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Transcriptional responses of heat shock protein 70 (*Hsp70*) to thermal, bisphenol A, and copper stresses in the dinoflagellate *Prorocentrum minimum*

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HIGHLIGHTS

- ▶ Toxicogenomic response of *Hsp70* in the dinoflagellate *Prorocentrum minimum.*
- ▶ Putative *PmHsp70* contained three signature patterns of the Hsp70 family.
- ▶ EC_{50}s are 1.1 mg L^{-1} copper and 1.5 mg L^{-1} biophenol A in *P. minimum.*
- ▶ *PmHsp70* was significantly upregulated by thermal, Cu, and BPA exposures.
- ▶ Demonstration of the *Hsp70* response in the dinoflagellate to thermal and toxic stress.

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ABSTRACT

The heat shock protein 70 (*Hsp70*) family is an important part of the cell's machinery for protein folding, and helps to protect cells from environmental stress. Although *Hsp70* functions have been discovered in various organisms, studies on dinoflagellate Hsps are limited, except for a few phylogenetic attempts. In this study, we sequenced the complete open reading frame of the dinoflagellate *Prorocentrum minimum Hsp70* (*PmHsp70*), and characterized its molecular functions. The putative *PmHsp70* protein contained 3 signature patterns of the Hsp70 family. Phylogenetic analysis revealed that *PmHsp70* belonged to the dinoflagellate clade. Real-time (RT)-PCR analyses revealed that *PmHsp70* was upregulated by thermal stress. Further, we examined the transcriptional response of *PmHsp70* to copper (Cu) and bisphenol A (BPA) exposures. In toxicity assays, Cu and BPA exhibited EC_{50}-72 h values of 1.07 ± 0.138 mg L^{-1} and 1.51 ± 0.110 mg L^{-1}, respectively, in *P. minimum*. Expression of *PmHsp70* was significantly upregulated in response to Cu and BPA exposures (one-way ANOVA, $P < 0.05$). *PmHsp70* displayed different expression patterns in response to different concentrations of Cu and BPA. This study evaluated typical characteristics and, for the first time, toxicant-related functions of *PmHsp70*. The results suggest that *Hsp70* genes may play a vital role in the environmental stress responses of dinoflagellates.

1. Introduction

Dinoflagellates are the primary producers in aquatic ecosystems; they are highly diversified, with more than 4500 species and 550 genera identified till date, according to the Tree of Life Web Project (http://tolweb.org/tree/). Dinoflagellates include organisms of interest (e.g., *Akashiwo*, *Alexandrium*, *Karenia*, *Pfiesteria*, and *Prorocentrum*) for eukaryotic evolutionary studies and, as toxic algae, for their impact on human health and fisheries. As unicellular eukaryotes, dinoflagellates have distinct genomic features (Taylor, 1987; Hackett et al., 2005; Moreno Díaz de la Espina et al., 2005; Moustafa et al., 2010). For instance, they possess a large amount of DNA, ranging from 1.5 to 225 pg per cell (LaJeunesse et al., 2005), and their chromosomes remain permanently condensed during the entire cell cycle (Taylor, 1987; Moreno Díaz de la Espina et al., 2005). The dinoflagellate nuclear DNA is extensively methylated, and 12–70% of thymine is replaced by 5-hydroxymethyluracil (Lin, 2011). The genes expressed in dinoflagellates are *trans*-spliced in nuclear mRNA processing reactions (Zhang et al., 2007). The expression of S-phase proteins in certain dinoflagellates (e.g., *Karenia brevis*) is independent of transcription upon entry into the S-phase, but appears to be under post-transcriptional control (Brunelle and Van Dolah, 2011). In recent years, dinoflagellate gene regulation and expression studies have been performed using expressed sequence tags (ESTs) or global gene expression profiles (Okamoto and Hastings, 2003; Hackett et al., 2005; Moustafa et al., 2010). These EST analyses indicate that many dinoflagellate genes possess a high copy number and display a certain degree of diversity between these copies (Bachvaroff and

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Place, 2008). In addition, genes involved in specific regulatory processes have been identified in dinoflagellates (Okamoto and Hastings, 2003; Moustafa et al., 2010).

