Proteomic analysis of metabolic pathways supports chloroplast–mitochondria cross-talk in a Cu-limited diatom

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Abstract
Diatoms are one of the most successful phytoplankton groups in our oceans, being responsible for over 20% of the Earth’s photosynthetic productivity. Their chimeric genomes have genes derived from red algae, green algae, bacteria, and heterotrophs, resulting in multiple isoenzymes targeted to different cellular compartments with the potential for differential regulation under nutrient limitation. The resulting interactions between metabolic pathways are not yet fully understood. We previously showed how acclimation to Cu limitation enhanced susceptibility to overreduction of the photosynthetic electron transport chain and its reorganization to favor photoprotection over light harvesting in the oceanic diatom Thalassiosira oceanica (Hippmann et al., 2017, 10.1371/journal.pone.0181753). In order to gain a better understanding of the overall metabolic changes that help alleviate the stress of Cu limitation, we have further analyzed the comprehensive proteomic datasets generated in that study to identify differentially expressed proteins involved in carbon, nitrogen, and oxidative stress-related metabolic pathways. Metabolic pathway analysis showed integrated responses to Cu limitation. The upregulation of ferredoxin (Fdx) was correlated with upregulation of plastidial Fdx-dependent isoenzymes involved in nitrogen assimilation as well as enzymes involved in glutathione synthesis, thus suggesting an integration of nitrogen uptake and metabolism with photosynthesis and oxidative stress resistance. The differential expression of glycolytic isoenzymes located in the chloroplast and mitochondria may enable them to channel both excess electrons and/or ATP between these compartments. An additional support for chloroplast–mitochondrial cross-talk is the increased expression of chloroplast and mitochondrial proteins involved in the proposed malate shunt under Cu limitation.

KEYWORDS
copper, cross-talk, Cu limitation, diatom, proteomics
1 | INTRODUCTION

Diatoms form an integral part of our oceans, influencing nutrient cycling and productivity of many marine foodwebs (Arntz, 2009). Annually, marine diatoms fix as much carbon dioxide through photosynthesis as all terrestrial rainforests combined (Field et al., 1998; Nelson et al., 1995), thus having a significant impact on atmospheric CO2 levels and global climate. One key to their success may lie in their complex evolutionary history (Moustafa et al., 2009; Oborník & Green, 2005), which resulted in a mosaic genome with genes derived from the original heterotrophic eukaryotic host cell, the engulfed green and red algal endosymbionts, and a variety of associated bacteria (Arntz, 2004; Bowler et al., 2008; Finazzi et al., 2010). As a result, diatoms possess multiple isoenzymes in many metabolic pathways, especially in carbon metabolism (Ewe et al., 2018; Gruber et al., 2009; Gruber & Kroth, 2014; Kroth et al., 2008; Smith et al., 2012).

The presence of multiple isoenzymes with different evolutionary histories also led to novel locations and interactions among metabolic pathways compared with green algal and animal ancestors (Allen et al., 2011; Gruber & Kroth, 2017). For example, in animals, the complete set of proteins involved in glycolysis is located in the cytosol, whereas in green algae, the first half of glycolysis (glucose to glyceraldehyde-3-phosphate, GAP) is located in the chloroplast and the second half (GAP to pyruvate) in the cytosol. In diatoms, an almost complete set of glycolytic proteins is found in both the cytosol and the chloroplast, with an additional set of proteins from the second half of glycolysis located in the mitochondria (Kroth et al., 2008; Río Bártulos et al., 2018; Smith et al., 2012). Furthermore, proteins involved in the ancient Entner–Dourodoff pathway, which is predominantly restricted to prokaryotes and catabolizes glucose to pyruvate, have also been identified in diatom genomes and are targeted to the mitochondria (Fabris et al., 2012; Río Bártulos et al., 2018).

A study by Allen et al. (2012) illustrates the complexity of isoenzymes in diatoms further: The genome of Phaeodactylum tricornutum encodes five different fructose-bisphosphate aldolase (FBA) isoenzymes, three targeted to the chloroplast and two to the cytosol (Allen et al., 2012). Each FBA has its own phylogenetic history. The expression pattern of these five isoenzymes changes depending on the nutritional status of the cell (Allen et al., 2012).

One of the most surprising discoveries from diatom genome sequencing was a complete urea cycle (Allen et al., 2011; Arntz et al., 2004). In contrast to the catabolic nature of the urea cycle in animals, in diatoms, it is an integral part of cellular metabolism and a hub of nitrogen and carbon redistribution within the cell. It is involved in amino acid synthesis, cell wall formation, and carbon and nitrogen recycling, and it interacts with the citric acid cycle (Allen et al., 2011; Arntz et al., 2004).

Most molecular studies on acclimation to nutrient limitation have focused on macronutrients, or on the essential micronutrient Fe, which limits phytoplankton in over 30% of the ocean (Moore et al., 2004). Some studies have shown an intricate interaction between Fe and Cu nutrition in phytoplankton (Annett et al., 2008; Guo et al., 2012; Maldonado et al., 2002; Maldonado et al., 2006; Peers & Price, 2006), but there are only a handful of studies on physiological adaptations to Cu limitation alone (Guo et al., 2012; Guo et al., 2015; Kong & Price, 2020; Lelong et al., 2013; Lombardi & Maldonado, 2011; Maldonado et al., 2006; Peers et al., 2005; Peers & Price, 2006).

Our recent comprehensive investigation on the physiological and proteomic changes to the photosynthetic apparatus of two strains of the open ocean diatom Thalassiosira oceanica in response to chronic Cu limitation revealed both similar and different strategies compared with those observed in response to low Fe (Hippmann et al., 2017). Acclimation to low Cu caused a bottleneck in the photosynthetic electron transport chain that was accompanied by major increases in the electron acceptors ferredoxin (Fdx) and Fdx:NADP+ reductase, which has major roles in counteracting reactive oxygen species (ROS). Along with changes in the composition of the light-harvesting apparatus, this resulted in a shift from photochemistry to photoprotection.

