Cellular Responses Associated with ROS Production and Cell Fate Decision in Early Stress Response to Iron Limitation in the Diatom Thalassiosira pseudonana

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Supporting Information

ABSTRACT: Investigation of how diatoms cope with the rapid fluctuations in iron bioavailability in marine environments may facilitate a better understanding of the mechanisms underlying their ecological success, in particular their ability to proliferate rapidly during favorable conditions. In this study, using in vivo biochemical markers and whole-cell iTRAQ-based proteomics analysis, we explored the cellular responses associated with reactive oxygen species (ROS) production and cell fate decision during the early response to Fe limitation in the centric diatom Thalassiosira pseudonana. Fe limitation caused a significant decrease in Photosystem (PS) II photosynthetic efficiency, damage to the photosynthetic electron transport chain in PS I, and blockage of the respiratory chain in complexes III and IV, which could all result in excess ROS accumulation. The increase in ROS likely triggered programmed cell death (PCD) in some of the Fe-limited cells through synthesis of a series of proteins involved in the delicate balance between pro-survival and pro-PCD factors. The results provide molecular-level insights into the major strategies that may be employed by T. pseudonana in response to Fe-limitation: the reduction of cell population density through PCD to reduce competition for available Fe, the reallocation of intracellular nitrogen and Fe to ensure survival, and an increase in expression of antioxidant and anti-PCD proteins to cope with stress.

KEYWORDS: diatom, iTRAQ-based proteomics, Fe-limitation stress, programmed cell death (PCD), reactive oxygen species (ROS), phytoplankton

INTRODUCTION

Diatoms are believed to be the most important primary producers in the ocean,1 accounting for approximately 40% of total marine primary productivity.2 These organisms are extensively distributed, are often dominant in well-mixed coastal and open-ocean upwelling regions,3 warm oligotrophic gyres,4 and the Southern and Arctic Oceans,5 and are even adapted to the subzero hypersaline conditions of brine channels within polar ice.6 Diatoms also grow rapidly with high turnover rates and often form massive blooms.1,7 It is of great interest to understand how and why diatoms have been able to achieve such remarkable prominence in contemporary oceans. It has been suggested that the ecological success of diatoms can be at least partially attributed to the unique mechanisms through which they cope with the wide range of fluctuations in the complex marine environment, such as variations in the supply of nutrients (most notably nitrogen, phosphorus, silicon, and iron).

The critical roles of iron (Fe) in diatom growth and primary production are now well established. Phytoplankton growth in major high-nutrient, low-chlorophyll (HNLC) waters can be limited by Fe deficiency (e.g., Martin et al., 1991).8 Open ocean Fe-enrichment experiments in HNLC areas9–11 together with laboratory studies have demonstrated that diatoms have multilevel strategies to endure long-term (or chronic) Fe limitation (herein referred to as acclimated response). For low Fe quota diatom species such as Phaeodactylum tricornutum,
**Thalassiosira oceanica** and *Pseudo-nitzschia multiseries*, several studies have indicated their unique high tolerance to chronic Fe-limitation stress and rapid responses to replenishment.\(^{12–16}\) However, *Thalassiosira pseudonana*, as a high Fe quota diatom species, has exhibited distinctive tolerance and different adaptation mechanisms in response to Fe-limitation conditions.\(^{13,17}\) For example the enhancement of photorespiration adaptation mechanisms in response to Fe-limitation could be distinguished from acclimated Fe-limitation, which is consistent with the markers of apoptosis and protein products involved in the early stress response to Fe-limitation (the first 3 or 4 days) and then “acclimate” to Fe limitation on the following days, showing thus a physiological adjustment to Fe limitation from an early stress response to latter acclimated response.\(^{19}\) To date, although many studies have focused on the fundamental cellular response of diatoms to various Fe conditions such as Fe long-term stress, Fe-acclimation, Fe-spiking, and Fe-enrichment (see list of previous studies in Nunn et al, 2013\(^{18}\)), studies of early stress responses in diatoms is very limited. Notwithstanding, it is likely that they often suffer from short-term Fe limitation (defined here as early stress response), particularly during bloom progression when they are likely to be subjected to rapid nutrient fluctuations, including rapid variations in Fe bioavailability.\(^{7,20}\) Therefore, it is necessary to investigate the cellular responses associated with ROS production and cell-fate decisions during the early stages of Fe limitation and the related cell-fate decision mechanisms, which is based on the balance of the functions of antioxidant and anti-PCD proteins and PCD-induced proteins. Furthermore, the data also provide a molecular-level understanding of the early stress response to Fe-limitation in *T. pseudonana*.

### MATERIALS AND METHODS

#### Culture Conditions

*Thalassiosira pseudonana* (Hust.) Hasle et. Heimdal (strain CCMP 1335) was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA, formerly known as the CCMP, https://ncma.bigelow.org/). The culture was grown in 1/2 medium at 18 °C under exposure to 60 micromol photons m\(^{-2}\) s\(^{-1}\) in a 12-h light/12-h dark regime. A starter culture of *T. pseudonana* was grown in 1/2 medium to the midexponential phase (\(~3.0 \times 10^8\) cells mL\(^{-1}\)). The cells were then pelleted via centrifugation (10 000 × g, 10 min) and washed once with filtered (0.45 \(\mu\)m pore-size) autoclaved seawater (FSW) for inoculation of duplicate 4-L cultures of either replete 1/2 medium (Fe-replete, +Fe) or 1/2 medium without added iron (Fe-limited, -Fe) at a cell concentration of \(1.5 \times 10^6\) cells mL\(^{-1}\). Seawater was collected from far offshore of Xiamen harbor and the seawater contained dissolved-Fe at very low concentrations. The duration of the experimental phase was 7 days.

#### Analysis of Physiological Parameters

Triplicate samples were collected daily and assessed for cell abundance and photosynthetic health. Cell numbers were counted using an Olympus microscope and a Qiajing hemocytometer at the same time every day.\(^{43}\) The cell density considered to be toxic and to be able to trigger PCD.\(^{37,38}\) For instance, Vardi et al. (1999) reported that CO\(_2\) limitation results in the formation of ROS to a level that induces PCD in the dinoflagellate *Peridinium gatunense*.\(^{25}\) ROS accumulation induced by Fe limitation was also demonstrated to be involved in the triggering of PCD in *T. pseudonana*.\(^{19}\) Moreover, the colocalization of PtNOA (nitric oxide-associated protein) and Mn-superoxide dismutase (MnSOD) in the chloroplast of the diatom *P. tricornutum* is likely to be of relevance for ROS metabolism.\(^{26,39}\) Although the diatom responses to various adverse environmental factors may show some commonalities, ROS generation triggered by various stresses is generally attributed to different mechanisms.\(^{40}\) Therefore, it is necessary to investigate the mechanisms of ROS production triggered by early stress of Fe limitation and their role in governing decisions related to cell-fate in diatoms.

