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INDUCTION OF PROGRAMMED CELL DEATH IN AGING *PROROCENTRUM DONGHAIENSE* CELLS AS WAS EVIDENCED PRELIMINARILY BY THE IDENTIFICATION OF ASSOCIATED TRANSCRIPTS

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Prorocentrum donghaiense caused large-scale red tides off Chinese coast in recent years. Expressed sequence tag (EST) analysis was carried out for this dinoflagellate in order to identify the genes involved in its proliferation and death. A cDNA library was constructed for *P. donghaiense* at late exponential growth phase, and 308 groups of EST were generated, which include 36 contigs and 272 singletons. Among 22 groups showed homologies with known genes, 2 matched significantly with caspase and proliferating cell nuclear antigen. Caspase and proliferating cell nuclear antigen are 2 key proteins involved in programmed cell death. Their identification evidenced preliminarily the induction of PCD in aging *P. donghaiense.* The identified included also calmodulin and protein phosphatase, two proteins involved in diverse cell processes including PCD by binding to or modifying others.

Keywords: Prorocentrum donghaiense – expressed sequence tag – programmed cell death

INTRODUCTION

Red tide, a widely observed phenomenon, is characteristic of the proliferation and fast death of some phytoplankton including dinoflagellate. The phytoplankton, which accounts only for less than 1% of the total biomass, contributes more than 50% of the primary productivity of the earth [10], which means phytoplankton must undergo a whole cycle of growth and death almost once a week. Therefore, the mechanism underlining the proliferation and death of phytoplankton is crucial for us to understand the breakout and extinction of associated red tides and global material cycling. In contrast to the intensive efforts to elucidate the conditions, mechanisms and strategies of controlling phytoplankton cell growth and further red tide formation, little

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attentions have been paid to how phytoplankton die. Previously, animal grazing and sedimentation were presumed to be the reason for cell disappearance of phytoplankton, and later, exogenous virus infection was presumed to be the reason for the high rate of lysis of phytoplankton. The impact of virus infection on red tide extinction of *Emiliania huxleyi* has even been evidenced by molecular biological studies and direct observation under transmission electron microscopy [19]. However, virus infection does not satisfy all experimental observations [5]. In recent years, it is found that programmed cell death (PCD) contributes to the cell death of both prokaryotic and eukaryotic phytoplankton [5].

PCD is characterized by distinct morphological changes of cells (apoptosis) that include cell shrinkage, chromatin condensation and DNA fragmentation and accompanied by the expressions of a set of genes. It is a biochemical cascade coordinated by receptors, adaptors, signal kinases, proteases and nuclear factors. PCD is a universal mechanism in metazoans. PCD has also been found in unicellular organisms including bacteria, yeast and protozoa, implying that it might be fundamental to both prokaryotic and eukaryotic microorganisms including phytoplankton [5, 9]. In response to nitrogen and phosphorus limitations, the mortality of *Ditylum brightwellii*, a diatom, was evidenced to undergo PCD in laboratory culture [7]. Similarly, in case of depletion of nitrogen, *Thalassiosira weissflogii* was found to lose selectively some important proteins including RuBisCO, markedly reducing photosynthetic efficiency [3]. *Perdinium gatunses*, a dinoflagellate, was shown to initiate PCD in response to carbon dioxide limitation and reactive oxygen stress resulted from such limitation [23]. When exposed to univalent-cation salts, freshwater cyanobacterium *Anabaena* spp. has been shown to initiate PCD with an increase in nonspecific protease activity [15]. PCD has also been observed in *Dunaliella tertiolecta* [20] in response to light deprivation and reactive oxygen stress, and prokaryotic filamentous cyanobacterium *Trichodesmium* sp. IMS101 [4] in response to aging, nutrient starvation, strong light irradiance and oxidative stress. However, these studies were only morphological observations, and to a certain extent, biochemical assaying of associated proteins. Researches have never reached the final decipherment of PCD in phytoplankton yet.

Cysteinyl-aspartate-specific proteases (caspases) are central to the PCD. As the final players of PCD cascade, they degrade various essential proteins selectively, causing PCD of cells [9]. Systematically, 2 other families of functional caspase orthologues have been distinguished. These include para-caspases from animals and slime mould and meta-caspases from plants, fungi, unicellular protozoa and various bacterial species [5]. The protein of caspase has only been isolated from multicellular animals. However, caspase activity has been detected in vascular plants, yeasts and trypanosomes. Analyses of whole genome sequences of both prokaryotic and eukaryotic phytoplankton have predicted wide existences of the functional homologues of caspases and metacaspases. These include the two unicellular eukaryotic algae, a chlorophyte *Chlamydomonas reinhardtii* and a diatom *Thalssiosira pseudonana*. However, the specific association of the expressions of these genes with caspase activity has not been demonstrated yet [5].

An appropriate approach of identifying functional genes of a species is to generate and analyse expressed sequence tags (EST). EST analysis has been implemented in functional genomic researches of a wide range of eukaryotes. ESTs are short cDNA sequences that serve as not only gene tags, but also the basis of multiple uses such as profiling transcripts, making cDNA arrays and most importantly, identifying functional genes. EST databases are exponentially expanding due to the generation of a large amount of EST for diverse organisms including eukaryotic phytoplankton such as unicellular green alga *Chlamydomonas reinhardtii* [21], diatom *Phaeodactylum tricornutum* [14, 18] and dinoflagellate *Alexandrium tamarense* [11], *Lingulodinium polyedrum* [22] and *Amphidinium carterae* [2]. Unfortunately, none of them was from *P. donghaiense*.