In our previous paper (Hippmann et al., 2017), we focused on the photosynthetic electron transport chain and light-harvesting antennas as well as a number of physiological parameters changed in response to Cu limitation but did not ask how carbon and nitrogen metabolism are affected and may interact when Cu is limiting. We now expand our proteomics analysis to include proteins involved in various carbon and nitrogen metabolic pathways (e.g., Calvin–Benson–Bassham [CBB] cycle, glycolysis, tricarboxylic acid [TCA] cycle, nitrogen acquisition and assimilation, urea cycle, malate shunt, and glutathione metabolism), taking into account their predicted cellular compartments (Table 1). Although the decrease in Rubisco activase suggests the CBB is downregulated, there appear to be complex effects on the three-compartment glycolysis machinery. Increased expression of enzymes involved in nitrogen acquisition and assimilation could act simultaneously as a sink for reducing equivalents and as a supplier of compounds needed to support dissipation of ROS. Finally, we present further evidence for cross-talk between chloroplast and mitochondria in form of an active malate shunt.

2 | RESULTS

2.1 | Overview of proteomic datasets

In our original study (Hippmann et al., 2017), we investigated two strains, CCMP 1003 and CCMP 1005, of the centric diatom T. oceanica (here referred to as TO03 and TO05, respectively). On further examining the proteomic datasets, it was clear that Cu limitation had a stronger and more comprehensive effect on proteins of carbon and nitrogen metabolism in TO03 than TO05 (this study), in line with observations for photosynthetic electron transport proteins. Although the proteomic dataset of TO05 contains twice as many distinct proteins as that of TO03 (1,431 vs. 724), TO03 has three times more significantly upregulated and 10 times more significantly downregulated proteins (Figure 1, overview Figures S5 and S6). For this reason, if not noted otherwise, we will focus on the TO03 results only (Tables 2 and...
A short discussion on the different adaptational strategies of the two strains can be found in Note S1. The data for all relevant proteins in both strains and both proteomic datasets (main and extended) are given in Tables S2–S9. Expression differences are classed as “highly regulated” (greater than or equal to 2-fold difference) or “regulated” (1.3- to 2-fold difference, see Section 5). All differential expression data discussed in the text are significantly upregulated or downregulated ($p < .05$), unless otherwise noted.

Of the 724 distinctive proteins in TO03, 525 have associated Kegg Orthology (KO) identifiers, and 52% of these were related to

| Abbreviation | Name | Abbreviation | Name |
|--------------|------|--------------|------|
| AAT          | Aspartate aminotransferase | GSS  | Glutathion synthetase |
| ACAS         | Acetyl-CoA synthase | GST  | Glutathione-S-transferase |
| ACC          | Acetyl-CoA carboxylase | IDH  | Isocitrate dehydrogenase |
| ACO          | Aconitasehydratase | LDH  | \(\beta\)-Lactate dehydrogenase |
| Agm          | Agmatinase | MDH  | Malate dehydrogenase |
| AMT          | Ammonium transporter | ME   | Malic enzyme |
| APX          | Ascorbate peroxidase | NAD(P)H-NiR | Nitrite reductase (NAD(P)H-dependend) |
| Arg          | Arginase | NR   | Nitrate reductase |
| argD         | \(n\)-Acetylornithine aminotransferase | NRT  | Nitrate/nitrite transporter |
| AsL          | Argininosuccinateyase | OCD  | Ommithine cyclodeaminase |
| AsuS         | Argininosuccinate synthase | OdC  | Ommithine decarboxylase |
| ATCase       | Aspartate carboxamoyltransferase | OGD  | 2-Oxoglutarate dehydrogenase |
| cbbX         | Rubisco expression protein | OTC  | Ommithine carboxamoyltransferase |
| CS           | Citrate synthase | PC   | Pyruvate carboxylase |
| CYS          | Cysteine synthase | PDH  | Pyruvate dehydrogenase |
| CYS2         | Cysteine synthase | PDH-E1 | Pyruvate dehydrogenase-E1 component |
| DHAR         | Dehydroascorbate reductase | PDH-E2 | Pyruvatedehydrogenase- E2 component (dihydrolipoamideaceteyltransferase) |
| DLDH         | Dihydrolipoamide dehydrogenase | PEPC | Phosphoenolpyruvate carboxylase |
| EDA          | 2-Keto-3-deoxy phosphogluconate aldolase | PEPK  | Phosphoenolpyruvate carboxykinase |
| EDD          | 6-Phosphogluconate dehydratase | PEPS  | Phosphoenolpyruvate synthase |
| ENO          | Enolase | PFK | Phosphofructokinase |
| F2BP         | Fructose-1,6-bisphosphatase | PGAM | Phosphoglycerate mutase |
| FBA I        | Fructose-bisphosphate aldolase class-I | pgCPSII | Carbamoyl-phosphate synthase |
| FBA II       | Fructose-bisphosphate aldolase class-II | PGK  | Phosphoglycerate kinase |
| Fd           | Ferredoxin | PGM  | Phosphogloucomutase |
| Fe-NiR       | Nitrite reductase (ferredoxin dependend) | PK   | Pyruvate kinase |
| FH           | Fumarate hydratase | PPDK  | Pyruvate |
| GAPDH        | Glyceraldehyde 3-phosphate dehydrogenase | rbCL | Ribulose-bisphosphate carboxylase |
| GCS          | Glutamate-cysteine ligase | rbcS  | Ribulose-bisphosphate carboxylase |
| GDCP         | Glycine decarboxylase p-protein | RPE   | Ribulose-5-phosphate epimerase |
| GDCT         | Glycine decarboxylase t-protein | RPI   | Ribose-5-phosphate-isomerase |
| GDH          | Glutamate dehydrogenase | RuBisCO | Ribulose-bisphosphate carboxylase |
| GDH          | Glutamates dehydrogenase | SRM   | Spermidine synthase |
| GOGAT        | Glutamate synthase | SUCLA | Succinate CoA synthetase |
| GPI          | Glucose-6-phosphate isomerase | TP    | Triosephosphate |
| GR           | Glutathione reductase | TPI   | Triosephosphate isomerase |
| GRX          | Glutaredoxin | TXN  | Thioredoxin |
| GSI          | Glutamine synthase | unCPS (CPSaseIII) | Carbamoyl-phosphate synthase |
| GSII         | Glutamine synthetase | Ure   | Urease |
| GSIII        | Glutamine synthetase | URT   | Na/urea-polymine transporter |
metabolism (Figure 2). Furthermore, 77%–78% of these metabolic proteins were particularly affected by Cu limitation, with general trends of downregulation of proteins involved in energy metabolism, upregulation of those in carbohydrate metabolism, and a modification of those in amino acid metabolism.