As a complement to previous studies of cellular responses to Fe availability in *T. pseudonana*, a proteomics analysis was employed here to investigate its cellular responses associated with ROS production and cell-fate decisions during the early stress response phase of Fe limitation. Because PCD has also been found to be initiated in aging cultures,\(^{21}\) cells in the exponential phase of growth were chosen for the proteomics analysis. We combined in vivo biochemical markers with the well-developed iTRAQ-based (isobaric tags for relative and absolute quantitation) proteomics approach\(^{11,42}\) for the detection of the concomitant induction of ROS, caspase-specific activity, externalization of phosphatidylserine, cell death, and the suite of proteins involved in ROS production and cellular responses to oxidative stress. Our results address the biochemical machinery of ROS production triggered during the early stages of Fe limitation and the related cell-fate decision mechanisms, which is based on the balance of the functions of antioxidant and anti-PCD proteins and PCD-induced proteins. Furthermore, the data also provide a molecular-level understanding of the early stress response to Fe-limitation in *T. pseudonana*.
(cells mL\(^{-1}\)) was calculated as follows: \( CD = (N/80) \times 400 \times 10^6 \), where \( CD \) is the cell density, and \( N \) is the cell abundance counted in 80 grids on the slide.

The photochemical yield of photosystem (PS) II \((F_\text{p}}/F_\text{m})\) was measured by XE-PAM (Waltz, Germany). The measurements were performed in duplicate. Fifteen microliters of cells were harvested onto 2-μm-pore-size polycarbonate filters, snap frozen in liquid nitrogen, and stored at −80 °C until \( F_\text{p}}/F_\text{m} \) analysis. Ten milliliters of the filtered medium (0.45-μm pore size) was sampled daily and stored at −20 °C for determination of nutrient concentrations.

**Determination of Nitrogen, Phosphorus, and Silicon Concentrations**

The concentrations of N—NO\(_3^-\), P—PO\(_4^{3-}\) and Si—SiO\(_2^{2-}\) in the medium were measured daily according to classic colorimetric methods using a Technicon AA3 Auto-Analyzer (BRAN+LUEBBE, GmbH, Germany). N—NO\(_3^-\) was analyzed using the copper–cadmium column reduction method (Method No. G-172-96 Rev.7, BRAN+LUEBBE, Germany), P—PO\(_4^{3-}\) was determined by the molybdenum blue method (Method No. G-175-96 Rev.8, BRAN+LUEBBE, Germany), and Si—SiO\(_2^{2-}\) was measured using silicon–molybdenum blue spectrophotometry (Method No. G-177-96 Rev.$\S$, BRAN +LUEBBE, Germany). The detection limits for N—NO\(_3^-\), P—PO\(_4^{3-}\), and Si—SiO\(_2^{2-}\) were 0.015, 0.024, and 0.03 μmol L\(^{-1}\), respectively.

**Transmission Electron Microscopy (TEM)**

The internal cell morphology of *T. pseudonana* cells was observed daily from day 2 by transmission electron microscopy (TEM). The cells were collected via centrifugation (4 °C, 10 000×g, 5 min) and fixed in 500 μL of 2.5% glutaraldehyde fixative (pH 7.4) for 2 h. The cells were then rinsed once, resuspended in 0.1 M PBS (pH 7.4), and stored at 4 °C (for less than 1 week) prior to the next treatment. The fixed cells were rinsed three times for 15 min in 0.1 M PBS (pH 7.4), postfixed for 2–3 h in 1% buffered OsO\(_4\), and washed three times with 0.1 M PBS (pH 7.4). After the supernatant was removed by centrifugation (4 °C, 10 000×g, 5 min), the pellets were dehydrated through a graded series of ethanol and then with propylene oxide. The cells were then embedded in an Epon812 Embedding medium (3 g of DDSA 3 g, 7 g of MNA, and 10 g of Epon812 in 0.32 mL of DMP). Sections were cut using a PowerTomo-XL ultramicrotome (RMC, U.S.A.), collected on 200-mesh copper grids, and stained with uranyl acetate and lead citrate. The stained sections were visualized and photographed using a H-7560 electron microscope (HITACHI, Japan).

**In Vivo Cell Staining and Flow Cytometry**

Cells were collected daily from day 2, pelleted via centrifugation (10 000×g, 10 min, 4 °C), resuspended in buffer, and stained with the following: SYTOX green (1 μM; Invitrogen) to visualize live/dead cells, z-VAD-FMK-FITC (20 μM; CaspACE; Promega, Madison, WI, U.S.A.) to detect activated caspasases, Annexin V (10 μL/100 μL cells; Invitrogen) to detect the externalization of phosphatidylserine, and CM-H2DCFDA [5 μM; 5- (and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; Invitrogen] to detect intracellular ROS. The detailed protocol was previously described. The percentage of positively stained cells (from a total of 10 000 cells) was determined at 520 nm after excitation with a 488 nm laser using a flow cytometer (BD Fortessa, U.S.A.). The gating and data analysis were performed using FlowJo analytical software.

**Protein Extraction and Preparation**

According to the physiological and biochemical results obtained for *T. pseudonana* during previous experiments, day 4 (middle exponential phase of growth) was chosen as the sampling time point for the iTRAQ-based proteomics analysis. Protein extraction was conducted according to the methods described by Du et al. (2014). Briefly, approximately 1 L of culture was collected through a 2 μm pore-size filter membrane for each sample. Cells on the membrane were then resuspended with 10 mL medium into a 15 mL centrifugal tube. After the cell pellets were collected by centrifuging at 3000×g for 5 min, 10 mL of TRizol Reagent (Invitrogen, Life Technologies) was added, and the protein was then extracted according to the manufacturer’s recommendations.

For protein preparation, the protein pellets were suspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris-HCl, pH 8.5), then incubated with 10 mM DTT at 56 °C for 1 h to reduce the disulfide bonds. After that, 55 mM IAM was added (final concentration), and the samples were incubated for 1 h in the dark to block the cysteine residues of the proteins (alkylate). The reduced and alkylated protein mixtures were precipitated by adding 4× volume of chilled acetone at −20 °C overnight. After centrifugation on at 4 °C, 30 000×g, the pellet was dissolved in 0.5 M TEAB (tris(hydroxymethyl)aminomethane bicarbonate, Applied Biosystems, Milan, Italy) and sonicated in ice. After centrifuging at 30 000×g at 4 °C, an aliquot of the supernatant was taken for determination of protein concentration by Bradford Protein Assay Kit according to the manufacturer’s protocol. The proteins in the supernatant were kept at −80 °C until further analysis.

