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Genome-Wide Profiling of Responses to Cadmium in the Diatom *Phaeodactylum tricornutum*

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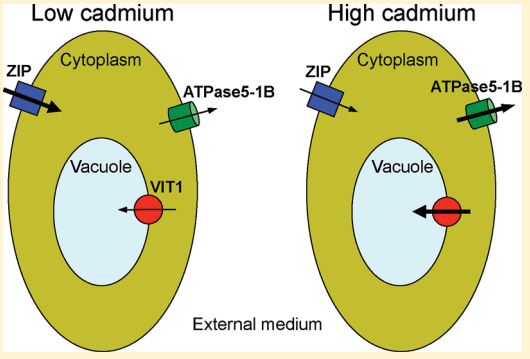
Supporting Information

ABSTRACT: The only group of organisms in which a biological function for cadmium has been shown is the diatoms, which are unicellular phytoplankton. Yet diatoms exhibit similar sensitivity to Cd as other groups of phytoplankton. We have investigated responses of Cd on molecular, metabolic, and physiological levels in the diatom *Phaeodactylum tricornutum*. *P. tricornutum* apparently has a high tolerance to Cd; only minor responses were observed on growth, pigment, and transcriptional changes at cadmium concentrations of 123 μg/L. No significant changes in chlorophyll and xanthophyll levels were observed, and the very few transcripts affected strongly indicate that the cells were able to respond to the increased Cd^{2+} levels without changing proteins levels. At 10 times this concentration, 1230 μg/L, a much clearer response was observed, including transcripts encoding proteins involved in metal transport, cell signaling, and detoxification processes. Our results point toward putative pathways for the removal or detoxification of Cd and its metabolites as well as a possible Cd uptake mechanism. We predict that ATPase5-1B is involved in removal of Cd by pumping Cd^{2+} ions out of the cell, whereas VIT1/CCC1 sequesters Cd^{2+} in the vacuole.



INTRODUCTION

Although cadmium (Cd) is known to be very toxic, the concentration of dissolved Cd in open ocean waters has a nutrient-type profile with depth.[1] The reasons for this distribution are unclear. Cadmium is generally not believed to have a biological function in the sea, in contrast to other heavy metals such as copper and zinc.[2] Price and Morel[3] have, however, proposed that the depletion of Cd from ocean surface water might be due to the utilization of Cd by phytoplankton in surface waters and sinking of particulate organic matter and subsequent remineralization at depth. Compared to pelagic ecosystems, coastal and estuarine ecosystems in proximity to human activity may experience much higher concentrations of Cd and other heavy metals as a result of terrestrial runoff from agriculture, mining, and industrial activity. Cd concentrations in coastal sediments outside of industrial plants in Northern China have reached concentrations of almost 500 mg kg^{-1}.[4]

The mechanisms behind cadmium toxicity are not fully understood. Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radicals, are often implicated in Cd toxicity, possibly through Cd depletion of glutathione and protein-bound sulfhydryl groups. Increased ROS levels in turn results in lipid peroxidation and DNA damage. In diatoms, high levels of Cd inhibit the epoxidation of diatoxanthin back to diadinoxanthin, thereby preventing effective photoprotection in high light by nonphotochemical quenching. This results in photoinhibition.[5] The photosynthetic process in general is known to be sensitive to Cd; it reduces growth, inhibits chlorophyll biosynthesis, and depresses the photosynthetic carbon assimilation.[6] There is one example of a biological function for Cd; the marine centric diatom *Thalassiosira weissflogii* contains a carbonic anhydrase that uses Cd^{2+} as cofactor.[7−10] At the oceanic sea surface, concentrations of biological trace elements such as the normal carbonic anhydrase cofactor zinc are extremely low. Thus, the ability to utilize Cd as a replacement is believed to be highly beneficial in these environments.

Phaeodactylum tricornutum has become a model system for pennate diatoms.[11] Whole-genome microarray studies of *P. tricornutum* responses to silicic acid starvation[12] and high light intensities[13] indicated that large and specific gene sets are regulated in a coordinated manner. Several previous studies have analyzed some of the responses of *P. tricornutum* to Cd.[5,14,15] *P. tricornutum* appears to be tolerant to Cd, with an IC50 value for growth at 96 h of 15.72 mg/L (139.8 μM) Cd^{2+},[16] which is substantially higher than what is observed for the centric pelagic diatom *Thalassiosira nordenskioeldii*[17] or the pennate benthic diatom *Navicula pelliculosa*.[18] Upon exposure to Cd and other heavy metals, *P. tricornutum* produces metal-binding thiol peptides, phytochelatins (PCs), with a positive correlation between PC and heavy metal concentrations.[15] However, other mechanisms are also likely to account for the Cd tolerance.

