that the redox step is most likely the common step in the uptake of Cu^{2+} and Fe^{3+}.

4. Conclusions

The BLM is a conceptual model that integrates metal speciation for the prediction of metal uptake and toxicity, particularly in aquatic systems (Paquin et al., 2002). The BLM has gained in complexity and representativeness throughout its development, leading to its current version (Adams et al., 2020; Paquin et al., 2002). Although important parameters such as pH, organic matter and major cations are now taken into account, several other factors still need to be incorporated into the BLM to better refine it given their impacts on metal bioavailability (Mebane et al., 2020; Wang and Dei, 2006). In this work, we showed that at environmentally relevant concentrations, changes in Fe growth conditions can modify physicochemical parameters of the Cu membrane transporters. Given the importance of these transporters in Cu uptake, taking into account factors that may affect them, should help improve Cu accumulation predictions by the BLM. Similar to Lavoie et al. (2012a) and Mebane et al. (2020), our results therefore plead in favor of a BLM that integrates physiological factors such as the one revealed here.

Competing interests statement

Authors have no competing interests to declare

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Tables

Table 1. List and functions of genes examined in *C. reinhardtii*.

Category	Genes
Oxidative stress	*cat, sodMn, sodFe, gst, gpx*
Mitochondrial metabolism	*cox1, nad5, 12S*
Photosystem	*d1, psaA*
Detoxification	*pcs1, mdr1*
Copper transporter systems	*ctr11, ctr2, ctr3, copt, mco*
NRAMP transporters	*nramp1, nramp2*
HMA transporters	*ctp1, ctp2, ctp3*
Iron transporter systems	*fre1, fox1, ftr1, fea1, fea2, fer1, fer2, irt1, irt2*

Abbreviations: *cat*: Catalase; *sodMn*: Mn superoxide dismutase; *sodFe*: Fe superoxide dismutase; *gst*: Glutathione S-transferase; *gpx*: glutathione peroxidase; *cox1*: cytochrome C oxidase subunit I; *nad5*: NADH dehydrogenase subunit 5; *12s*: mitochondrial ribosomal RNA 12S; *d1*: D1 protein; *psaA*: PsaA protein; *pcs*: Phytochelatin synthase; *mdr1*: Multidrug-resistance P-glycoprotein; *ctr/copt*: Copper transporters; *nramp1/2*: Natural resistance-associated macrophage proteins subunit 1 and 2; HMA: heavy metal P-type ATPase; *ctp*: Copper-transporting P-type ATPases; *fre1*: ferric reductase subunit 1; *fox1*: ferroxidase subunit 1; *mco*: multi-copper oxidase; *ftr1*: Fe transporter subunit 1; *fea1/2*: Fe-assimilation protein subunit 1 and 2; *isip2a*: Iron starvation inducible protein subunit 2a; *fer1/2*: Ferritin subunit 1 and 2; *irt*: Involved in Fe assimilation.

Table 2. Stability constants (K_M) for the binding of Cu to membrane transport sites of *C. reinhardtii* grown in the low Fe medium (L-alga) and Cu maximum fluxes (J_{max}).

Exposure Media	K_M (M^{-1})	J_{max} (amol· μm^{-2}·min^{-1})
-Fe	$10^{11.9}$ [$10^{11.65}$ – $10^{12.02}$]	0.0502 ± 0.0020
+Fe	$10^{11.6}$ [$10^{11.40}$ – $10^{11.79}$]	0.0498 ± 0.0002

