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# Genome-wide analysis of transcription and photosynthesis inhibition in the harmful dinoflagellate *Prorocentrum minimum* in response to the biocide copper sulfate

Ruoyu Guo [a], Weol-Ae Lim [b], Jang-Seu Ki [a,*]

[a] Department of Life Science, College of Natural Sciences, Sangmyung University, Seoul 03016, Republic of Korea
[b] Oceanic Climate & Ecology Research Division, the National Institute of Fisheries Science (NISF), Busan 46083, Republic of Korea

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ABSTRACT

Copper is an essential trace metal for organisms; however, excess copper may damage cellular processes. Their efficiency and physiological effects of biocides have been well documented; however, molecular transcriptome responses to biocides are insufficiently studied. In the present study, a 6.0 K oligonucleotide chip was developed to investigate the molecular responses of the harmful dinoflagellate *Prorocentrum minimum* to copper sulfate ($CuSO_4$) treatment. The results revealed that 515 genes (approximately 8.6%) responded to $CuSO_4$, defined as being within a 2-fold change. Further, KEGG pathway analysis showed that differentially expressed genes (DEGs) were involved in ribosomal function, RNA transport, carbon metabolism, biosynthesis of amino acids, photosystem maintenance, and other cellular processes. Among the DEGs, 49 genes were related to chloroplasts and mitochondria. Furthermore, the genes involved in the RAS signaling pathway, MAPK signaling pathway, and transport pathways were identified. An additional experiment showed that the photosynthesis efficiency decreased considerably, and reactive oxygen species (ROS) production increased in *P. minimum* after $CuSO_4$ exposure. These results suggest that $CuSO_4$ caused cellular oxidative stress in *P. minimum*, affecting the ribosome and mitochondria, and severely damaged the photosystem. These effects may potentially lead to cell death, although the dinoflagellate has developed a complex signal transduction process to combat copper toxicity.



## 1. Introduction

Dinoflagellate algae are a large group of aquatic protists; approximately half are photosynthetic, playing a crucial role in the marine and freshwater ecosystems. Some dinoflagellates are notorious for forming harmful algal blooms (HABs, commonly referred to as red-tide), and can produce biotoxins, severely affecting aquatic environments and aquaculture industries (Van Dolah et al., 2001; Landsberg, 2002; Shumway et al., 2003). To date, harmful dinoflagellates and HABs are controlled by several biological, chemical, and/or physical methods (e.g., algicide bacteria, ultrasonication, biocide agents, etc.). Generally, the yellow loess is the most commonly employed technique for the mitigation of HABs in the field; however, it is difficult to investigate its cellular and molecular effect on cells owing to its chemical characteristics. In recent studies, the physiological and biochemical responses of the dinoflagellate *Prorocentrum minimum* when exposed to certain chemicals (e.g., oxidizing chlorine, yellow loess, and $CuSO_4$) were tested; the results showed that the cells were very sensitive to $CuSO_4$, as judged by the biocidal efficiency and the observed decrease in both pigment content and chlorophyll autofluorescence of the cells (Ebenezer and Ki, 2013). Additionally, *P. minimum* exhibited increased levels of reduced glutathione when exposed to $CuSO_4$, as well as increased transcriptional levels of some stress-related or antioxidant genes, including heat shock proteins, catalase-peroxidase, and glutathione *S*-transferase (Guo et al., 2012, 2014; Guo and Ki, 2012a, 2013). These results suggest that $CuSO_4$ affects various physiological and biochemical pathways, and could even alter genome-wide gene expression in dinoflagellates. The biocide $CuSO_4$ could be a potential candidate to investigate the HAB termination mechanism.

As a microeukaryote, dinoflagellates have distinct genomic characteristics, which include permanently condensed and liquid-crystalline chromosomes, large nuclear genome size, fewer histones, and ~70% replacement of thymine with 5-hydroxymethyluracil. Gene transcription and regulation also differ from those of other typical eukaryotes. Recent studies show the TTTT motif, followed by (TATG)2, may serve as a core promoter motif,

* Corresponding author. Tel.: +82 2 2287 5449; fax: +82 2 2287 0070.
*E-mail address:* kijs@smu.ac.kr (J.-S. Ki).

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replacing the typical TATA box; tandemly arranged genes are not polycistronically transcribed in dinoflagellates (Hackett and Bhattacharya, 2006; Beauchemin et al., 2012; Lin et al., 2015). Additionally, spliced-leader *trans*-splicing (SL *trans*-splicing) is the main transcription method for the nuclear genes of many species of dinoflagellates (Lidie and Van Dolah, 2007; Zhang et al., 2007), but not all nuclear genes are SL *trans*-spliced in the dinoflagellate *Symbiodinium minutum* (Shoguchi et al., 2013). Furthermore, genes involved in the cell cycle and bioluminescence were not altered at the transcriptional level, but were altered at the protein level, indicating that some dinoflagellate genes are post-transcriptionally or translationally regulated (Mittag et al., 1998; Brunelle and Van Dolah, 2011). Genome studies in *Symbiodinium kawagutii* provided evidence for microRNA-based gene regulatory machinery (Lin et al., 2015). These extraordinary features suggest a highly complex and likely gene specific regulation mechanism in dinoflagellates (Mittag et al., 1998; Lidie et al., 2005; Erdner and Anderson, 2006; Morey et al., 2011; Guo and Ki, 2012a; Lin et al., 2015; Ponmani et al., 2015).