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Only a few functional genomics studies of microalgal responses to heavy metals have been published. The green alga *Chlamydomonas reinhardtii* was exposed to Cd^{2+} concentrations between 7.8 nM and 9 μM for 2 h, and microarray and differential display techniques were used to identify differentially expressed genes.[19] Only a small set of 10 genes showed a significant change in expression as a result of these treatments. A PCR-based subtractive cDNA approach was used to identify 17 copper (Cu)-responsive genes in *Thalassiosira pseudonana*.[20] A subset of the genes was analyzed in Cd- and H_2O_2-treated cells; however, no large changes in expression were observed, indicating that these genes are involved in a Cu specific response.

We set out to investigate the responses of *P. tricornutum* to Cd on the molecular, metabolic, and physiological level. Growth and pigment profiles and concentrations were compared for cells cultured at Cd concentrations of 123 μg/L and 1230 μg/L to study effects on physiological processes. A genome-wide transcriptional analysis using full-genome microarrays was performed on cells exposed to 123 μg/L and 1230 μg/L Cd^{2+} for 24 h as a method of global analyses of Cd induced responses. Exposure to the highest concentration of Cd affected a large number of genes encoding proteins involved in metal transport, cell signaling, and detoxification processes, suggesting that previously uncharacterized pathways in diatoms are involved in the response to cadmium exposure.

MATERIALS AND METHODS

Diatom Cultures. *P. tricornutum* Bohlin clone CCMP632 was obtained from the culture collection of the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, USA and maintained in f/2 medium.

Axenic *P. tricornutum* cells were transferred from f/2 medium to artificial seawater base AK modified,[21] with added f/2-medium nutrients. No EDTA was used; Fe-EDTA was replaced with an equal molar amount of ferric chloride. The prepared artificial seawater was sterile filtrated through a 0.2 μm pore filter before nutrients were added by sterile filtration through 0.2 μm pore filter. The cultures were maintained at 14 $\pm$ 1 °C under continuous cool white fluorescent light (54 μmol photons m^{-2} s^{-1}) on a rotary table and acclimated for one week prior to the exposure to cadmium. Axenity was monitored by occasional inoculation into peptone-enriched f/2 medium to check for bacterial growth. Cells for the experiments were grown in batch cultures; all vessels were acid washed and rinsed with Milli-Q water prior to use. The cells were first grown axenically in a batch culture to reach an approximate density of 10^6 cells mL^{-1}, and then aliquots were transferred to 75 cm^2 sterile Falcon polystyrene flasks to reach cell densities of $1-3 \times 10^6$ cells mL^{-1} on the day of the experiment. Cadmium was added as $CdCl_2$ to final Cd^{2+} concentrations of 123 μg/L and 1230 μg/L from 1000× stock solutions. For growth rate analysis and cadmium quantification, cell density was adjusted to 1.13×10^5 at the beginning of the experiment. A 1:9 dilution was performed after three days to ensure exponential growth.

Cadmium Quantification. Cells in exponential growth phase were harvested through filtering onto PVDF filters using moderate vacuum. The filtered cells were resuspended in AK modified seawater supplied with 1 mM diethylenetriaminepentaacetic acid and incubated for 10 min in order to remove metals bound/associated with the cell wall,[22] followed by three rinses with AK modified seawater. 1.5 mL of medium was added, and the cells were carefully resuspended and centrifuged at 16 000 g for one minute. The algal pellets were dried at 80 °C for 2 h. Total cadmium concentrations were determined by graphite furnace atomic adsorption spectrometry (AAS, Perkin-Elmer) after a HNO_3 acid digestion. The program used with the graphite furnace AAS (WinLab32 for AA, Perkin-Elmer) automatically calculated statistical values necessary to assess the relevance of the results obtained.

Cell Growth. Cell number was obtained daily using a Bürker-Türk chamber, counting 4−500 cells per volume-unit. Cell-specific growth rate was calculated as described in ref 23.

Pigment Analysis. HPLC pigment analyses were performed as described in Rodriguez et al.[24] using a Hewlett-Packard HPLC 1100 Series system. The pigment values from the HPLC analyses were calculated as femtogram (fg) pigment per cell.

Spectrofluorometry. Fluorescence excitation (fl-ex) spectra (400−700 nm) were measured by spectrofluorometry (Hitachi F-3000). Prior to measurements, DCMU was added to the samples to ensure that all reaction centers were closed. Fluorescence emission at 730 nm was registered using excitation light at 440 and 550 nm in time-scan mode. The raw data of the *in vivo* fl-ex spectra were quantum-corrected from 400 to 700 nm using Basic Blue 3 as a quantum correcting dye. The *in vivo* and quantum-corrected fl-ex spectra were scaled as described by Johnsen and Sakshaug[25] to obtain the final results.