2.2 | Carbon fixation, glycolysis, and the citrate (TCA) cycle

In diatoms, the enzymes of glycolysis are found in all three major compartments: chloroplast stroma, cytosol, and mitochondria (Gruber & Kroth, 2017; Kroth et al., 2008; Río Bártulos et al., 2018; Smith et al., 2012). Four (or seven, if the three triose isomerase isoenzymes are counted) of the 15 proteins involved in the carbon fixing CBB cycle are part of the chloroplast glycolytic pathway (Table 2, Figure 3 [CBB and TCA cycle], Figure 4 [glycolysis], Table S4). In the initial step of CO2 fixation, the large and small subunits of Rubisco were not affected by Cu limitation, but the essential Rubisco activator protein cbbX (To24360) was downregulated by 2.3-fold. Six proteins were upregulated: phosphoglycerate kinase (PGK, To07617) by 6.8-fold, the two FBA class II (FBA II) proteins by 1.4- and 2-fold (To00388 and To12069), and the three triose phosphate isomerase (TPI) isoenzymes (To02438, To35826, and To32006) by 3.3-, 1.9-, and 1.5-fold, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, To13085) and phosphoglycerate mutase (PGAM, To21902, 2.3-fold) were the only proteins downregulated, by 4.2- and 2.3-fold, respectively. Of the nine expressed proteins targeted to the chloroplast, only FBA class I (FBA I, To02112) was not affected by Cu limitation.

The upregulation of TPI (Figures 3 and 4) combined with the downregulation of GAPDH and PGAM could lead to an increase in triose-phosphates and their subsequent export from the chloroplast. Probing the genome for gene models containing the triose-phosphate transporter Pfam domain identified seven candidate genes (Table S3) of which only two were expressed. Neither of them was differentially expressed.

Nine expressed proteins involved in the citrate cycle in the mitochondria were identified (Figure 3, Table 2, Table S5). Malate dehydrogenase (MDH1, To03405) was the only one upregulated (1.6-fold). Aconitase hydratase (ACO, To20545) and two isocitrate dehydrogenases (IDHs, To37807 and To34595) were all downregulated by 4.7-, 3.0-, and 1.6-fold, respectively. Of the proteins considered to be part of mitochondrial glycolysis (Figure 4), GAPDH (To33331) and enolase (ENO, To34936) were both upregulated by 3.8- and 1.5-fold respectively, while pyruvate kinase (PK, To07097) was downregulated by 1.3-fold.

Of the eight expressed cytosolic proteins detected, three were downregulated: phosphoglucomutase (PGM, To06412) by 3.2-fold, phosphofructokinase (PFK, To16559) by 1.8-fold, and FBA I (To24978) by 1.6-fold (or 2.7-fold considering expression of a contig associated with the same gene). The only cytosolic protein that was upregulated was PK (To34937, by 1.6-fold).

2.3 | Nitrogen metabolism

Twenty-two proteins involved in the urea cycle, nitrogen acquisition and assimilation, and also four membrane transporters were identified
At the plasma membrane, the urea (URT, To31656) and nitrate/nitrite (NRT, To04919) transporters were both significantly upregulated (6.9- and 11-fold, respectively). However, the expression of the two transporters putatively located in the chloroplast envelope, the formate/nitrate (NiRT, To00240) and ammonium (AMT, To07247) transporters, was not affected by Cu limitation.

Within the chloroplast, three nitrite reductases were identified. Of these, the NAD(P)H-dependent isoenzyme was not differentially expressed (NAD(P)H-NiR, To35252), whereas two Fdx-containing nitrite reductases (Fe-NiRs, To00016 and To02363) were upregulated by 1.3- and 2.3-fold, respectively, in concert with the massive 40-fold increase of Fdx in the chloroplast (Table 3; Hippmann et al., 2017). Both glutamine (GSII, To31900) and glutamate synthases (GOGAT, To13288) that both require Fdx cofactors were upregulated (1.7- and 1.6-fold, respectively). In contrast, aspartate aminotransferase (AAT, To16827) was the only chloroplast protein involved in core nitrogen metabolism that was downregulated (2.3-fold).

In the mitochondria, the pattern was reversed: The glutamine synthase isoform (GSIII, To06032) was 5.3-fold downregulated, while the GOGAT isoform (TO04828) expression did not change, and the mitochondrial AAT (To15049) was upregulated by 3.6-fold. Glycine decarboxylase t- and p-proteins (GDCT/P, To17688 and To36273), involved in photorespiration, were not affected. In the urea cycle, six proteins were identified, but only ornithine carbamoyltransferase (OTC, To05385) was upregulated by 1.7-fold.

In the cytosol, glutamate dehydrogenase (GDH, To06254) was upregulated by 2.09-fold ($p = .05$), and nitrate reductase (NR, To34460) was upregulated by 1.5-fold. The only other cytosolic protein upregulated was spermidine synthase (SRM, To22108; by 2.3-fold), which is essential for silica deposition.