**iTRAQ Labeling and SCX Fractionation**

A total of 100 μg proteins from each sample were digested with Trypsin Gold (Promega, Madison, WI, U.S.A.) with the ratio of protein/trypsin = 30:1 at 37 °C for 16 h. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer’s protocol for 8-plex iTRAQ reagent (Applied Biosystems, Foster City, CA, U.S.A.). Four samples (two biological replicates for Fe-replete and Fe-limited, respectively) were labeled with different iTRAQ tags: 113- and 114-iTRAQ tags for Fe-replete replicate 1 and 2, respectively; and 119- and 121-iTRAQ tags for Fe-limited replicate 1 and 2, respectively. The peptides were labeled with the isobaric tags, and then incubated at room temperature for 2h. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation.

**SCX (Strong Cationic Exchange) chromatography**

was performed using a Shimadzu LC-20AB HPLC pump system (Shimadzu, Kyoto, Japan). The fractionated peptides were first reconstituted in 4 mL of buffer A (25 mM NaH\(_2\)PO\(_4\) in 25% ACN, pH 2.7) and then loaded onto a 4.6 × 250 mm\(^2\) Ultremex SCX column containing S-μm particles (Phenomenex). The peptides were eluted at a flow rate of 1 mL/min with a gradient of buffer A for 10 min, 5–60% buffer B (25 mM NaH\(_2\)PO\(_4\), 1 mL KCl in 25% ACN, pH 2.7) for 27 min, 60–100% buffer B for 1 min. The system was then maintained at 100% buffer B for 1 min before equilibrating with buffer A for 10 min before the next injection. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every minute. The eluted peptides were then pooled...
into 20 fractions, desalted with a Strata X C18 column (Phenomenex), and vacuum-dried.

**LC-ESI-MS/MS Analysis**

Each fraction was resuspended in solution A (2% ACN, 0.1% FA) and centrifuged at 20 000×g for 10 min. The final concentration of peptide was about 0.5 μg/μL on average. Ten μL supernatant was then loaded on a Shimadzu LC-20AD nano HPLC by the autosampler onto a C18 trap column, and the peptides were eluted onto an analytical C18 column (inner diameter of 75 μm) packed in-house. The samples were collected at a rate of 8 μL/min for 4 min. A 44 min gradient was then run at a rate of 300 nL/min starting from 2% to 35% solution B (98% ACN and 0.1% FA), followed by a 2 min linear gradient to 80% solution B, maintenance at 80% solution B for 4 min, and return to 5% in 1 min.

The peptide samples were then subjected to nano-electrospray ionization for tandem mass spectrometry (MS/MS) in a Q Exactive (Thermo Fisher Scientific, San Jose, CA, U.S.A.) coupled online to the HPLC. Intact peptides were detected in the Orbitrap at a resolution of 70 000. The peptides were chosen for MS/MS using the high-energy collision dissociation (HCD) operating mode with a normalized collision energy setting of 27.0 and a stepped NCE of 12.0%. The ion fragments were detected in the Orbitrap at a resolution of 17 500. A data-dependent procedure that alternated between one MS scan followed by 15 MS/MS scans was applied for the 15 most abundant precursor ions above a threshold ion count of 20 000 in the MS survey scan with subsequent dynamic exclusion duration of 15 s. The electrospray voltage applied was 1.6 kV. Automatic gain control (AGC) was used to optimize the spectra generated by the Orbitrap. The AGC target for full MS was 3e6 and 1e5 for MS2. For MS scans, the m/z scan range was 350 to 2000 Da. For MS2 scans, the m/z scan range was 100–1800.

**Proteomic Data Analysis**

Raw data files acquired from the Orbitrap were converted into MGF files using Proteome Discoverer 1.2 (PD 1.2, Thermo), and the MGF file was searched. The peptide and protein identifications were performed through the Mascot search engine (ver. 2.3.02; Matrix Science, London, U.K.) against Joint Genome Institute database for the *T. pseudonana* genome (http://genome.jgi-psf.org/Thaps3/Thaps3.home.html, October, 2012, which contains a total of 34 736 sequences including the Thaps3 finished chromosomes and Thaps3_bd unmapped sequence data (386 sequences)) and NCBI.

For protein identification, a mass tolerance of 0.05 Da (the tolerance was “± 0.05 Da”) was permitted for intact peptide masses and ±0.1 Da for fragmented ions, with allowance for one missed cleavage in the trypsin digests. Gln → pyro-Glu (N-term Q), Oxidation (M), Deamidated (NQ) as the potential variable modifications, and Carbamidomethyl (C), iTRAQ 8plex (N-term), iTRAQ 8plex (K) as fixed modifications. The charge states of peptides were set to +2 and +3. For reducing the probability of false peptide identification, only peptides at the 95% confidence interval by a Mascot probability analysis greater than “identity” were counted as identified.

For protein quantitation, it was required that a protein contains at least two unique peptides. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. The student’s t test was performed using the Mascot 2.3.02 software. We only used ratios with p-values <0.05, and only fold changes of >1.5 were considered as significant.

**Function Annotation Description**

Functional annotations of the proteins were conducted using Blast2GO program against the nonredundant protein database.
(NR; NCBI). GO (Gene Ontology) enrichment analysis was carried out for the differentially regulated proteins to determine the affected cellular metabolism. Cluster of Orthologous Groups (COG, http://www.geneontology.org), a system for automated detection of homologues among the annotated genes of several completely sequenced eukaryotic genomes, was used to compare identified proteins to COG database for predicting the function of identical proteins and for functional classification and statistics. The analysis was performed by matching the responsive proteins to the proteins annotated with a COG term and then comparing the frequencies of the responsive proteins in each COG term subset to determine the statistical authenticity of the involvement of that COG term in the Fe-limited responses. A P-value of 0.05 was set as the threshold. KEGG pathway database (KEGG, http://www.genome.jp/kegg/), a collection of manually drawn pathway maps representing knowledge on molecular interaction and reaction networks, was used for protein functional and interaction analysis in different metabolic pathways.

**Quantitative RT-PCR Analysis**

The expression of 11 randomly selected genes encoding identified proteins was performed by quantitative RT-PCR analysis. The primers were designed using Primer-BLAST (Supporting Information, SI, Table S1). Each primer pair was designed to span at least one exon-exon junction such that genome DNA digestion was not needed. At day 4, approximately 30 mL of culture was filtered for Fe-replete and Fe-limited conditions. Samples were rapidly placed in liquid nitrogen with the filter, ground to powder in a precooled traditional mortar, and immediately transferred to TRIzol Reagent (Invitrogen, Life Technologies). Total RNA was then extracted according to the manufacturer’s recommendations. The cDNA was synthesized immediately after the RNA was extracted (ReverTra Ace qPCR RT Kit, TOYOBO, Japan). Quantitative PCR was performed with a Rotor-Gene 6000 system (Corbett Life Science) using the SYBR Green Real-time Quantitative PCR was performed with a Rotor-Gene 6000 system (Corbett Life Science) using the SYBR Green Real-time

**RESULTS AND DISCUSSION**

**General Physiological and Biochemical Responses**

The cells were cultured under Fe-limited conditions (f/2+Si medium without Fe added) and Fe-replete conditions (f/2+Si medium). The photosynthetic efficiency of PSII ($F_v/F_m$), an indicator of Fe limitation in the laboratory, was used as a rapid measure of the physiological status of *T. pseudonana*. On day 4, which corresponds to the exponential phase (Figure 1A), the value of $F_v/F_m$ of the cells exposed to Fe-limitation conditions was markedly reduced, being 20% lower ($\sim 0.50$) compared to Fe-replete cells ($\sim 0.63$). After day 4, the value of $F_v/F_m$ slightly increased, indicating that the cells were exhibiting some physiological adjustment to Fe limitation on days 5 and 6. The growth rate and maximum cell abundances of the Fe-limited cells on day 4 were much lower than those of the Fe-replete cells: 17% lower growth rate ($\sim 0.50$ in limited versus $\sim 0.63$ in replete) and 41% fewer cells ($\sim 2.33 \times 10^7$ in limited versus $\sim 3.93 \times 10^7$ in replete), respectively (Figure 1B).