RNA Isolation and Microarray Experiments. Harvesting of cells and total RNA isolation was performed as described in Nymark et al.[13] The RNA (0.13−0.4 μg) was reverse transcribed, amplified, and labeled using the Quick Amp Labeling Kit, Two-Color (Agilent p/n 5190−0444). Hybridization on 4 × 44K *P. tricornutum* whole-genome 60-mer oligonucleotide microarrays (Agilent Technologies), scanning, and image processing were performed as described in Nymark et al.[13] Microarray data have been deposited at the NCBI/GEO database and are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31131.

Statistical Analyses of Microarray Data. Statistical analyses of the microarray data was performed as described in Nymark et al.[13] Genes are represented by 1−5 different probes on each microarray. We observed that expression ratios generally decreased with increasing probe hybridization distance from the 3′ end of the transcript. This is probably an artifact caused by cDNA synthesis using oligo dT primers, where cDNA synthesis efficiency decreases with increasing distances from the polyA tail. Expression ratios discussed in the text are therefore an average of values obtained from the two probes closest to the 3′ end for each gene. BLASTP searches[26] were performed for the putative products of each of the differentially expressed genes; they were classified into 13 putative categories as described in ref 27 based on similarity to known genes.

Quantitative Real-Time PCR. A two-step quantitative real-time PCR (qRT-PCR) was performed on the same total RNA that was used for the microarray experiments. Reverse transcription of the RNA was performed with the QuantiTect Reverse Transcription kit (QIAgen) following the recommended protocol for synthesis of real-time PCR template using random primers. 195 ng of total RNA was used in each reaction. Remaining genomic DNA contamination in the RNA samples was removed using gDNA wipeout buffer (QIAgen). Twenty μL qRT-PCR mixtures were prepared containing forward and reverse primers listed in Supporting Information Table S1, with a final concentration of 0.5 μM each, 5 μL of cDNA template diluted 1:10, and

2x LightCycler 480 SYBR Green I Master mix (Roche). The qRT-PCR reactions were run in a LightCycler 480 Multiwell Plate 96 (Roche) in a LightCycler 480 instrument (Roche). Detailed information on qRT-PCR and statistical analyses of the data is available as Supporting Information.

RESULTS

Cadmium Uptake. *P. tricornutum* cultures were grown for 4 days in medium containing 123 μg/L and 1230 μg/L Cd^{2+}, and Cd uptake was measured using atomic adsorption spectrometry (Figure 1A). Interestingly, cells exposed to 1230 μg/L Cd^{2+} contain only about five times more Cd^{2+} than cells exposed to 123 μg/L Cd^{2+}, suggesting either active Cd import under the lower concentration or active Cd export under the higher concentration in *P. tricornutum*.

Even High Concentrations of Cd^{2+} Have Little Effect on P. tricornutum Growth. Previous studies have suggested that a Cd concentration of several mg per liter medium is necessary for significant inhibition of growth of *P. tricornutum*.[14,16] We tested the growth response of *P. tricornutum* to 123 μg/L and 1230 μg/L Cd^{2+} (Figure 1B). After 24 h, no significant change in growth rate (μ) was observed in the cadmium-treated cultures (results not shown). The cell-specific growth rate over 96 h was moderately but insignificantly reduced in cultures treated with 123 μg/L Cd^{2+} compared with untreated cultures. In contrast, cultures treated with 123 μg/L Cd^{2+} grew significantly slower compared with cultures grown on 123 μg/L Cd^{2+} or control medium (Student's *t* test; $p < 0.05$).

Pigment Analysis. The pigment contents of *P. tricornutum* cultures were analyzed by HPLC 24 h after exposure to Cd (Figure 1C). The light-harvesting pigments Chl *a*, Chl *c*, and fucoxanthin as well as the photoprotective pigment diadinoxanthin were detected. Diatoxanthin, which is formed by deepoxidation of diadinoxanthin in response to high light intensities, was not detected, probably because of the relatively low light intensities used in the experiment. No significant differences in pigment levels were observed. The *in vivo* fluorescence excitation spectra were analyzed (Supporting Information, Figure S1). No significant changes in the spectra were observed 24 h after exposure to Cd, supporting the results from the pigment analysis.