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2.4 Malate shunt

In plants, malate transfers excess NAD(P)H-reducing equivalents from one compartment (i.e., the chloroplast) to another (i.e., the mitochondria) (reviewed by Scheibe, 2004). Metabolite antiporters and two isoenzymes for MDH and amino AAT are involved (Table 2, Figure 6, Table S8). In diatoms, the malate shunt has been proposed to connect the chloroplast with the mitochondria (Bailleul et al., 2015; Prihoda et al., 2012). In P. tricornutum, both MDH1 and MDH2 are targeted to the mitochondrion (Ewe et al., 2018), but in T. pseudonana, MDH2 is predicted to be targeted to the chloroplast (Smith et al., 2012). Aligning the T. oceanica model with Smith’s extended TpMDH2 model (Figure S2), supports the conclusion that ToMDH2 is also targeted to the chloroplast.

In our proteomic datasets, we found evidence for reciprocal regulation of both isozyme sets in the putative malate shunt (MDH1, MDH2, AAT, AAT2), as well as two isoenzymes of pyruvate carboxylase (PC) that could be feeding into this metabolic pathway (Figure 6). Of these six enzymes, four were upregulated: both plastidial PC (To31413) and MDH2 (To30817) by 2.6-fold and mitochondrial MDH1 (To03405) and AAT2 (To15049) by 1.6- and 3.6-fold, respectively. Only the plastidial AAT (To16827) was downregulated (by 2.3-fold).

**Table 3** Proteins involved in nitrogen and stress response metabolism that are significantly upregulated or downregulated in *Thalassiosira oceanica* (CCMP 1003) under chronic Cu limitation

| Gene name | Protein name | Fold change expression under low Cu | Pathway | Compartment |
|-----------|--------------|------------------------------------|---------|-------------|
| THAOC_15049 | AAT, aspartate aminotransferase | 3.6 | N | M |
| THAOC_16827 | AAT, aspartate aminotransferase | −2.3 | N | Chl |
| THAOC_00263 | Fe-NiR, nitrite/sulfite reductase ferredoxin-like half-domain | 2.3 | N | Chl |
| THAOC_00016 | Fe-NiR, nitrite/sulfite reductase ferredoxin-like half-domain | 1.3 | N | Chl |
| THAOC_31900 | GSII, glutamine synthetase | 1.7 | N | Chl |
| THAOC_06032 | GSII, glutamine synthetase | −5.3 | N | M |
| THAOC_34460 | NR, nitrate reductase | 1.5 | N | Cyt |
| THAOC_00016 | NR, nitrate reductase | 1.3 | N | Chl |
| THAOC_04919 | NRT, nitrate/nitrite transporter | 11.0 | N | Trans |
| THAOC_04380 | OCD, ornithine cycloaminase | 1.2 | N | Cyt |
| THAOC_05385 | OTC, ornithine carbamoyltransferase | 1.7 | N | M |
| THAOC_22108 | SRM, spermidine synthase | 2.3 | N | Cyt |
| THAOC_31656 | URT, Na/urea-polyamine transporter | 6.9 | N | Trans |
| THAOC_13288 | GOGAT, glutamate synthase | 1.6 | N, ROS | Chl |
| THAOC_37364 | APX, ascorbate peroxidase | 1.4 | ROS | Cyt |
| THAOC_27524 | CPS, cysteine synthase | 2.5 | ROS | Chl |
| THAOC_10442 | CPS2, cysteine synthase | 1.5 | ROS | Chl |
| THAOC_07268 | GR, glutathione reductase | 2.5 | ROS | Chl |
| THAOC_07269 | GRX glutathione reductase | −1.3 | ROS | Chl |
| THAOC_18234 | GRX glutathione reductase | −1.3 | ROS | Chl |
| THAOC_09062 | GST, glutathione-S-transferase | 7.3 | ROS | Cyt |
| THAOC_02860 | MnSOD, Mn/Fe binding superoxide dismutase | 1.8 | ROS | Chl |
| THAOC_10112 | NISOD, nickel-dependent superoxide dismutase | 1.4 | ROS | Cyt |
| THAOC_05213 | TXN, thioredoxin | 4.2 | ROS | Cyt |
| THAOC_13865 | TXN, thioredoxin | 1.8 | ROS | M |
| THAOC_31425 | TXN, thioredoxin | 1.5 | ROS | Chl |
| THAOC_25559 | Fdx, ferredoxin | 43.79 | — | Chl |

* Gene name as per Lommer et al. (2010).
* Average fold change in Cu-limited compared to control cultures, bold indicates highly differentially expressed (> ± 2-fold, p < .05), otherwise differential expression ratio of ±1.3- to 2-fold (p < .05).
* Metabolic pathway in which the protein is involved: N, nitrogen metabolism; ROS, reactive oxygen species metabolism; M, multiple.
* Predicted cellular localization of protein: Chl, chloroplast; Cyt, cytoplasm; M, mitochondrion; TRANS, transmembrane.
2.5 | Glutathione and antioxidant metabolism strongly upregulated

Glutathione is a small tripeptide (Glu-Cys-Gly) that is involved in redox sensing and counteracting ROS. Twenty-one expressed proteins involved in glutathione metabolism and other antioxidant agents (e.g., three thioredoxins [TXNs], three glutaredoxins [GRXs], and three superoxide dismutases [SODs]) were identified (Table 3, Figure 7, Table S7). Nine proteins are predicted to be targeted to the chloroplast. Six of these were upregulated: two isoenzymes for cysteine synthase (CYS, To27524 by 2.5-fold, To10442 by 1.5-fold), GOGAT (To13288 by 1.6-fold), glutathione reductase (GR, To07268, by 2.5-fold), TXN (To13864, by 1.5-fold), and the Mn-containing SOD (MnSOD, To02860, by 1.8-fold). Two GRX isoenzymes (To07269, To18234) were only mildly downregulated proteins (both by 1.3-fold).