Heat shock proteins (Hsps) are ubiquitously expressed and highly conserved molecular chaperones involved in transport, folding, unfolding, assembly, and disassembly of multistructured units, and degradation of misfolded or aggregated proteins (Sørensen et al., 2003; Stephanou and Latchman, 2011). In addition, Hsps are involved in defense mechanisms against various environmental stresses, such as elevated temperature, heavy metals, endocrine disrupting chemicals (EDCs), UV light, xenobiotics, and hypoxia (Mukhopadhyay et al., 2003; Sørensen et al., 2003; Planelló et al., 2008; Rhee et al., 2009; Gupta et al., 2010; Morales et al., 2011). Overall, the Hsps are divided into five major families, including small Hsp, Hsp60, Hsp70, Hsp90, and Hsp100, depending on their apparent molecular weights, amino acid sequence homologies, and functions (Mukhopadhyay et al., 2003). Among these Hsps, the Hsp70 family is the most conserved and largest family, and the first to be induced under stress conditions (Gupta et al., 2010). Furthermore, a variety of environmental stresses and toxic chemicals can induce *Hsp70* expression (Piano et al., 2004; Rhee et al., 2009; Morales et al., 2011). *Hsp70* genes and proteins have been extensively studied from bacteria to humans, and the genes exhibit various expression patterns depending on the type of environmental stress (Morales et al., 2011). Given that Hsps have important roles in the cellular defense response, Hsp70 has been used as a biomarker in many organisms, such as green algae (Bierkens et al., 1998), fish (Washburn et al., 2002), and mollusks (Wepener et al., 2005), for monitoring aquatic pollution.

In dinoflagellates, the complete open reading frame (ORF) sequence of the *Hsp70* gene was first determined in *Crypthecodinium cohnii* (Fast et al., 2002), followed by *Prorocentrum minimum* (Zhang et al., 2007) and *Symbiodinium* sp. (Rosic et al., 2011). In addition, at least 7 partial *Hsp70* sequences of dinoflagellates (e.g., *Lepidodinium chlorophorum*, *Noctiluca scintillans*, *P. minimum*, and *Symbiodinium* sp.) have been recorded in the public GenBank database (searched in January 2012). Most sequences of the dinoflagellate *Hsp70* were studied for phylogenetic implications (Fast et al., 2002; Minge et al., 2010). Even though 2 complete *Hsp70* ORF sequences have been revealed from cDNAs of *P. minimum* (CCMP 696), they were studied through spliced leader sequence analysis (Zhang et al., 2007). Okamoto and Hastings (2003) attempted to analyze genome-wide expression in the dinoflagellate *Pyrocystis lunula* using cDNA microarrays, and detected upregulation of *Hsp70* in cells that were exposed to $NaNO_2$. Recently, Rosic et al. (2011) studied *Hsp70* expression in the symbiotic dinoflagellate *Symbiodinium* sp. exposed to thermal stress and different light:dark cycles. The authors suggested that the dinoflagellate Hsps may have functions similar to those of defense/stress response proteins and molecular chaperones; however, other functions of dinoflagellate *Hsp70*, such as its role in protection against toxic chemicals, have not been thoroughly investigated.