Microarray Analyses. In order to study Cd^{2+} responses on transcriptional level, we isolated total RNA from cells harvested after 24 h exposure to 123 μg/L and 1230 μg/L Cd^{2+} as well as unexposed cells and hybridized cDNA derived from this RNA against an oligonucleotide microarray representing all known transcribed genes in *P. tricornutum*. The resulting data sets were subjected to a strict statistical filtering; defining only genes with adjusted p-values less than 0.01 as being differentially expressed. Using these filtering criteria, only five genes were found to be differentially expressed after treatment with 123 μg/L Cd^{2+} compared to untreated cells; four of these were upregulated (Table 1). Using less stringent filtering ($p < 0.05$) only three more probes, representing three different genes, were included as differentially expressed (Supporting Information, Table S2).

Not surprisingly, exposure to 1230 μg/L Cd^{2+} resulted in a much stronger response; 672 genes were differentially expressed (at $p < 0.01$), of which 412 were upregulated and 260 were downregulated (Supporting Information, Table S2). These genes were assigned to functional categories based on similarity to known proteins (Figure 2). For several categories, the distribution between upregulated and downregulated genes was clearly skewed. Of the

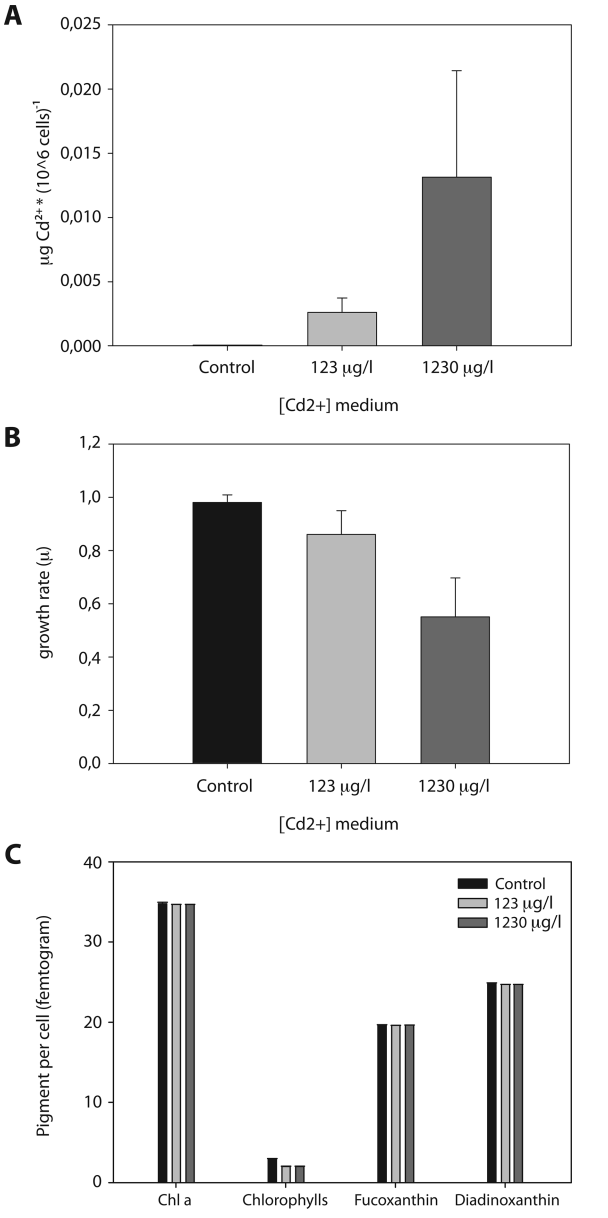

Figure 1. Effects of cadmium exposure on *P. tricornutum*. (a) Cadmium concentrations (μg 10^6 $cell^{-1}$) in *P. tricornutum* grown 96 h in medium supplemented with different concentrations of Cd^{2+} (123 and 1230 μg/L). (b) Cell-specific growth rate (μ) of cultures grown 96 h in medium supplemented with different concentrations of Cd^{2+}. (c) Photosynthetic pigments in *P. tricornutum* exposed to different concentrations of Cd^{2+} for 24 h. Chl a; Chlorophyll a, Chl c; Chlorophyll c. Values are presented with ± SD bars ($n = 3$).

differentially expressed genes encoding proteins putatively involved in protein synthesis, 41 were downregulated whereas only 4 were upregulated, suggesting that translation is reduced by high concentrations of Cd^{2+}. Of the 32 downregulated genes, 17 encoded ribosomal proteins. In contrast, the functional categories for energy production, transporters, and disease/defense were enriched for upregulated genes.