Of the nine cytosolic proteins, glutathione-S-transferase (GST, To09062) and TXN (To05213) were highly upregulated (by 7.3- and 4.2-fold, respectively), while one of the two Ni-dependent SOD (NiSOD) isoenzymes was moderately upregulated (To10112, by 1.4-fold). Glutamate cysteine ligase/γ-glutamylcysteine synthase (GCL/GCS, To23355) was only identified in one of the biological triplicates but with a 4.5-fold increase in expression. In contrast to the chloroplastic CYS isoenzymes, the expression of cytosolic CYS (To05931) did not change. The TO03 GST (To09062) has its closest homologs in the polyp Hydravulgaris, the anemone Nematostella, and the brachiopod Lingula, and not in other diatoms (Table S7).

Only two of the expressed proteins involved in glutathione metabolism are predicted to be targeted to the mitochondria; TXN (To13864) was upregulated by 1.8-fold, whereas GRX (To02323) was not differentially expressed in Cu-limited TO03.

3 | DISCUSSION

In response to low Cu, T. oceanica (CCMP1003) restructures the photosynthetic electron transport proteins, resulting in a decrease in carbon assimilation (mg C mg Chl a⁻¹ h⁻¹), and increased susceptibility to overreduction of the photosynthetic electron transport chain.
Susceptibility to overreduction of the photosynthetic electron transport chain at saturating light intensities was suggested by (i) the 17% reduction in photochemical quenching ($F_{q0}/F_{v0}$) and (ii) the light response curves that indicated that the light saturation point decreased well below the actual growth light of 155 µmol quanta m$^{-2}$ s$^{-1}$. Growth at saturating light conditions could therefore lead to an increase in ROS. Consequently, there would be an increased need to safely dissipate excess energy, for example, through additional electron sinks (Niyogi, 2000). Our findings of a ~40-fold increase in Fdx (petF) and a 2.5-fold increase in Fdx:NAD(P)H oxidoreductase (FNR) under Cu limitation (Hippmann et al., 2017) suggested that there is indeed a surplus of reduced Fdx (Fd$^{red}$) and NAD(P)H in the chloroplast.

Here, on the basis of our now expanded proteomic analysis, we hypothesize how the interaction between various metabolic pathways (e.g., nitrogen assimilation, glycolysis, citrate, and the urea cycle) and the sophisticated coordination between the chloroplast and the mitochondria may facilitate the re-oxidation of Fd$^{red}$ and NAD(P)H in the chloroplast. Protein abundance alone is not always indicative of protein activity, and where known, we have included information on posttranslational activity modulation. We discuss our results with this in mind while suggesting a plausible restructuring of key metabolic pathways in T. oceanica in response to copper limitation.

3.1 Carbon metabolism: The CBB cycle is downregulated via its activase, and glycolysis is used to redistribute ATP and NAD(P)H within the cell

The three most thoroughly annotated diatom genomes (T. pseudonana, Armbust et al., 2004; P. tricornutum, Bowler et al., 2008; Fragilariopsis cylindrus, Mock et al., 2017) revealed many isoenzymes, particularly those involved in C metabolism. Indeed, homologous C metabolism isoenzymes exist among and between these diatoms (Gruber & Kroth, 2017; Kroth et al., 2008; Smith et al., 2012), and their differential expression is thought to manage cellular carbon flow. Furthermore, given that within the chloroplast, more than 50% of the proteins involved in glycolysis are also part of the CBB cycle (Smith et al., 2012), to regulate C flow, some isoenzymes might be preferentially involved in glycolysis over carbon fixation. For example, in P. tricornutum, the three plastidial FBAs are differently targeted and regulated under low versus high Fe
conditions (Allen et al., 2012). Here, we hypothesize that to overcome Cu limitation, *T. oceanica* downregulates the CBB cycle, while modulating glycolysis to promote the redistribution of ATP and NAD(P)H-reducing equivalents among cellular compartments. Similarly to *P. tricornutum* under Fe limitation, Cu-limited *T. oceanica* also regulates the expression of FBA homologs (Table 4), albeit in a different way. While the chloroplast FBA (FbaC2 homolog, To12069) is upregulated, one of the pyrenoid-associated FBAs is only mildly upregulated (FbaC1 homolog, To00388). This suggests that FbaC2 is preferentially involved in glycolysis over C assimilation, for the following reasons: (i) C assimilation decreased by 66% in Cu-limited cultures compared with the control (Hippmann et al., 2017), suggesting it is less likely for the C fixation proteins to be upregulated; (ii) the three significantly upregulated proteins involved in the CBB cycle can also be part of glycolysis (i.e., PGK, TPI, and FBA, Table 2); (iii) none of the distinct CBB cycle proteins (i.e., Rubisco, ribose-5-phosphate-isomerase [RPI], and ribulose-5-phosphate epimerase [RPE]) were differentially expressed; (iv) the red algal-type Rubisco activase (cbbX) was downregulated by 2.25-fold. The downregulation of cbbX results in slower carbon fixation and activity of Rubisco although Rubisco levels remain unchanged (Mueller-Cajar et al., 2011). Since RPI and RPE abundance remain constant, ribulose-bisphosphate would be bound to Rubisco. Consequently, once nutrient conditions are favorable, only the cbbX would need upregulation for C fixation to proceed. This strategy might be advantageous in nutrient limited environments with short-lived nutrient-rich conditions.