The notable differences in the degree of in vivo cell staining for ROS production (oxidative stress), externalization of phosphatidylserine (a morphological characteristic of the early stages of PCD), caspase activity (caspases are a specific class of intracellular cysteinyl aspartate-specific proteases that initiate and execute PCD in metazoans), and cell mortality (visualization of live/dead cells) between cells exposed to Fe-replete and -limiting conditions during days 2–7 are shown in Figure 2 and Table 1. After 4 days of exposure, 24% of the Fe-limited cells stained positive for ROS compared with 6% of the Fe-replete cells (Figure 2A). This observation indicates that Fe-limitation caused a most marked increase in ROS (more than 4-fold) on day 4 compared with the Fe-replete cultures, although the percent of positive stained cells for intracellular ROS was not
the highest on day 4. This result was consistent with the most marked decrease in $F_v/F_m$ data on day 4 shown in Figure 1A, which indicates that the cells suffered from acute oxidative stress (early stress response) before attempting to physiologically adjust to the Fe-limited conditions after day 4. Correspondingly, the percent of positively stained cells for the externalization of phosphatidylserine (Annexin) (16%) and caspase activity (CaspACE) (25%) increased significantly by around 3-fold on day 4 in the Fe-limited cultures (Figure 2B,C). However, the ratio of dead cells was not significantly different between the Fe-limited and the Fe-replete cultures from days 2−6 (Figure 2D). These findings suggest that some cells were undergoing PCD due to intense oxidative stress on day 4 but were not actually dead.

### Table 1. Percentage of Cells Positively-Stained for Intracellular ROS, Externalization of Phosphatidylserine, Caspase Activity, and Dead Cells Based on the in Vivo Staining of *T. pseudonana* Cells from Days 2 to 7 in Fe-Replete (+Fe) or Fe-Limited (−Fe) Conditions

| time (D) | culture | ROS (%) | annexin (%) | caspases (%) | SYTOX (%) |
|----------|---------|---------|-------------|--------------|-----------|
| 2        | +Fe     | 4.1     | 2.6         | 6.5          | 11.9      |
| 3        | +Fe     | 5.1     | 4.8         | 7.0          | 10.3      |
| 4        | +Fe     | 6.6     | 5.7         | 20.0         | 13.0      |
| 5        | +Fe     | 4.4     | 5.1         | 9.6          | 26.6      |
| 6        | +Fe     | 13.8    | 12.6        | 39.2         | 30.7      |
| 7        | +Fe     | 18.8    | 15.5        | 35.5         | 28.1      |
|          | −Fe     | 40.4    | 28.0        | 47.8         | 31.7      |
| 3        | +Fe     | 24.4    | 16.0        | 25.4         | 22.5      |
| 4        | +Fe     | 18.8    | 15.5        | 35.5         | 28.1      |
| 5        | +Fe     | 4.4     | 5.1         | 9.6          | 26.6      |
| 6        | +Fe     | 13.8    | 12.6        | 39.2         | 30.7      |
| 7        | +Fe     | 18.8    | 15.5        | 35.5         | 28.1      |
|          | −Fe     | 40.4    | 28.0        | 47.8         | 31.7      |

### Figure 3. Electron micrographs of cells grown in Fe-replete and Fe-limited conditions. Morphological changes in *T. pseudonana* cells grown in Fe-replete conditions on day 2 (A), day 4 (B) and day 7 (C), and Fe-limited conditions on day 2 (D), day 4 (E), and day 7 (F). n: nucleus; c: chloroplast; m: mitochondria; d: dictyosome; v: vacuole; a: autophagosome; and bars: 1 μm.

To find further evidence that ROS induced PCD on day 4, we examined morphological changes based on TEM observations. The most common hallmarks of PCD previously recorded in *T. pseudonana*,21 such as marked vacuolization, internal degradation (unrecognizable organelles with integral membranes), and chromatin condensation, were indeed observed, as shown in Figure 3E. Morphological characteristics of PCD were also found in the cells on day 7 (Figure 3C,F) under both Fe-replete and Fe-limiting conditions, due to the fact that culture age also induces PCD.21 However, the nucleosomal laddering of DNA, the strongest evidence for PCD in higher eukaryotes, could not be detected (data not shown), which is consistent with the absence of ladders observed in previous studies of PCD in the same species,21 the dinoflagellate *Peridinium gatunense*,25 and yeast.49

### General Results from the iTRAQ Analysis

To unravel the cellular responses associated with ROS production and cell-fate decision during the early stress response to Fe limitation, we harvested cells for iTRAQ-based proteomics analysis on day 4. We labeled and mixed two biological replicates of Fe-replete and Fe-limited samples directly for proteomic analysis. Reproducibility of the proteomics analysis for two comparison samples is shown in Figure S1 (SI), and Pearson correlation coefficients were used to evaluate the similarity between the two replicates. The delta error in the figure represents the difference between the quantitative values of two biological replicates of each treatment (Fe-replete or Fe-limited). The difference was plotted against the percentage of proteins identified, and the comparisons showed that approximately 50% of the proteins had differences with a delta error of less than 0.1, and more than 95% of the proteins had differences of less than 0.5 in both
treatments, suggesting good analytical reproducibility (SI Figure S1).

A total of 883 differentially regulated proteins between the Fe-replete and Fe-limited conditions were confidently identified in both biological replicates using the criteria described in the experimental methods (SI Table S2). Among these proteins, 510 were subjected to Cluster of Orthologous Groups (COG) enrichment analysis to determine the cellular metabolisms most impacted by Fe limitation (data shown in SI Table S3; 75 out of 510 proteins have more than one description). Functional classification of the proteins identified showed that they were involved in almost every aspect of <i>T. pseudonana</i> metabolism (Figure 4). Several key cellular processes, such as post-translational modification, protein turnover, and chaperones (14.9%), energy production and conversion (13.9%), translation, ribosomal structure and biogenesis (10.8%), and amino acid transport and metabolism (10.3%) were significantly affected by the Fe-limiting conditions. Furthermore, most of the “translation, ribosomal structure, and biogenesis” proteins identified were found to decrease in abundance, suggesting an overall slowdown of protein biosynthesis and a possible slowdown of metabolism.