Adding an expression ratio cutoff (mean $|\log2(\text{ratio})| > 1.0$) to the filtered gene set reduced the number of genes differentially expressed after 1230 μg/L Cd^{2+} exposure to 41, of which 28 were upregulated and 13 were downregulated. These genes are listed

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Table 1. *P. tricomutum* Genes Regulated by 24 h Exposure to 123 μg/L and 1230 μg/L Cd^{2+}

		Log2 ratios microarray		
Phatr2 protein ID[a]	description	123 μg/L Cd^{2+}	1230 μg/L Cd^{2+}	functional category
43350	YeeE/YedE family integral membrane protein	2.01	-	unclear classification
42755	ZIP family transporter, putative zinc transporter	1.60	-	transporters
49253	CoADR; putative coenzyme A disulfide reductase	1.25	-	disease/defense
45591	unknown protein; contains DUF1122 domain	−0.95	-	unclassified
54574	TPR1; TPR domain protein	-	1.94	unclear classification
54954	calcium binding protein; contains two EF hands	-	1.44	signal transduction
46597[b]	TPR2; TPR domain protein	-	1.40	unclear classification
43799	GFA; glutathione-dependent formaldehyde-activating protein	-	1.37	disease/defense
49199	unknown protein	-	1.36	unclassified
44077	NAD-dependent epimerase/dehydratase family protein	-	1.35	metabolism
39769	no hits found, specific for Phaeodactylum	-	1.34	unclassified
49729	conserved unknown protein	-	1.31	unclassified
39969	unknown protein	-	1.29	unclassified
43687	no hits found, specific for Phaeodactylum	-	1.20	unclassified
30246	zinc-containing alcohol dehydrogenase family protein	0.92	1.19	metabolism
44652	conserved hypothetical protein	-	1.18	unclear classification
25433	papain family cysteine protease	-	1.15	protein destination/storage
43314	Vacuolar Iron Transporter (VIT1) homologue	-	1.14	transporters
34132	DUF3291 domain containing protein	-	1.11	unclear classification
29488	PTD6; delta 6 fatty acid desaturase D6	-	1.09	metabolism
45324	no hits found, specific for Phaeodactylum	-	1.09	unclassified
48899	no hits found, restricted to diatoms	-	1.03	unclassified
12662	c-Myc-binding protein	-	1.02	signal transduction
52367	ATPase5-lB; putative Zn/Cd heavy metal transporter	-	1.02	transporters
37482	calcium binding domain protein; contains two EF-hands	-	1.00	signal transduction
36435	weak similarity to Saccharopine dehydrogenase	-	0.94	unclear classification
43531	OXR1; oxidation resistance 1 (OXR1) homologue	-	0.94	disease/defense
33120	conserved hypothetical protein	-	0.92	unclear classification
50486	no hits found, restricted to diatoms	-	0.88	unclassified
35939	short chain type dehydrogenase, fabG type	-	0.87	metabolism
42605	no hits found, restricted to diatoms	-	0.86	unclassified
47783	hypothetical protein; contains SAM domain	-	0.86	unclear classification
51134	HSP101; heat shock protein 101/ClpB protease	-	−0.93	protein destination/storage
22319	putative importin alpha	-	−0.97	intracellular transport
49933	putative elicitin	-	−0.98	unclear classification
54656	HSP20; heat shock protein HSP20	-	−1.01	protein destination/storage
13244	cytochrome *c* peroxidase	-	−1.02	disease/defense
12783	CDC20_l; coactivator of the anaphase-promoting complex	-	−1.02	cell growth and division
PhtrCp016	uncharacterized protein ycf90 (chloroplast genome)	-	−1.02	unclear classification
34754	no hits found, specific for Phaeodactylum	-	−1.05	unclassified
51291	ribosomal protein S20	-	−1.05	protein synthesis
39559	no hits found, specific for Phaeodactylum	-	−1.14	unclassified
8686	ribosomal protein S12	-	−1.24	protein synthesis
PhtrCp099	50s ribosomal protein 12 (chloroplast genome)	-	−1.29	protein synthesis
49020	ankyrin repeat protein	-	−1.46	unclear classification

[a] Protein IDs are based on the *P. tricomutum* genome database at Joint Genome Institute (http://genomejgi-psf.org/Phatr2/Phatr2.home.html). [b] Phatrl protein ID, not included in Phatr2.

in Table 1, together with the genes differentially expressed after exposure to 123 μg/L Cd^{2+}. The regulation of selected transcripts was validated using RT-qPCR (Supporting Information, Table S3).