In general, most reactions facilitated by proteins in glycolysis can proceed in either directions, that is, glycolysis or gluconeogenesis. Smith et al. (2012) suggest that gluconeogenesis prevails in the mitochondria. However, assuming that the required metabolite transporters are present in the mitochondria (e.g., aspartate/glutamate shuttle, malate/2-oxoglutarate shuttle, citrate/malate shuttle, and fumarate/succinate shuttle), modeling flux balances in *P. tricornutum* predict that glycolysis would indeed be more favorable than gluconeogenesis in the mitochondria (Kim et al., 2016). In *T. oceanica*, in each cellular compartment, different subsets of glycolytic proteins were upregulated or downregulated under Cu

**FIGURE 4**  Relative expression of proteins involved in glycolysis in the three compartments (chloroplast, mitochondrion, cytosol), including Entner–Doudoroff pathway. Boxes indicate proteins with their abbreviated name and known *Thalassiosira pseudonana* (Tp) and *Thalassiosira oceanica* (To) homologs. Colors and shading as in Figure 3. Metabolites: 1,3-bisPG, 1,3-bisphosphoglycerate; 2K3D-PG, 2-keto-3-deoxyphosphogluconate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; 6PG, 6-phosphogluconate; DHAP, dihydroxyacetone phosphate; Fru-1,6-bis-P, fructose 1,6-bisphosphate; Fru-6P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate; Glu 6-P, glucose 6-phosphate; HCO$_3^-$, bicarbonate; OAA, oxaloacetate; PEP, phosphoenolpyruvate. Other abbreviations in Table 1
limitation (Figure 4, Table S4, overview Figure S5). Focusing on the upregulated proteins (Figure S3), a pattern emerges relating the possibility of increased ATP formation in the chloroplast and cytosol with NAD(P)H consumption in the chloroplast and its coupled formation in the mitochondria. By reducing chloroplast GAPDH (To13085) and increasing mitochondrial GAPDH (To33331), NAD(P)H-reducing equivalents would be generated in the mitochondria, whereas increasing PGK (To07617) in the chloroplast would increase ATP in this compartment. Therefore, an increased ATP/NAD(P)H ratio in the plastid would be predicted under Cu limitation.

The contrasting differential expression of GAPDH in plastid and mitochondrial compartments suggests the possibility of a key role for triose-phosphate transporters. Several of them have been identified in *T. pseudonana* and shown to be located in the chloroplast or its bounding membranes (Moog et al., 2015). A search of our *T. oceanica* proteome showed one potential mitochondrial transporter, but so far, there is no experimental evidence that any of these is located in the mitochondrial outer membrane.

Interestingly, Hockin et al. (2012) postulated that *T. pseudonana* increases glycolytic activity when nitrogen starved. However, when we mapped the involved proteins in *T. pseudonana* to their cellular target compartments, a regulation of isoenzymes similar to the response of Cu-limited *T. oceanica* emerged (i.e., PK downregulated in mitochondria and upregulated in the cytosol, Figure S3, Table S4). Thus, the coordinated regulation of particular glycolytic isoenzymes to distribute NAD(P)H-reducing equivalents and/or ATP production might be a general trait in diatoms.

### 3.2 Nitrogen metabolism is essential for Fd<sup>red</sup> oxidation

Another striking feature in the response to Cu limitation in *T. oceanica* was the upregulation of nitrogen acquisition and assimilation as seen in the increased expression of six out of eight proteins involved as well as the electron donor/acceptor Fdx (Figure 5, Table S6, overview Figure S5). In plants, nitrogen assimilation is an important sink for excess NAD(P)H (Hoefnagel et al., 1998). In *T. oceanica*, the increased expression may alleviate the stress incurred by low Cu, namely, by re-oxidizing Fd<sup>red</sup> in the chloroplast. This could be achieved via upregulation of only those NiR isoenzymes that use Fd<sup>red</sup> as their cofactor (To00016, To02363). Glutamine synthase (GSII, To31900) and the Fd<sup>red</sup>-dependent GOAT (To13288) were also upregulated, thereby potentially easing the chloroplast electron pressure. The
3.3 | Counteracting ROS: Glutathione, TXN, and SODs

An enhanced nitrogen assimilation increases glutamate, which can be incorporated into (or be a precursor of) glutathione (GSH, \(\gamma\)-glutamyl-L-cysteine-glycine) to detoxify ROS via either direct scavenging or the ascorbate-glutathione cycle (Foyer & Noctor, 2011). Glutathione biosynthesis involves (i) the cytosolic GCL (also known as GCS) that combines glutamate and cysteine to \(\gamma\)-glutamyl-L-cysteine and (ii) the plastid glutathione synthase (GSS) that adds glycine. Strikingly, both proteins were upregulated in Cu-limited *T. oceanica*. However, in plants, the rate-limiting step in glutathione production is cysteine biosynthesis (Zechmann, 2014). Under Cu limitation, two chloroplast CYS isoenzymes were upregulated (CS, To27524 and To10442; Figure 7, Table 3, Table S7) suggesting an increase in glutathione production. Furthermore, GST was one of the most highly upregulated proteins (To09062), which would be able to add glutathione to nucleophilic groups to detoxify oxidative stress (Gallogly & Mieyal, 2007). The upregulation of GR (To07268), which oxidizes the overabundant NAD(P)H in the chloroplast further supports our hypothesis that in *T. oceanica*, glutathione counteracts ROS.

TXNs are important redox regulators in plants, especially in the chloroplast (Balmer et al., 2003), although their role in diatoms is unclear (Weber et al., 2009). In *T. oceanica*, the levels of three TXNs were increased, and each one was targeted to a different compartment: the chloroplast (TXN, To31425), the cytosol (To05213), and the mitochondria (To31425).

Another defense mechanism against ROS is the production of SODs, which catalyze the conversion of superoxide radicals into hydrogen peroxide and oxygen. Of the three SODs identified in Cu-limited cultures, two were upregulated: chloroplast Mn/Fe-SOD (To02860) and cytosolic Ni-SOD (To10112). Thus, under Cu limitation, cells may be able to control ROS levels by increasing the expression of both glutathione and SODs. The increase of TXN isoenzymes in all three major cellular compartments (i.e., cytosol, chloroplast, and mitochondria) points to their involvement in sensing the cellular redox state and regulating excess NAD(P)H.