In the present study, we determined the complete ORF sequence of the dinolagellate *P. minimum Hsp70* (*PmHsp70*), and characterized its phylogenetic relationships to those of other alveolates, and transcriptional responses to certain environmental stressors, such as thermal shock, and toxic chemicals. Particularly, we evaluated toxic, genomic effects of copper (Cu) and bisphenol A (BPA) to *P. minimum*, because these toxic chemicals were generally considered as common environmental contaminants under representative metals or EDCs (Staples et al., 1998; Grosell et al., 2007; Ebenezer and Ki, 2012). As baseline data, we measured the median effective concentration (EC_{50}) values for Cu and BPA in *P. minimum*, which is a phototrophic, free-living, and armored dinoflagellate that belongs to an important group of phytoplankton living in marine and freshwater environments (Hackett et al., 2004). *P. minimum* produces a potent neurotoxin that causes diarrhetic shellfish poisoning (DSP), and is responsible for harmful algal blooms (Hackett et al., 2004).

2. Materials and methods

2.1. Cell culture

A strain (D-127) of *P. minimum* was obtained from the Korea Marine Microalgae Culture Center (Pukyung National University, Busan, Korea), which was originally isolated from surface coastal waters at Tongyeong, Korea in 1997. The cells were routinely maintained in f/2 medium, and were grown at 20 °C in 12:12 h light:dark cycle with a photon flux density of about 65 μmol photons/m^{-2}/s^{-1}.

2.2. PmHsp70 gene sequence determination

Partial *PmHsp70* sequences were obtained from the *P. minimum* EST data (773 K sequence reads, 291 Mb) in our laboratory, where DNA sequences were determined by 454 pyrosequencing (GS-FLX Titanium; 454 Life Sciences, Roche, Branford, CT). For determining the complete ORF sequence of *PmHsp70*, we designed a dinoflagellate-specific primer using the conserved dinoflagellate spliced leader (SL) sequence (5′-DCC GTA GCC ATT TTG GCT CAA G-3′, where D = T, A, or G) that was identified by Zhang et al. (2007), and a *PmHsp70*-specific reverse primer (Hsp70-R1) using the available partial sequences of the *PmHsp70* gene (Table 1). In this case, the Hsp70-R1 was located downstream from the Hsp70 stop codon. First, PCR amplification was carried out with a set of SL and Hsp70-R1 primers to generate amplicons that covered the entire ORF of *PmHsp70*. However, the target molecules were insufficiently amplified in the primary PCR; therefore, we diluted the PCR products 100-fold for use as templates in the secondary PCR. We designed 2 additional PCR primers (Hsp70-F1, and Hsp70-R2) that targeted the complete ORF of *PmHsp70* (Table 1). Thus, we successfully amplified the complete ORF of *PmHsp70* from the cDNA of *P. minimum*. PCR conditions for the primary and secondary PCRs were as follows: pre-denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 54/55 °C for 30 s, 72 °C for 100 s; and extension at 72 °C for 10 min. Secondary PCR products were cloned into RBC TA cloning vector (RBC Bioscience, Taipei, Taiwan), transformed into competent cells, and subjected to DNA sequencing. The complete ORF sequence of *PmHsp70* was deposited into the GenBank database (accession number JN401970).

The online tools PROSITE and PSORT program (http://prosite.expasy.org/; http://psort.hgc.jp/) were used for protein motif and location analysis, respectively.

Table 1
Primers used in this study.

Gene	Primer	Remarks	Nucleotide sequence (5′ → 3′)
PmHsp70	SL	cDNA amplification	DCCGTAGCCATTTTGGCTCAAG
PmHsp70	Hsp70-F1	cDNA amplification	ASMCATGTCGAAGAAGACCG
PmHsp70	Hsp70-R1	cDNA amplification	GCGAAGTCTATGAGTCTGTGG
PmHsp70	Hsp70-R2	cDNA amplification	TTAGTCCACCTCCTCCACAG
α-Tubulin	TUA1	RT-PCR	GCGTGCTGCATGATGTATCGTG
α-Tubulin	TUA2	RT-PCR	ATCCGGTAGGGCACCAATCAAC
PmHsp70	Hsp70F	RT-PCR	TGATCGGTCGCAAATTCGCCG
PmHsp70	Hsp70R	RT-PCR	TCTCCTCGCCCTGTGATGTCAC