Using a significance cutoff of 1.5-fold change and a P-value less than 0.05 to assess changes in abundance, we determined that a total of 127 proteins showed significant differences. Of these, 41 and 86 unique proteins, respectively, were found to be present in significantly higher and lower relative abundances in the Fe-limited cells compared with the cells exposed to Fe-replete conditions (SI Table S4). The most interesting proteins are summarized in SI Table S5.

### Metabolic Mechanisms Involved in ROS Production

#### Downregulation of Photosynthesis and Accumulation of ROS

Light-harvesting complexes (LHCs) are generally used to harvest and transfer light energy into the reaction centers to drive photosynthesis. Diatoms contain a branch of genes encoding the LHC superfamily known as fucoxanthin-chlorophyll a/c-binding proteins (FCPs). Recent studies have demonstrated that LHCX protein members have a dual role in light energy harvesting and excess expression levels were included, as well to cover a range of important regulatory processes. According to the iTRAQ proteomics analysis, the abundance of seven of the proteins encoded by the seven genes were decreased (i.e., Thaps3|255232, Thaps3|2152, Thaps3|8571, Thaps3|25206, Thaps3|24591, and Thaps3|11118), and four of the proteins encoded by the selected genes were increased in abundance (i.e., Thaps3|20362, Thaps3|21534, Thaps3|3353, and Thaps3|2404). Eight of our quantitative RT-PCR results (Thaps3|255232, Thaps3|2152, Thaps3|8571, Thaps3|25206, Thaps3|24591, Thaps3|3353, Thaps3|2404, and Thaps3|11118) were somewhat consistent with the iTRAQ data, whereas three (Thaps3|20362, Thaps3|21534, and Thaps3|11118) were not (Figure S5), indicating that their protein levels may not necessarily correlate with their mRNA levels. In particular, death-specific protein 1 (Thaps3|11118), an important protein associated with cell death and PCD regulation in phytoplankton (see below), was found at increased mRNA levels but decreased protein abundance. Another two proteins, a cyclin-dependent kinase (Thaps3|20362) and an RNA-binding protein (RRM domain) (Thaps3|21534), both showed decreased abundance of mRNA and increased abundance of protein.

### Quantitative RT-PCR Analysis

A subset of 11 genes encoding the proteins detected in iTRAQ analysis was selected for quantitative RT-PCR analysis. The genes were selected based on the abundance levels of their encoded proteins to ensure that genes with a wide range of expression levels were included, as well to cover a range of important regulatory processes. According to the iTRAQ proteomics analysis, the abundance of seven of the proteins encoded by the seven genes were decreased (i.e., Thaps3|255232, Thaps3|2152, Thaps3|8571, Thaps3|25206, Thaps3|24591, and Thaps3|11118), and four of the proteins encoded by the selected genes were increased in abundance (i.e., Thaps3|20362, Thaps3|21534, Thaps3|3353, and Thaps3|2404). Eight of our quantitative RT-PCR results (Thaps3|255232, Thaps3|2152, Thaps3|8571, Thaps3|25206, Thaps3|24591, Thaps3|3353, Thaps3|2404, and Thaps3|11118) were somewhat consistent with the iTRAQ data, whereas three (Thaps3|20362, Thaps3|21534, and Thaps3|11118) were not (Figure S5), indicating that their protein levels may not necessarily correlate with their mRNA levels. In particular, death-specific protein 1 (Thaps3|11118), an important protein associated with cell death and PCD regulation in phytoplankton (see below), was found at increased mRNA levels but decreased protein abundance. Another two proteins, a cyclin-dependent kinase (Thaps3|20362) and an RNA-binding protein (RRM domain) (Thaps3|21534), both showed decreased abundance of mRNA and increased abundance of protein.

#### Metabolic Mechanisms Involved in ROS Production

**Figure 4.** Functional category (COG) coverage of the proteins identified in <i>T. pseudonana</i> under Fe-limited conditions based on the iTRAQ-LC−MS/MS analysis.
Moreover, the increased protein abundance and transcript level of FCP in Fe-limited T. oceanica cultures have been hypothesized by Lommer et al. (2012) to be associated with light-mediated oxidative stress. One gene of the LHCX family was also found to be significantly up-regulated in Fe-limited P. tricornutum cells. Thus, the increased levels of these FCP proteins could infer that Fe-limited T. pseudonana cells were undergoing oxidative stress.

The abundance of proteins involved in PS II, such as photosystem II reaction center protein D2 (Thaps3|bd|1272, 1.39 fold, \( p > 0.05 \)), photosystem II oxygen-evolving complex 23K protein (Thaps3|2185, 1.22 fold, \( p > 0.05 \)), and photosystem II 44-kDa reaction center protein (Thaps3|bd|1244, 1.23 fold), were increased slightly in our study (Figure 6), resulting potentially in a slight enhancement of PS II activity. The electrons produced in PS II must then be transferred to PS I by plastocyanin and the cytochrome b6f complex (cyt b6f), to ultimately lead to ATP production. However, in this study, most of PS I-related proteins and cytochrome f, a component of cyt b6f (Thaps3|bd|106, 0.64 fold, \( p > 0.05 \)), were decreased in abundance in the Fe-limited T. pseudonana cells. In addition, the abundance of ferredoxin-NADP+ reductase (Thaps3|25892), which catalyzes the last electron transfer from PS I to reduce NADP+

Figure 5. Comparison of iTRAQ and qPCR results for 11 genes. Comparison of the protein (iTRAQ) and mRNA (qPCR) levels of Thaps3|11118, Thaps3|20362, Thaps3|255232, Thaps3|2152, Thaps3|8571, Thaps3|25206, Thaps3|24591, Thaps3|21534, Thaps3|3333, Thaps3|2404, and Thaps3|1669 under Fe-limited conditions. \( \beta \)-actin was used as a housekeeping marker. Fold changes of either more than 1 or less than 1 from iTRAQ and qPCR indicate that they are consistent (red dotted line).