An important mechanism to prevent intracellular accumulation of toxic metals is ion transporters that pump the metal ions out of the cell or into storage compartments such as the vacuole.[28] Treatment with 123 μg/L Cd^{2+} induced a gene (Phatr2_42755) encoding a putative zinc transporter with similarity to the ZIP family. Treatment with 1230 μg/L Cd^{2+} did not induce this gene; however, the expression level of two other transporters increased

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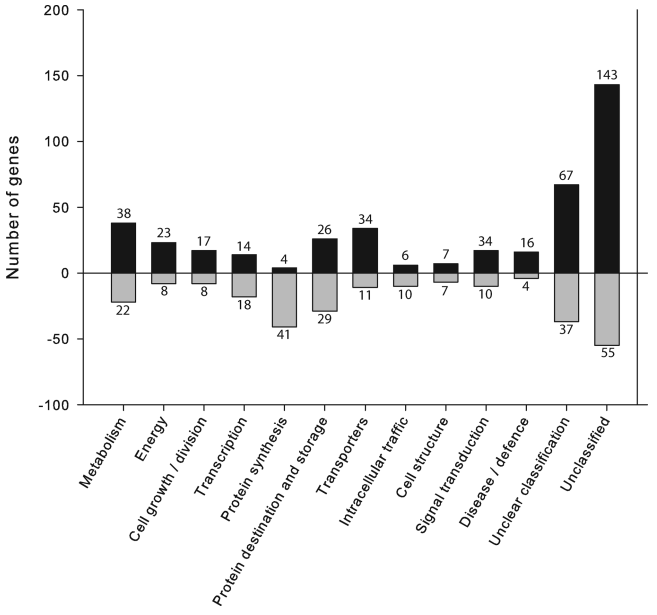

Figure 2. Distribution of up- and downregulated genes among functional categories in response to 24 h exposure to 1230 μg/L Cd^{2+}. Up- and downregulated genes are indicated by black and gray bars, respectively.

about 2-fold at this concentration. ATPase5-1B (Phatr2_52367) encodes a P_{1B}-type ATPase with high similarity to cadmium/zinc transporters such as *Arabidopsis* HMA4, which are known to confer cadmium resistance in hyperaccumulating *Arabidopsis* species.[29] RT-qPCR validation produced very similar results for both genes (Supporting Information Table S3). Also induced by 1230 μg/L Cd^{2+} was a gene encoding a transporter (Phatr2_43314) with similarity to the *Arabidopsis* vacuolar Mn^{2+} and Fe^{2+} transporter VIT1.[30] No subcellular localization could be predicted using the TargetP prediction server.[31]

Surprisingly, genes encoding antioxidant proteins like superoxide dismutase, catalase, glutathione peroxidase, and ascorbate peroxidase did not appear to be induced by any of the Cd^{2+} treatments. Similarly, none of the phytochelatin synthase-encoding genes were responsive. 123 μg/L Cd^{2+} induced the expression of a coenzyme A disulfide reductase (CoADR; Phatr2_49253) with strong similarity to bacterial homologues. Reduced CoA has antioxidant activity, being able to reduce H_2O_2.[32] CoADR expression may therefore be activated in response to increased ROS levels. However, CoADR was not induced after exposure to 1230 μg/L Cd^{2+}. A gene encoding a putative glutathione-dependent formaldehyde-activating protein (GFA; Phatr2_43799), which catalyzes the condensation of formaldehyde and glutathione to S-hydroxymethylglutathione,[33] was induced by Cd^{2+}.

Three genes encoding enzymes possibly involved in fatty acid biosynthesis were upregulated by 1230 μg/L Cd^{2+} exposure. A NAD-dependent epimerase/dehydratase (Phatr2_44077), a delta 6 fatty acid desaturase (PTD6; Phatr2_29488), and a fabG family 3-ketoacyl-(acyl-carrier-protein) reductase (Phatr2_35939) were all induced about 2-fold. Expression of a zinc-dependent alcohol dehydrogenase (Phatr2_30246) with unknown substrate specificity was also induced.

No transcription factors were induced or repressed beyond the expression ratio cutoff. In the $p < 0.01$ data set, nine genes encoding heat shock transcription factors were found to be moderately upregulated by 1230 μg/L Cd^{2+} (Supporting Information, Table S2), suggesting that this gene family may be responsible for regulation of the stress response. Few components of signal transduction pathways were affected by the Cd treatments. A gene encoding a c-Myc binding protein homologue (Phatr2_12662) was induced 2-fold by 1230 μg/L Cd^{2+}. This protein is conserved throughout Eukaryota and also binds other signaling components such as S-AKAP84 and AKAP95 in mammalian cells.[34] Expression of an oxidation resistance 1 homologue (OXR1; Phatr2_43531) proposed to protect mitochondria from oxidative damage through activation of antioxidant enzyme expression[35,36] increased almost 2-fold at 1230 μg/L Cd^{2+}. Among the gene products most induced by the 1230 μg/L Cd^{2+} treatment were two proteins (Phatr2_54574 and Phatr1_46597) containing a tetratricopeptide (TPR) domain. TPR domains are believed to mediate protein−protein interactions.