Figures

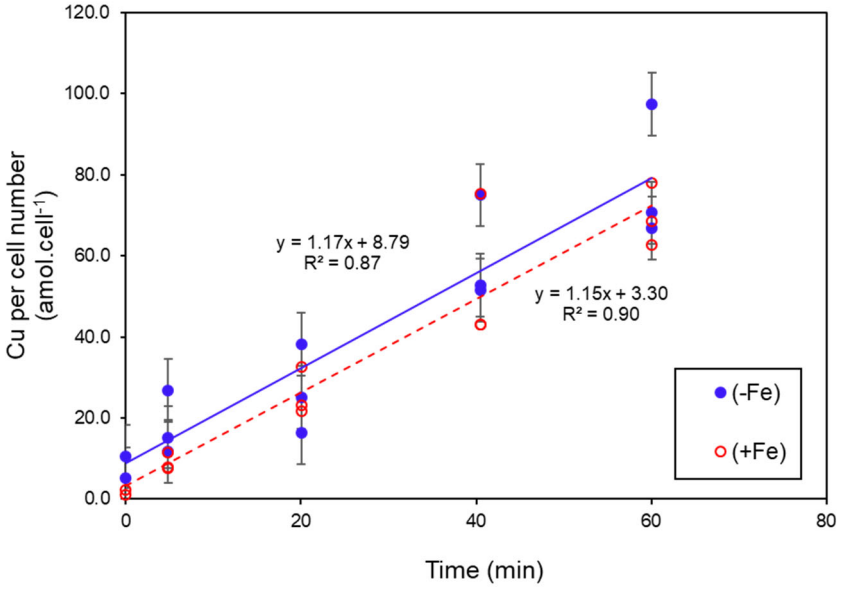

Figure 1. Copper uptake kinetics by *C. reinhardtii* previously grown in low iron conditions (L-algae) and exposed to ^{65}Cu ($[Cu^{2+}]_{free}$ = 27.5 nM) in the presence (+Fe, $[Fe^{3+}]_{free}$ = 4.4 x 10^{-18} M) and absence (-Fe) of Fe. Each point is the average of three replicates per exposure time from two independent experiments. Error bars represent standard deviations (n = 3). (Results for M-algae are shown in Figure SI2 A and B).

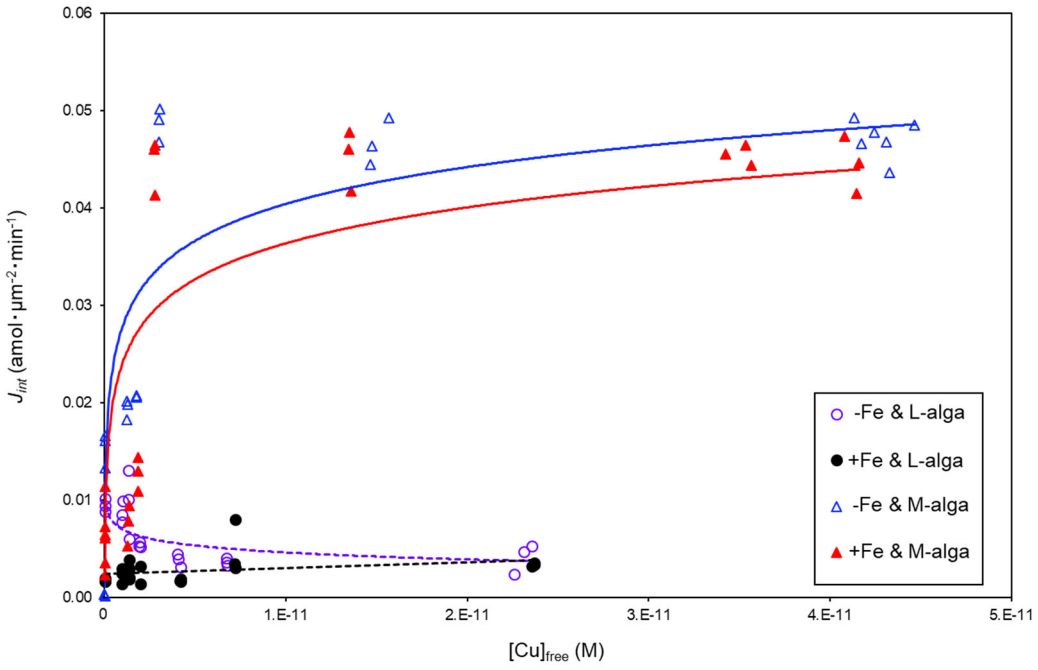

Figure 2. Internalization fluxes of free $^{65}Cu^{2+}$ in *C. reinhardtii* grown in low Fe^{3+} media (L-algae; $[Fe^{3+}]_{free}$ = 1.00 x 10^{-19} M) and exposed to $^{65}Cu^{2+}$ (see the detailed concentrations in Table SI3) in the presence (+Fe, $[Fe^{3+}]_{free}$ = 4.4 x 10^{-18} M) and absence (-Fe) of Fe for 60 min. For M-algae (cells grown in Fe replete media ($[Fe^{3+}]_{free}$ = 1.00 x 10^{-18} M), when $[Cu^{2+}]$ was varied the internalized flux observed over 60 min remained constant whether the exposure media were +Fe or -Fe.