With modern technological advances, recent molecular methods (e.g., microarray and next generation sequencing) could allow analyses of the genomes and transcriptomes of dinoflagellates (Okamoto and Hastings, 2003; Moustafa et al., 2010; Toulza et al., 2010; Yang et al., 2011; Johnson et al., 2012; Shoguchi et al., 2013; Zhang et al., 2014a,c; Lin et al., 2015; Xiang et al., 2015). This has increased our understanding of the extraordinary genomic features of dinoflagellates, particularly the evolution of the eukaryotic genome. These high throughput technologies have sometimes been used to clarify the single-gene and genomic responses of HAB species. Few previous molecular studies were related to toxin-producing genes and nutrient effects on gene regulation (Moustafa et al., 2010; Morey et al., 2011; Zhang et al., 2014a,c). The effects of gene expression and regulation of nutrients on dinoflagellate growth have been studied (Moustafa et al., 2010; Morey et al., 2011). Therefore, further studies of the dinoflagellate genome and transcription might explain the molecular mechanisms underlying dinoflagellate bloom initiation and termination.

In the present study, a 6.0 K oligonucleotide chip was developed for the dinoflagellate *Prorocentrum minimum*, and it was used to determine the transcriptomic responses in bloom termination caused by copper treatment. The experimental dinoflagellate *P. minimum* is a potential HAB-forming species, which can produce diarrhetic shellfish poisoning (DSP) toxin. It has also been used in genomic, ecotoxicological, and evolutionary studies (Guo et al., 2012, 2014; Guo and Ki, 2012a,b, 2013), because the cells grow well under laboratory conditions, and have distinct genomic features.

## 2. Materials and methods

### 2.1. Cell culture

A strain (D-127) of *Prorocentrum minimum* was obtained from the Korea Marine Microalgae Culture Center (Pukyong National University, Busan, Korea). The *P. minimum* cultures were maintained in f/2 medium at 20 °C in a 12 h:12 h light–dark cycle with a photon flux density of approximately 65 μmol photons/$m^2$/s.

### 2.2. Stress treatment and RNA extraction

Using data from a previous study on median effective concentration ($EC_{50}$) for copper sulfate (Guo et al., 2012), exponential-phase *Prorocentrum minimum* cells were treated with $CuSO_4$ (Cat. No. C1297, Sigma, MO) at a final concentration of 0.5 mg $L^{-1}$. The treated cultures were harvested at 12 h, 24 h, and 48 h. The cell cultures were harvested by centrifugation at 1000 × *g* for 10 min, frozen immediately in liquid nitrogen, and stored at −80 °C until RNA extraction. Preserved cells were physically broken by freeze-thawing in liquid nitrogen, and further homogenization was performed using zirconium beads (diameter 0.1 mm) with Mini-Bead beater (BioSpec Products Inc., Bartlesville, OK). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA quality and quantity were analyzed by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA), and the same amount of RNA was used in the microarray analysis.

### 2.3. Photosynthetic efficiency and reactive oxygen species (ROS) measurement

Two physiological parameters, photosynthetic efficiency and ROS production, were analyzed. Photosynthetic efficiency is used as an evaluation of the photosystem; and ROS production is one indicator of oxidative stress in cells. Photosynthetic efficiency was evaluated by chlorophyll fluorescence, and measured by Handy PEA (Hansatech Instruments Ltd, Norfolk, UK). Parameters *Fv* (variation in fluorescence) and *Fm* (maximal fluorescence in the dark-adapted state) were measured at 0 h, 12 h, 24 h, and 48 h after $CuSO_4$ treatment. Subsequently, the *Fv*/*Fm* was calculated. The ratio of *Fv*/*Fm* indicated the efficiency of the photosynthetic apparatus. To measure ROS, the cells were stained with dihydrorhodamine 123 (DHR123, D1054, Sigma, MO) for 1 h. The DHR123 stock solutions were directly added into cell cultures at a final concentration of 5 μmol/L. After incubation, ROS was quantified by measuring fluorescence (at a wavelength of 485/530 excitation/emission, respectively) using a fluorescence spectrophotometer (Model LS-55, PerkinElmer, CA).

### 2.4. Oligonucleotide chip development

An expressed sequence tag (EST) database of *Prorocentrum minimum* (773K sequence reads, 291 Mb) was constructed using the DNA sequences obtained by a 454 GS FLX Titanium system, and contig assembly was carried out using Newbler (Roche, Branford, CT; Macrogen Inc., Seoul, Korea). The EST databases were annotated by the Blastx alignment with an *E*-value <0.001 against the NCBI non-redundant protein (NR) database. Functional annotation of these sequences by gene ontology (GO) was carried out with Blast2go software (Conesa et al., 2005). To design oligonucleotide probes and real time PCR primers, short EST fragments, those less than 250 bp in length, and certain bacteria-originated genes were removed. Exactly 7058 unique gene fragments were identified (3744 contigs and 3314 singletons) from 104,889 fragments (20,519 contigs and 84,370 singletons). From this, 97,931 unmatched fragments were left out, as they were most likely untranslated regions (UTR) or non-coding RNAs. From the above-identified genes, the dataset was further minimized, by excluding certain overlaps of identical genes, unknown genes, and genes less than 200 bp in size; furthermore, the genes assigned to bacteria in NR database were also removed (according to the taxonomy of best hit). Finally, a total of 6.0 K oligonucleotide probes (each of 35–40 nucleotides in length) were designed commercially (CombiMatrix, Mukilteo, WA). The 6.0 K oligonucleotide probes were then synthesized on a plate, provided by CombiMatrix, according to the manufacturer's protocol.

### 2.5. Microarray analysis

For the hybridization experiments, RNA samples from triplicates were labeled using biotin. The mRNA was purified from total RNA and used in further analysis. Pre-hybridization was performed