Figure 6. Hypothetical cellular pathways and processes in the diatom T. pseudonana under iron-limited growth conditions. All red words or arrows represent proteins with increased abundance or enhanced pathways, respectively. All green words or arrows represent proteins with decreased abundance or inhibited pathways, respectively. Lhc: Light harvesting complex; PS: Photosystem; OEM: Oxygen-evolving complex; Cyt: Cytochrome; FNR: Ferredoxin-NADP+ reductase; Fd: Ferredoxin; Fd-NiR: Ferredoxin nitrite reductase; ROS: Reactive oxygen species; PCD: Programmed cell death; RUBP: Ribulose-1,5-bisphosphate; TCA: Tricarboxylic acid cycle; FET3: Ferroxidase; TyDSP: T. pseudonana death-specific protein; COX6B1: Cytochrome c oxidase subunit 6B1; EC 5.3.1.1: Triosephosphate isomerase; EC 2.7.2.3: 3-phosphoglycerate kinase; EC 1.2.4.1: Pyruvate dehydrogenase (E1) component; EC 2.3.3.1: Citrate synthase; EC 1.1.1.37: Malate/lactate dehydrogenases; EC 1.6.5.3: NADH-ubiquinone reductase; EC 1.10.2.2: Cytochrome c1; EC 3.6.3.14: ATP synthase; EC 6.3.5.5: Carbamoyl-phosphate synthase; EC 6.3.4.5: Argininosuccinate synthase; and EC 3.5.3.1: Arginase.
to NADP, also decreased 0.56-fold. Therefore, the photosynthetic electron transport chain could be damaged in PS I, which could result in the accumulation of electrons and a concomitant increase in ROS generation. Furthermore, the abundance of some proteins associated with photosynthesis were also found to be slightly decreased, such as oxygen-evolving enhancer protein 1 precursor (Thaps316430, 0.29 fold, \( p > 0.05 \)), high light-induced protein 2 (Thaps321467, 0.65 fold, \( p > 0.05 \)), and ferredoxin (Thaps3_bd11258, 0.55-fold), which further supports the existence of insufficient photosynthetic electron transport in Fe-limited \( P. \) tricornutum cells. In other words, Fe limitation caused the downregulation of photosynthesis and may block electron flow in PS I to result in the excess production of highly toxic ROS, which is consistent with previous results from the diatom \( P. \) tricornutum.

Response of Cellular Respiration and ROS Accumulation. Under Fe-limited conditions, it was found that the three proteins involved in glycolysis, through which glucose is broken down into pyruvate with generation of a small amount of ATP and NADH, were markedly increased in abundance (Figure 6). These proteins are triosephosphate isomerase (EC 5.3.1.1, Thaps316639, 1.68-fold) which catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and \( \alpha \)-glyceraldehyde 3-phosphate, and 3-phosphoglycerate kinase (EC 2.7.2.3, Thaps314980, 1.55-fold and Thaps3126275, 1.54-fold), an enzyme that catalyzes the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate (1,3-BPG) to adenosine diphosphate (ADP) and then produces 3-phosphoglycerate (3-PG) and ATP. Thus, it could be inferred that more pyruvate would enter the mitochondrion and be fully oxidized to produce GTP, FADH\(_2\), and NADH through the TCA cycle. At the same time, three proteins related to the TCA cycle were also significantly increased in protein abundance in the Fe-limited cells, including pyruvate dehydrogenase E1 (Thaps315834, 1.51-fold), citrate synthase (Thaps311411, 1.79-fold), and malate/lactate dehydrogenases (Thaps320726, 1.57-fold). The first protein (Thaps315834) is a component of the pyruvate dehydrogenase complex (PDC), which converts pyruvate into acetyl-CoA and links the glycolysis metabolic pathway to the TCA cycle. Citrate synthase catalyzes the first step of the TCA cycle, and the last protein, namely malate/lactate dehydrogenase, is an enzyme that reversibly catalyzes the oxidation of malate to oxaloacetate by the reduction of NAD\(^+\) to NADH. The increased abundance of these proteins could suggest that \( P. \) pseudonana may prefer to produce more NADH and FADH\(_2\) and other bioenergy molecules by accelerating the decomposition of glucose in response to iron-limited stress.

The respiratory chain consists of a series of protein complexes that transfer electrons from NADH (or FADH\(_2\)) to \( O_2 \) via redox reactions. This chain includes coenzyme Q, cytochrome \( c \), and four membrane-bound complexes (Complexes I, II, III, and IV) (Figure 6). In the present study, it was found that NADH-ubiquinone reductase (H\(^-\)translocating) (EC 1.6.5.3, Thaps3_bd1380) in Complex I was markedly increased in abundance (1.7-fold), which may suggest that more electrons could be delivered to the respiratory chain in Fe-limited cells. However, both abundance of cytochrome \( c_1 \) (Thaps325564, 0.56-fold) of Complex II and a possible cytochrome \( c \) oxidase subunit 6B1 (COX6B1, Uniprot_Swissprot, Thaps31996, 0.48-fold) of Complex IV were significantly decreased, which could indicate that Complexes III and IV were inhibited and thus that the respiratory chain was blocked and electrons were undeliverable. In general, the major sites for ROS generation are Complexes I and III of the electron transport chain. Previous studies have also indicated that the oxidation of either Complex I or Complex II substrates when Complex III is inhibited by antimycin A may increase ROS accumulation. Thus, it can be proposed that under the Fe-limited conditions in the present study, increases in ROS production are likely to occur when Complex III is inhibited. Although cytochrome \( c \) oxidase is not a source of ROS, the inhibition of cytochrome \( c \) oxidase due to the decreased abundance of COX6B1 may facilitate ROS production from Complex I or III. Therefore, our findings also suggest that the observed decreased abundance of COX6B1 could also be considered to be related with the overproduction of ROS. It was found that mitochondrial alternative oxidase (AOX) is used as an alternative electron acceptor to remove excess electrons in Fe limited \( P. \) tricornutum cells because it uses less iron. However, we did not find AOX protein expression in this Fe limited \( P. \) pseudonana based on our proteomic data.

In addition, the decrease in ATP production via the respiratory chain coincided with the blockage of the respiratory chain when \( P. \) pseudonana suffered from Fe-limited stress. In contrast, the abundance of three proteins belonging to the F1 region of FoF1-type ATP synthase (an important enzyme that catalyzes the synthesis of ATP) were found to increase in response to Fe-limited stress in this study, indicating an increase in ATP production. These proteins are F0F1-type ATP synthase \( \alpha \) subunit (Thaps313863, 1.52-fold), \( \beta \) subunit (Thaps317981, 1.67-fold, \( p > 0.05 \)), and \( \epsilon \) subunit (Thaps3_bd1254, 1.60-fold, \( p > 0.05 \)), and all possess the core catalytic functions for the synthesis of ATP. Thus, it could be concluded that the increase in ATP synthesis observed in the present study may be a compensatory mechanism in response to the decrease in ATP production through the respiratory chain.

To conclude, evidence for the accumulation of ROS under Fe-limited conditions has been found to stem from inefficient activity of the electron transport chains in both the photosynthetic and respiratory processes (Figure 6). These results coincide with the high levels of intracellular ROS detected in vivo (Figure 2A), indicating that the cells were in a state of oxidative stresses.