3.4 | The malate shunt drains NAD(P)H-reducing equivalents from the chloroplast to the mitochondria, thus integrating the nitrogen and carbon metabolisms

The efficiency of photosynthesis (both electron transport and carbon fixation) depends on an adequate supply of ATP/ADP and NAD(P)H/NAD(P)\(^+\) (Allen, 2002). In plants, the malate shunt can channel excess NAD(P)H-reducing equivalents from the chloroplast to other cellular compartments, via the differential regulation of MDH isoenzymes.
In this process, NAD(P)H in the chloroplast reduces oxaloacetate (OAA) to malate, a compound that can be transported across membranes and re-oxidized, resulting in the production of NAD(P)H in the target compartment. NAD(P)H can then be used in reactions such as nitrate reduction in the cytosol or ATP production in the mitochondria.

In diatoms, the interaction between the chloroplast and mitochondria is expected to be multifaceted, possibly with direct exchange of ATP/ADP (Bailleul et al., 2015) and indirect exchange of NAD(P)H via the ornithine/glutamate shunt (Broddrick et al., 2019; Levering et al., 2016) and the malate/aspartate shunt (Bailleul et al., 2015; Prihoda et al., 2012). Some support for the spatial interconnectedness between chloroplast and mitochondria in diatoms has been reported recently (Fiori et al., 2017). However, the location of the potential transporters needed (e.g., malate/2-oxoglutarate antiporter and glutamate/aspartate antiporter) have yet to be proven (Bailleul et al., 2015; Kim et al., 2016).

The proteomic patterns we present here support the existence and activation of the malate shunt in *T. oceanica* in response to low Cu. We observe the increased expression of chloroplast and mitochondrial MDH (MDH2, To30817; MDH1, To03405), as well as mitochondrial AAT (AAT2, To15049, Figure 6). As described by Kim et al. (2016) in *P. tricornutum*, chloroplast OAA is reduced to malate via MDH2. Malate is then transported into the mitochondria via a putative malate/2-oxoglutarate antiporter. NAD(P)H-reducing equivalents are released in the mitochondria via the re-oxidation of malate to OAA by mitochondrial MDH1. In turn, mitochondrial AAT2 transfers an amine group from glutamate to OAA, thereby releasing aspartate and 2-oxoglutarate into the mitochondria. To close the cycle, aspartate is transported back, via a glutamate/aspartate antiporter, into the chloroplast where the plastidial AAT isoenzyme would resupply OAA (Kim et al., 2016). However, in *T. oceanica*, chloroplast AAT was significantly downregulated. We suggest that chloroplast OAA, the substrate for MDH2, would be resupplied in the chloroplast via the ATP-dependent carboxylation of pyruvate due to the significant upregulation of PC. This would lead to a net decrease of NAD(P)H in the chloroplast and a net increase of NAD(P)H in the mitochondria. Furthermore, the channeling of NAD(P)H-reducing equivalents towards respiration, instead of the CBB cycle, is supported by a 66% decrease in C fixation, while respiration rates remained constant (Hippmann et al., 2017).
Fructose-bisphosphate aldolase (FBA) isoenzymes in *Phaeodactylum tricornutum* (Pt) and homologs in *Thalassiosira oceanica* (To, CCMP 1003): Information on *P. tricornutum* as per Allen et al. (2012)

| Gene name (Pt)a | FBA classb | Phylogenetic ancestryc | Location in Pt*d | Pt id* | Pt mRNA lowFe† | To homologg | Protein ratio lowCuhh |
|----------------|-----------|------------------------|------------------|--------|---------------|-------------|----------------------|
| FbaC1          | Class II  | Chromalveolate specific gene duplication of FbaC2 prior to diversification | Chloroplast, Pyrenoid | Bd825  | ↑ > 25       | To00388     | (1.4)               |
| FbaC2          | Class II  | Endosymbiotic gene transfer from prasinophyte-like green algal ancestor | Chloroplast, diffuse | Pt22993 | ↓ < 20       | To12069      | (2.0)               |
| Fba3           | Class II  | Heterokont host of secondary endosymbiosis | Cytosol           | Pt29014 | ↑ > 10       | To24977      | ±                   |
| Fba4           | Class I   | Bacterial like (unknown in non-diatom eukaryotes) | Cytosol, putative cytoskeletal interaction | Pt42447 | ~ 1          | To24978      | (2.0)               |
| FbaC5          | Class I   | Endosymbiotic gene transfer from red algal ancestor (with selective gene loss in some centric diatoms) | Chloroplast, Pyrenoid | Pt51289 | ↑ > 80       | To02112      | ±                   |

Abbreviations: FBA, fructose-bisphosphate aldolase; Pt, *Phaeodactylum tricornutum*; To, *Thalassiosira oceanica*.

aAs per Allen et al. (2012).
bClass I uses a metal co-factor, Class II uses a Schiff base.
cAs per Allen et al. (2012).
dAs per Allen et al. (2012) using GFP-fusion proteins.
eNCBI identifier.
fFold change of mRNA transcript levels in acute Fe limited versus Fe replete cultures; arrows indicating upregulation and downregulation.
gAs per Allen et al. (2012).
hFold change of protein levels in chronic Cu limited versus Cu replete cultures.

The expected increase in 2-oxoglutarate and aspartate in the mitochondria, due to an upregulation of mitochondrial AAT2, could be helpful for the cell. If the putative malate/2-oxoglutarate antiporter is indeed involved in the malate shunt, 2-oxoglutarate would be transported back into the chloroplast. As chloroplast AAT is downregulated, 2-oxoglutarate could be used as a substrate for the upregulated Fdx-dependent GOGAT in nitrogen assimilation. Any surplus 2-oxoglutarate in the mitochondria could feed into the citrate cycle. Fittingly, aconitase (To20545) and IDH (To34595), the two proteins involved in the citrate cycle immediately before 2-oxoglutarate, were both significantly downregulated (Figure 3).

Mitochondrial aspartate can be channeled into the urea cycle, where it will produce argininosuccinate, which can then be diverted back into the mitochondrial citrate cycle as fumarate via the aspartate/fumarate shunt (Allen et al., 2011). Thus, even though two of the first three steps in the citrate cycle were downregulated, we hypothesize that the malate shunt in combination with the urea cycle would ensure the continuation of this vital metabolic pathway by supplying it with essential carbon skeletons, that is, 2-oxoglutarate and fumarate.