DISCUSSION

Our results suggest that *P. tricornutum* is highly tolerant to cadmium. Doses as high as 123 μg/L of Cd^{2+} did not significantly alter cell growth, and only a few and moderate responses could be measured in the genome-wide transcriptional analysis. Exposure to 1230 μg/L Cd^{2+} retarded cell growth after 96 h, and although growth rate was not significantly affected after 24 h, a clearly modified transcriptional signature was evident. Such results are in line with previous studies showing that *P. tricornutum* is tolerant to Cd and growth rates only slightly affected.[14,16] The transcriptional signature suggests that the metabolic load of Cd^{2+} doses of 1230 μg/L is reflected in the downregulation of genes involved in protein synthesis. An upregulation of genes involved in energy processes indicates a higher energy demand in the cells, possibly because of the need for transport/export and detoxification of Cd (Figure 2).

The cell pigment content (Figure 1C) and the *in vivo* fluorescence spectra (Supporting Information, Figure S1) showed no significant differences between the control and the treatment groups, which indicates that the *P. tricornutum* cells are able to perform normal pigment synthesis and photosynthesis 24 h after exposure to the given Cd^{2+} concentrations. In a similar experiment performed on *T. weissflogii*, Chl a levels increased only at the highest Cd concentration where growth rate was reduced.[23] These results are supported by the fact that only one gene encoding an enzyme involved in pigment biosynthesis (HemN; Phatr2_51528) is significantly regulated by 1230 μg/L Cd^{2+} (Supplementary Table S2). Together, the cell growth, pigment, and transcriptome data at 24 h exposure are indicative of a relatively early response to Cd, where intracellular Cd have not yet accumulated to levels affecting the growth rate, but where responses are observed at the molecular level.

One of the main strategies in algae for detoxification of metals involves generation of phytochelatins (PCs), which are synthesized from glutathione by phytochelatin synthase (PCS).[37] A recent analysis of PC production in *P. tricornutum* showed that cadmium induces PC production at Cd^{2+} concentrations down to 0.2 nM,[38] and PC levels increase rapidly upon Cd exposure in *Thalassiosira nordenskioeldii*.[39] Transcription of PCS is not induced by Cd exposures in our study. However, PCS enzyme activity is stimulated by heavy metal ions, whereas PCS expression appears not to be regulated by heavy metal exposure in plants,[40] indicating that PCS activation generally is post-transcriptional.

Most genes known to be involved in oxidative stress responses were not transcriptionally activated, one exception being the

OXR1 homologue Phatr2_43531. OXR1 is believed to protect against oxidative damage in mitochondria and the nucleus.[35,41] Recently, an OXR1 homologue in malaria mosquito (*Anopheles gambiae*) was shown to regulate gene expression of the antioxidant enzymes catalase and glutathione peroxidase.[36] The lack of transcriptional activation of antioxidant enzymes in Cd treated cells does not indicate a similar role in *P. tricornutum*, but an upregulation of these genes may be observed after longer exposures.

Mitochondria contain high levels of ROS as byproduct of respiration; further oxidative stress caused by cadmium may therefore be deleterious for mitochondrial function. Effects of cadmium on mitochondria include disturbance of respiratory activity, alteration of inner membrane permeability, and increased lipid peroxidation.[42] Products of lipid peroxidation are aldehydes such as malondialdehyde. In a recent study, exposure of the freshwater diatom *Nitzschia palea* to cadmium led to higher intracellular concentrations of malondialdehyde.[43] Furthermore, treatment of tobacco plants with aluminum chloride resulted in increased levels of aldehydes; the greatest absolute increase was that of formaldehyde.[44] It is therefore interesting that a glutathione-dependent formaldehyde-activating protein (GFA) is among the most upregulated after 1230 μg/L Cd^{2+} treatment. GFA catalyzes the first step in metabolism of formaldehyde in both prokaryotes and eukaryotes, forming S-hydroxymethyl-GSH (GS-CH_2OH). A GSH-dependent formaldehyde dehydrogenase (FALDH) oxidizes GS-CH_2OH to S-formyl-GSH (GS-CHO), which is converted to formate ($CHOO^-$) by S-formyl-GSH hydrolase (SFGH). In diatoms and ciliates, these two enzyme activities are combined in a fusion protein.[45] Finally, *P. tricornutum* encodes a putative formate/nitrite channel (FocA), which facilitates cellular export of formate.[46] A FocA homologue was found to be overexpressed in a Cd-resistant culture of the freshwater protozoan *Euglena gracilis*, compared to a control culture.[47] Thus, the *P. tricornutum* genome encodes a complete metabolic pathway for detoxification of formaldehyde, and the transcriptional analysis suggests that at least parts of this pathway is upregulated by exposure to high Cd concentrations.