Response to Oxidative Stress and Cell Fate Decision

Response to oxidative stress. Because oxidative stress can trigger PCD, \( P. \) pseudonana must have effective mechanisms for removing excess electrons and for scavenging ROS. On the other hand, due to Fe limitation, some typical ROS defense proteins that require iron as a cofactor showed limited abundance or even a decreased abundance. For example, superoxide dismutase (SOD, Thaps317168, 0.95-fold), which is a cambialistic (Fe/Mn)-SOD that has been previously cloned and characterized in the diatom \( Thalassiosira weissflogii \), and catalase (peroxidase I) (Thaps3257595, 0.68-fold), which is a tetramer of four polypeptide chains containing four porphyrin heme (iron) groups that allow the enzyme to react with hydrogen peroxide, both showed decreased abundance. Notwithstanding, some other iron-free antioxidant enzymes were found to increase in abundance, presumably to replace these ROS scavenging functions. These included peroxiredoxins (Thaps332681, 1.64-fold), a ubiquitous family of antioxidant enzymes (peroxidasins) to detoxify various
peroxide substrate,64 which notably represent the second most highly abundant group of proteins found in this proteomics analysis. Moreover, glutaredoxin-related proteins (Thaps3l261282, 1.26-fold) are thiol-disulfide oxidoreductases that can combat oxidative stress by detoxifying H2O2 to H2O using glutathione and NADPH, and some members have been shown to exert anti-apoptotic effects.65 The expression of thiol-disulfide isomerase and thioredoxins (Thaps3l5491, 1.47-fold, Thaps3l23961, 1.22-fold), the key antioxidant proteins in T. pseudonana,66 were also elevated, which is consistent with the upregulation of the genes detected by Thamtrakoln et al. (2012).19 Our results indicate rather that an iron-free ROS scavenging machinery replaced the iron-rich systems to respond to the oxidative stress caused by Fe limitation.

In addition, some heat shock proteins showed differences in their expression levels under Fe-limited conditions. For example, chaperonin GroES (HSP10) (Thaps3l24914, 0.29-fold), molecular chaperone GrpE (Thaps3l18072, 0.64-fold), and some coexpression proteins, such as FKBP-type peptidyl-prolyl cis–trans isomerase 1 (Thaps3l18543, 0.43-fold) and trypsin-like serine proteases (Thaps3l875, 0.59-fold), all decreased in abundance. The decreased abundance of these proteins could reduce the tolerance of T. pseudonana cells to Fe limitation, inhibit growth, and other physiological processes, and eventually induce PCD. However, the increased abundance (1.56-fold) of chaperonin GroEL (HSP60 family, Thaps3l31353) suggested that this anti-apoptotic protein is also present in the Fe-limited cells to resist the apoptotic process induced by Fe limitation. Furthermore, chaperonin GroEL, which is involved in protein folding after its post-translational translocation to the mitochondrion/chloroplast, has been suggested to play a key role in preventing the stress response and apoptosis68 in the cytoplasm.

Photosynthesis is thought to play an important role in excess energy dissipation under stress conditions, such as high light or low temperature.69 A similar function was also detected in Fe-limited cells of P. tricornutum.70 Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the key enzyme used to fix CO2 in photosynthesis,70 also participates in photorespiration by consuming the O2 created by photosynthesis (O2 is a competitive inhibitor of CO2).71 In the present study, the abundance of RubisCO large subunit (Thaps3l3bdl216s) (that has catalytic activity) (1.33-fold, p > 0.05), whereas the abundance of the RubisCO small subunit (Thaps3l3_bdl1314) (that can functions as a CO2 reservoir72) decreased (0.87-fold, p > 0.05). This finding indicates that photorespiration may be strengthened to alleviate oxidative stress under Fe limitation.

PCD Induction. Internally triggered cell death has been identified in unicellular organisms and may play a major role in phytoplankton bloom succession and collapse.24–26 ROS are one of the factors that can induce PCD. When oxidative stress exceeds the antioxidant capacity of cells, PCD pathways can be induced. In this study, based on the in vivo staining data and TEM observations, a markedly higher number of cells underwent PCD under Fe-limited conditions compared with Fe-replete conditions. Moreover, some proteins that are closely related to PCD processes were also detected in Fe-limited T. pseudonana cells.

As a case in point, the family of serine proteases comprises enzymes that cleave peptide bonds in proteins74 and exhibit caspase-specific activity.19 Our results showed that a serine protease inhibitor (Thaps3l23814) decreased in abundance (0.56-fold), which could suggest an increase in caspase-specific activity and the induction of PCD under Fe-limited conditions. In addition, the protein adenine nucleotide translocator has received much attention due to its participation in altering the integrity of the mitochondrial membrane, which promotes apoptosis.76 The increased abundance (1.6-fold) of adenine nucleotide translocator (Thaps3l260967) found in this study was in accordance with the typical features of PCD, as determined based on our morphological TEM observations (Figure 3E). Therefore, the protein data further confirmed that some cells were undergoing PCD in response to Fe limitation on day 4.

Furthermore, death-specific protein (DSP), which was first identified in the diatom S. costatum, has been suggested to play a role in the molecular mechanism of PCD in phytoplankton under stress.32 T. pseudonana has two DSP-like proteins (TpDSPs, namely TpDSP1 and TpDSP2), both of which have a transmembrane domain and calcium-binding EF-hand motifs.19 A recent investigation indicated that TpDSPs have a dual role in stress-acclimation and cell death based on the finding that the transcript levels of TpDSP1 and TpDSP2 genes are all markedly increased in response to sublethal and lethal ROS.19 Only one TpDSP, namely TpDSP1 (Thaps3l111181), was detected in this study. Together with the in vivo staining results, which showed no difference in the ratio of dead cells between Fe-limited and Fe-replete cultures on day 4, the decreased abundance (0.72-fold, p > 0.05) of TpDSP1 implied that the TpDSP1 protein may play a role in cell death under Fe-limited conditions. According to the different expression patterns obtained for the TpDSP1 gene19 and protein (this study), which are also supported by our quantitative RT-PCR findings, we hypothesize that TpDSP may be subject to post-transcriptional regulation. However, more work is needed to confirm this hypothesis.

Unicellular organism PCD is considered an altruistic adaptation designed to benefit a population, as has been demonstrated in bacteria and yeast.77–80 Bidle et al. (2004) suggested that PCD may also have evolved in phytoplankton cells as a strategy to relieve a population from nutrient stress and remove aging and/or damaged cells from a population to ensure that they do not become a burden,24 further supported by studies in P. tricornutum (Vardi et al. 2006). Therefore, in our study, PCD may be employed as a strategy to reduce the number of cells in the population, thereby decreasing the Fe demands of the whole population. Ultimately, the surviving cells would be able to grow better with an increased Fe supply under what would otherwise be Fe-limited conditions.