In addition to the malate shunt, other pathways have been proposed to alleviate electron pressure in diatoms. In *P. tricornutum*, modeling experiments suggest the prevalence of the glutamine-ornithine shunt over the malate shunt (Broddrick et al., 2019). However, none of the homologs involved in this shunt were identified in Cu-limited *T. oceanica* (e.g., *n*-acetyl-γ-glutamyl-phosphate reductase; *n*-acylornithine aminotransferase). Furthermore, the activation of alternative oxidase (AOX) in Fe-limited *P. tricornutum* to alleviate electron stress in the impaired mitochondrial respiration (Allen et al., 2008) was not observed in Cu-limited *T. oceanica* (Hippmann et al., 2017). Future research is needed to elucidate the regulation of shuttle system/compartmental cross talks in diatoms.

**4 | CONCLUSIONS**

The success of diatoms in the modern ocean is thought to be due to their complex genomic makeup and their successful integration and versatility of metabolic pathways. This was exemplified in the present study, where our proteomic data suggest how interaction among metabolic pathways act to maximize growth in *T. oceanica* (CCMP 1003) acclimated to severe Cu-limiting conditions. The differential expression of glycolytic isoenzymes located in the chloroplast and mitochondria may enable them to channel both excess electrons and/or ATP between these compartments. We found additional evidence for chloroplast–mitochondrial cross-talk in the reciprocal expression of chloroplast and mitochondrial isozymes involved in the proposed malate shunt, which could result in transferring both NAD(P)H-reducing equivalents and carbon skeletons from the chloroplast to the mitochondria. The upregulation of Fdx was correlated with upregulation of plastidial Fdx-dependent isoenzymes involved in nitrogen assimilation as well as enzymes involved in glutathione synthesis, thus integrating nitrogen uptake and metabolism with photosynthesis and oxidative stress resistance.
5 | METHODS

Cultures of the centric diatom *T. oceanica* strains CCMP 1003 and CCMP 1005 (here referred to as TO03 and TO05, respectively) were grown under Cu-replete and Cu-limited conditions. Proteins were extracted, purified, and analyzed by LC–MS/MS as detailed in Hippmann et al. (2017). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD006237.

5.1 | Statistical analysis of differential protein expression

As described in Hippmann et al. (2017), peptides were labeled with different isotopologues of formaldehyde depending on their nutrient regime (i.e., control, lowCu, lowFeCu), then mixed in a 1:1:1 ratio and analyzed by LC–MS/MS. Differential expression is then derived from the ratio of the intensities (area under the curve) of the light and heavy peaks for each peptide (see schematic in Figure S1). All three nutrient regimes (control, lowCu, lowFeCu) were processed together to be as consistent as possible and to decrease the number of false positives. However, in the present study, we discuss the lowCu regime (i.e., control, lowCu, lowFeCu), then mixed in a 1:1:1 ratio and different isotopologues of formaldehyde depending on their nutrient regime (Armbrust et al., 2004; Oudot-Le Secq et al., 2007).

We defined two levels of statistically significant difference in expression: (i) greater than or equal to 2-fold (highly regulated) or (ii) between 1.3- and 2-fold (regulated). In addition, the result must be found in at least two of the three biological replicates and result in a *p* value of <.05 for the z test, determining significant difference of the average ratios between treatments, taking the variance into account. Additionally, any protein that had a differential expression ratio of >10 (upregulated) or <0.1 (downregulated) in at least one biological replicate was considered to be an all-or-nothing response and was included in the “significantly changed” set.

5.2 | Protein annotation and targeting prediction

Predicted proteins from both the publicly available genome of TO05 (CCMP1005) and our transcriptome of TO03 (CCMP 1003) were searched against a comprehensive sequence database, PhyloDB for functional annotation using BlastP. PhyloDB version 1.076 consists of 24,509,327 peptides from 19,962 viral, 230 archaeal, 4,910 bacterial, and 894 eukaryotic taxa. It includes peptides from the 410 taxa of the Marine Microbial Eukaryotic Transcriptome Sequencing Project (http://marinemicroeukaryotes.org/), as well as peptides from KEGG, GenBank, JGI, ENSEMBL, CAMERA, and various other repositories. To predict gene localization for proteins involved in carbon and nitrogen metabolism, four in silico strategies were followed: (i) sequences of candidate genes were compared with the publicly available chloroplast genomes of *T. oceanica* (CCMP 1005) (Lommer et al., 2010) and *T. pseudonana* (Armburst et al., 2004; Oudot-Le Secq et al., 2007); (ii) the diatom-specific chloroplast targeting sequence software ASAFind (Gruber et al., 2015) was used in conjunction with SignalP 3.0 (Petersen et al., 2011) to find nuclear encoded, chloroplast targeted proteins; (iii) SignalP and TargetP (Emanuelsson et al., 2007) were used for mitochondrial targeting; and (iv) comparison with curated subcellular locations of the closest homologs in *T. pseudonana*, *P. tricornutum*, and *F. cylindricus* genomes. We acknowledge that deducing cellular targeting via comparison to predicted or experimentally verified proteins in other diatoms can be challenging, as homologs can be found in different compartments depending on species (Gruber et al., 2015; Gruber & Kroth, 2017; Schober et al., 2019). The corresponding names of all protein abbreviations used throughout the present study (text and figures) are given in Table 1.

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AUTHOR CONTRIBUTIONS

AAH, AEA, LJF, BRG, and MTM planned and designed the research. AAH performed cell culturing, protein purification, statistical analysis, curated protein annotation, and targeting prediction. KMM performed protein purification and LC–MS/MS. JPM, under the guidance of AEA, created the RNAseq dataset and provided bioinformatic assistance to AAH. AAH, BRG, and MTM wrote the manuscript.

CONFLICT OF INTEREST

The Authors did not report any conflict of interest.

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SUPPORTING INFORMATION
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