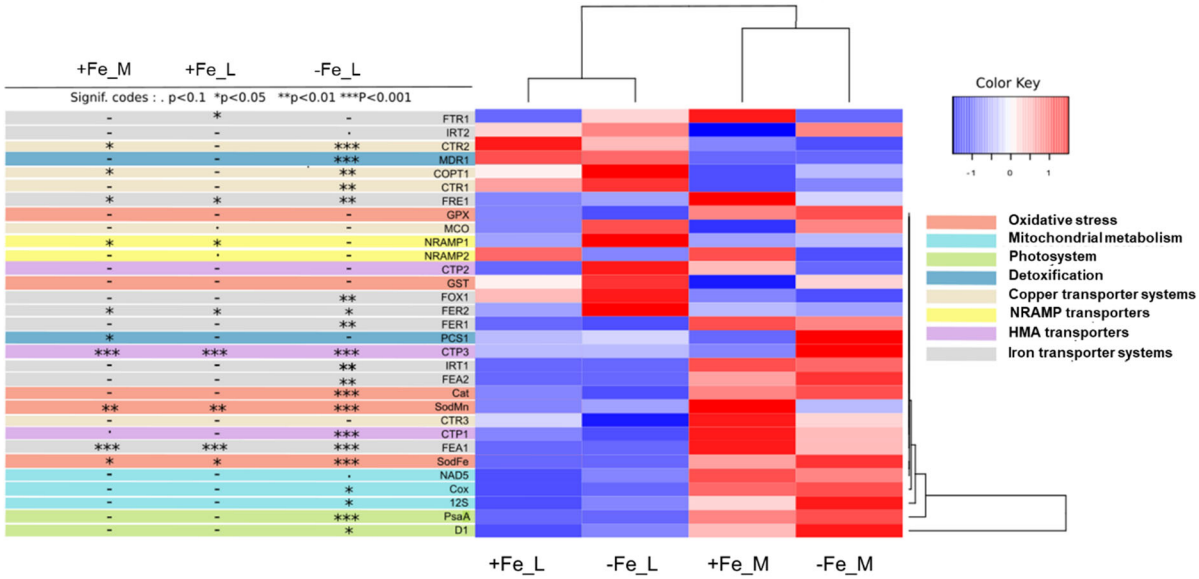

Figure 3. Significant differences in gene expression depending on the experimental conditions. L = Algae grown in –Fe media; M = Algae grown in +Fe media; Fe = iron element; ± = addition/absence of Fe^{3+} in the exposure media. Red color means less expression and yellow or white color means high expression levels. The level of significance corresponds to: 0.001 '***' 0.01 '**' 0.05 '*' 0.1 '.'.

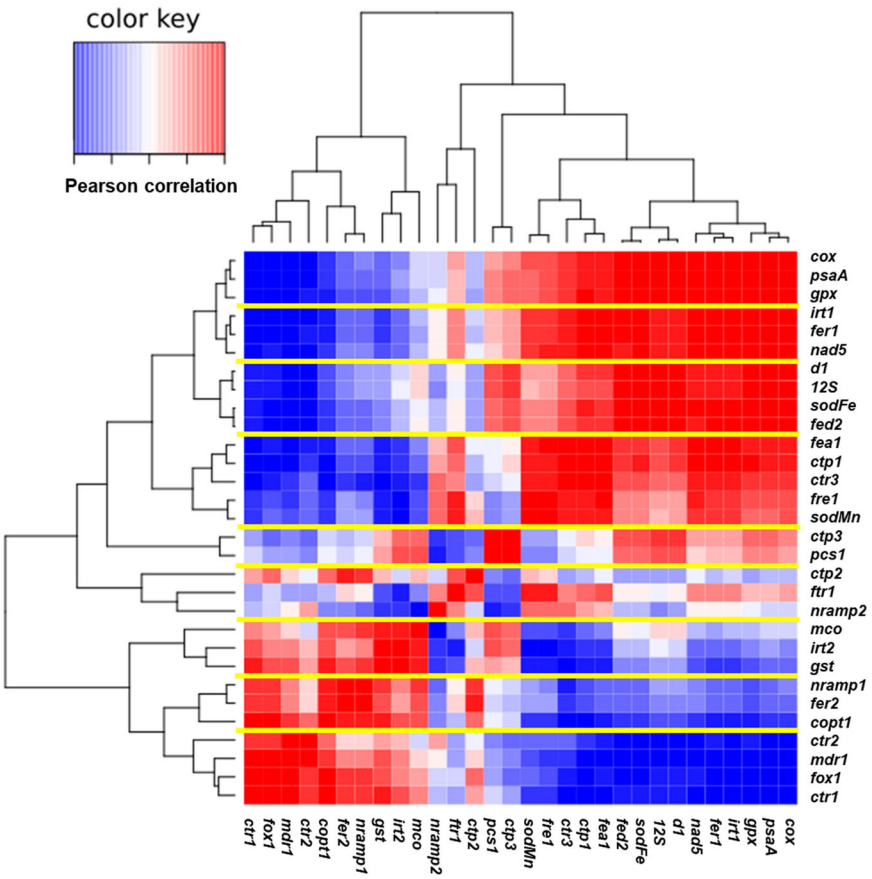

Figure 4. Heatmap of gene expression correlation in *C. reinhardtii*. The heatmap was constructed using the Pearson correlation coefficient of genes expression. Positive correlations are marked in yellow and negative ones in red. The yellow lines delimit groupings of co-regulated genes. Clusters defined by hierarchical clustering are indicated at top and left.