Other Cellular Responses to Fe Limitation

Intracellular Nitrogen Metabolism in Fe-Limited Cells. The use of nitrate can be restricted by Fe bioavailability because the enzymes involved in nitrate assimilation (nitrate reductase and nitrite reductase), require Fe as a cofactor. However, analysis of the nitrogen content in the medium showed that the assimilation of nitrate was not influenced by Fe limitation in our study, even showed an increase according to the consumption of per cell (Figure 1C,D). The proteomics data nonetheless showed that the abundance of ferredoxin subunits of nitrite reductase (Thaps3l2673) were significantly decreased (0.60-fold), whereas the nitrate reductase of T. pseudonana was not detected (only one nitrate reductase [NADH] fragment (Thaps3l24484) was detected and showed a slightly increased abundance (1.24-fold)). It can be inferred that T. pseudonana...
cells exposed to early Fe limitation stress may continue to take up nitrate despite the decrease in reducing power (inactive nitrite reductase) and build up an internal pool of NO$_3^-$, which is in agreement with the findings reported by Milligan and Harrison (2000) and Allen et al. (2008). Furthermore, it is well-known that nitrite reductase requires five Fe atoms per active enzyme, whereas nitrate reductase only requires two. Thus, we speculate that nitrite reductase is affected prior to nitrate reductase upon exposure of T. pseudonana cells to early Fe limitation stress, whereas both enzymes may be affected during chronic Fe limitation. However, the genes for nitrate assimilation, such as nitrate reductase, two forms of nitrite reductase, and a plastid-targeted nitrite transporter, were all down regulated in Fe-limited P. tricornutum cells, suggesting differences in the responses of individual species to Fe limitation.

Moreover, some enzymes involved in intracellular amino acid metabolism, specifically cysteine synthase (Thaps314952, 1.82-fold, p > 0.05), methenyltetrahydrofolate cyclohydrolase (Thaps324746, 1.56-fold, p > 0.05), and glutamate dehydrogenase/leucine dehydrogenase (Thaps3262098, 1.63-fold, p > 0.05), were found to increase in abundance in Fe-limited T. pseudonana cells. In addition, multiple aminotransferases, such as ornithine/acetolornithine aminotransferase (Thaps314668, 1.69-fold), aspartate/tyrosine/aromatic aminotransferase (Thaps314577, 1.63-fold), and serine-pyruvate aminotransferase/archaeal aspartate aminotransferase (Thaps322208, 1.58-fold), which are involved in amino acid reorganization and intracellular recycling, were also found to increase in abundance. The increased expression of the above-mentioned proteins could suggest that the intracellular recycling of N-containing compounds may be enhanced due to Fe-limitation stress. It is interesting that the aminotransferases detected in this study are totally different from the types of aminotransferases found in T. pseudonana cells when they underwent chronic Fe limitation stress. Thus, it can be hypothesized that different intracellular nitrogen-containing compounds may be reallocated due to different responses between the early and the acclimated stress response to Fe limitation.

Three enzymes associated with the ornithine-urea cycle (OUC) were also detected, specifically carbamoyl phosphate synthase large subunit (Thaps312265, 0.96-fold, p > 0.05), argininosuccinate synthase (Thaps325328, 1.27-fold), and arginase (Thaps316456, 1.78-fold, p > 0.05). However, other enzymes such as ornithine transcarbamoylase and argininosuccinase were not detected, and only arginase was found to be significantly increased in abundance in Fe-limited cultures compared with Fe-replete cultures. Arginase is an enzyme that catalyzes the final step in the urea cycle and converts L-arginine into L-ornithine and urea. In addition, the difference in the expression levels of the urease enzyme between the Fe-replete and Fe-limited cultures was not significant. Thus, the accumulation of urea may be as a nitrogen reserve to alleviate nitrogen deficiency caused by iron limitation in T. pseudonana based on the hypothesis that urea can also provide nitrogen to diatoms. However, arginase was not detected and carbamoyl phosphate synthase was found to increase in abundance when T. pseudonana was acclimated to Fe limitation. These different responses of the OUC between the early stress response and the acclimated response indicate that different cellular strategies are employed.

**Intracellular Iron Metabolism in Fe-Limited Cells.** Fe acquisition by T. pseudonana occurs via a Fe(II) ferroxidase/permease pathway, the molecular components of which consist of two ferric reductases (FRE1 and FRE2), two iron permeases (FTR1 and FTR2), a ferroxidase (FET3), and the divergent metal transporter NRAMP. However, only the plasma membrane iron permease (FTR1, Thaps320774) was detected and found to increase in abundance (1.54-fold) in this early stress response of Fe-limited cells, which is consistent with the acclimation response of T. pseudonana to Fe limitation (Nunn et al, 2013). This result suggests that a similar cellular strategy may be employed between these two responses, namely that T. pseudonana could increase production of FTR1 for Fe acquisition by endocytic recycling when limited by Fe.

Moreover, the cells reduced their iron protein demands by using iron-free proteins with similar functions, e.g., ferredoxin was replaced by flavodoxin. In addition, proteins that do not require heme as a coenzyme or iron as a cofactor were found to increase in abundance during the antioxidant and anti-apoptotic processes reported here. Thus, we propose that the cell regulates its own protein expression to reduce the consumption of iron such that it is able to adapt to the environmental stress associated with iron limitation.

**Intracellular Silicon Metabolism in Fe-Limited Cells**

It is interesting that silicon uptake was also found to be enhanced by Fe-limitation (Figure 1). A higher silicon concentration in the medium was consumed under Fe-limited compared to Fe-replete conditions on day 4. Even the medium of Fe-replete cultures was completely depleted of silicon by day 5, while approximately 100 μm of silicon was still present in the medium of Fe-limited cultures. More silicon was taken up by Fe-limited cells than by Fe-replete cells on day 5, which was calculated based on the cell number. Our result is consistent with the previous finding that Fe limitation induced silica deposition in T. pseudonana, as demonstrated by the upregulation of the gene encoding silafin 1 (a protein associated with silica deposition, SIL 1), as well as with the observation that the frustules of Fe-limited cells are thicker and more heavily siliciated than Fe-replete cells (Mock et al, 2008). Our finding therefore support a possible link between iron bioavailability and silica deposition (Mock et al., 2008).

**CONCLUSIONS**

In this study, iTRAQ proteomic profiles, in vivo biochemical markers and physiological characteristics represent not only a comprehensive systematic analysis of the early stress response of diatoms to Fe limitation, but also a large amount of information about previously uncharacterized cellular metabolism processes and proteins involved in ROS accumulation and PCD induction in response to short-term Fe limitation. Our observations provide a new molecular view about the diatom response to oxidative stress and control of cell fate: reduction of the cell population by PCD to reduce the consumption of Fe, enhancement of the turnover and reallocation of intracellular nitrogen and Fe into key cellular components to ensure the most basic survival and defense, and the increased abundance of antioxidant and anti-PCD proteins to ensure cell survival. These results significantly increase our understanding of how diatom cells control their life and death during blooms, and the molecular mechanism of diatoms involved in their survival and competition strategies to cope with trace metal fluctuations in the marine environment.
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