Ion transporters appear to play an important role in Cd responses in *P. tricornutum*. Among the transporter genes significantly regulated by 1230 μg/L Cd^{2+}, more than 75% are upregulated by the treatment, including several putative metal transporters (Figure 2). ABC transporters belonging to the HMA (Heavy metal ATPase) family are important for Cd tolerance in plants. The Cd hyperaccumulator *Arabidopsis halleri* encodes three copies of the HMA4 gene compared to a single copy in the closely related *A. thaliana*; the cis-regulatory sequences of these genes have also been modified.[29] The *A. thaliana* HMA2 and HMA4 transporters are localized to the plasma membrane and are believed to act in Cd efflux as a part of root-to-shoot translocation, whereas HMA3 has a vacuolar localization and therefore seems to be involved in vacuolar Cd storage.[48] *P. tricornutum* ATPase5-1B (Phatr2_52367), which is upregulated by 1230 μg/L Cd^{2+}, shows higher similarity to *A. thaliana* HMA2/HMA4 (results not shown). Another gene induced by 1230 μg/L Cd^{2+} encodes a homologue (Phatr2_43314) of the vacuolar iron transporter VIT1, which is important for proper localization of iron in *A. thaliana* seeds.[30] In yeast the CCC1 transporter *Saccharomyces cerevisiae* performs a similar role.[49] The *P. tricornutum* genome encodes two VIT1/CCC1 homologues in addition to Phatr2_43314 (results not shown); one of these genes (Phatr2_43313) is also significantly upregulated by 1230 μg/L Cd^{2+} (Supporting Information, Table S2). We therefore suggest that ATPase5-1B is involved in removal of Cd by pumping Cd^{2+} ions out of the cell, whereas VIT1/CCC1 sequesters Cd^{2+} in the vacuole. However, the increased cellular Cd levels in treated cells indicate that if such a mechanism is present in *P. tricornutum*, it is not sufficient to counterbalance the Cd influx under the concentrations used in our experiments.

One of the few genes induced by low concentrations of Cd also encodes a putative ion transporter (Phatr2_42755), with similarity to the ZIP family of metal transporters, which facilitate uptake of Zn and other divalent metals. A member of the mouse ZIP family, ZIP8/SLC39A8, has been identified as a locus for Cd sensitivity. ZIP8 expression levels were correlated with the degree of Cd tolerance or sensitivity in different cell lines.[50] Cd^{2+} is believed to be able to displace the endogenous cation and thereby enter the cell.[51] However, nothing is known about ZIP8/SLC39A8 homologues in algae. Why should a treatment with moderate levels of Cd^{2+} induce expression of a transporter that may lead to increased uptake of Cd^{2+}? After a 24 h exposure, Cd levels may not yet have accumulated to levels that are perceived as harmful to the cell. It is tempting to speculate that Cd^{2+} could have a biological function in *P. tricornutum* and that Cd^{2+} in low concentrations therefore would be actively imported into the cell, maybe to act as a cofactor for some enzyme similar to the function of cadmium for carbonic anhydrase (CdCA1) in *T. weissflogii*.

Our results point to metabolic pathways, signaling components, and ion transporters that are induced by moderate or high levels of Cd. Although techniques for classical genetic studies have not been established in diatoms, molecular tools for studies of intracellular localization, overexpression, and gene silencing have been developed.[52] Functional analyses of key genes identified in this study are likely to provide important knowledge on the molecular mechanisms used by diatoms for utilization of or protection against cadmium.

■ ASSOCIATED CONTENT

Supporting Information. *In vivo* fluorescence excitation spectra of Cd-treated *P. tricornutum* cultures are shown in Figure S1. PCR primers used in qRT-PCR analyses are listed in Table S1. A full list of *P. tricornutum* genes significantly regulated by 24 h exposure to 123 μg/L and 1230 μg/L Cd^{2+} are listed in Table S2. qRT-PCR results are shown in Table S3. Information on qRT-PCR and statistical analyses of the data. This material is available free of charge via the Internet at http://pubs.acs.org.

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