P. donghaiense, one of dinoflagellate, has caused the most notorious red tides off Chinese coast in recent years, bringing local environments serious impacts. Currently, very rich publications concerning its life history, ecology and taxonomy are available [13, 17, 25]. In contrast, molecular biological studies of *P. donghaiense* are very scarce [26, 27]. In order to initiate the molecular biological research of *P. donghaiense*, a cDNA library was constructed for the artificial culture of this species at late exponential growth phase. Here we reported the identification of PCD associated transcripts which evidenced preliminarily the induction of PCD in aging *P. donghaiense*.

MATERIALS AND METHODS

Algal culture

P. donghaiense pure culture was isolated and maintained by The First Institute of Oceanography, National Oceanic Administration of China. Cells were cultured using *f*/2 medium prepared with seawater sterilized through filtration at 20 °C, under the irradiance of 150 μmol photons m^{-2} sec^{-1} and at the regime of 12 h light/12 h dark. Light source is cool white fluorescent lamps 20 cm above the flask, and the irradiance was measured at the bottom of the flask. The biomass was frequently reduced (once two weeks) to maintain normal growth of *P. donghaiense.* Five milliliters of culture at logarithm growth phase ($8.0 – 10.0 \times 10^3$ cells mL^{-1}) were inoculated into 200 mL medium and culture for the isolation of total RNA. During cultivation, the culture was sampled frequently, and fixed with 5% formalin (final concentration) for cell counting under microscope. The growth curve was made always in order to sample culture at late exponential growth phase for the isolation of Total RNA.

cDNA library construction

Cells were collected by centrifugation at 4000 rpm and 4 °C, frozen in liquid nitrogen and ground into powder. Total RNA was isolated from ca. 10 mg of cells (fresh

weight) using UNIQ-10 Trizol RNA Preparation Kit following manufacturer's instruction (Sangon, Shanghai, China). Quality and concentration were estimated according to the brightness of 28S and 18S rRNA bands and yeast tRNA reference in 0.8% agarose gel stained with EtBr. Double-stranded cDNA was synthesized from about 1 μg of total RNA using M-MLV RTase cDNA Synthesis Kit (Takara, Dalian, China) following manufacture's instruction. Briefly, total RNA was pretreated at 65 °C for 5 min and placed on ice. The reaction mixture was made as the suggested of kit manufacturer. The first strand was synthesized at 30 °C for 10 min followed by 42 °C 1 h and 80 °C 5 min. The second strand was synthesized at 16 °C for 2 h and 70 °C for 10 min. cDNA was amplified using cDNA PCR Library Kit (Takara, Dalian, China) after purification, end-blunting at 37 °C for 10 min and adaptor-adding at 22 °C overnight (>8 h) and 65 °C for 5 min. Oligonucleotides (5'-AGC GCG TGG TAC CAT GGT CTA GAG TCG ACT AAG TAG GT-3' and 3'-(NH2)-TTC ATC CA-(P)-5') were synthesised and annealed to yield the adaptor. Those fragments >400 bp were retrieved from 1.0% low-melting point agarose gel. The PCR amplification of cDNA was carried out at 95 °C for 4 min followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min and a final extension step at 72 °C for 10 min. The primers used were RA (5'-CTG ATC TAG ACC TGC AGG CTC -3') and CA (5'-CGT GGT ACC ATG GTC TAG AGT-3'). cDNA was ligated into pMD18-T vector (Takara, Dalian, China) and electroporated into *E. coli* JM109 using Eppendorf Electroporator 2510 set at 1500 V and 5 ms. Clone were picked randomly and subjected to single pass sequencing after insert orientation was determined using primer combinations of adaptor corresponding one and two sequencing primers each (one each side). The clone was sequenced suing either M13 (–47) or M13 (–48) sequencing primer depending on the orientation of the insert contained. The remaining were washed off the medium and stored at –80 °C in 15% of glycerol.

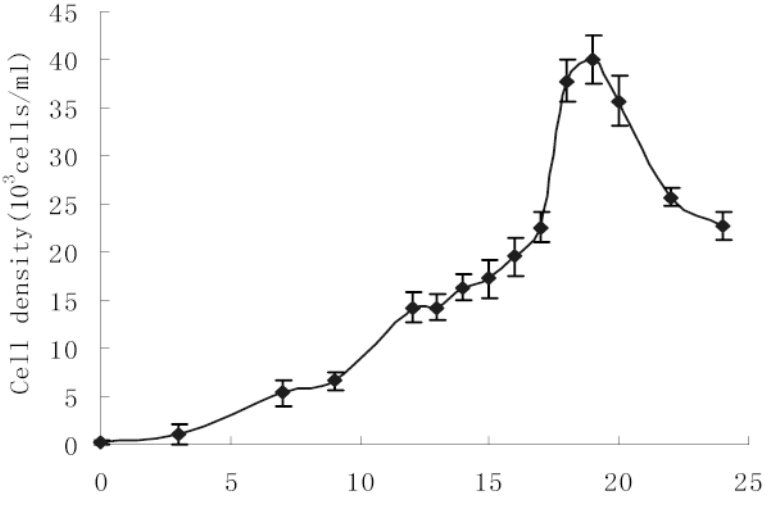

Fig. 1. Growth curve of *P. donghaiense* under laboratory cultivation condition

Sequence analysis

ABI3730 automatic sequencer was used to read the sequences of the clones selected. Each clone was sequenced from the 5' end of mRNA corresponding strand in order to avoid pre-termination of reading caused by poly (A) tail. The sequencing was done through commercial service provided by Sangon, Shanghai, China. After trimming the sequence portions with low quality or corresponding to vector off the original, usable reads generated were assembled using CAP3 program [12] into contigs and singletons, each was called an EST group.

The consensus sequence of each EST groups was subjected to searching against database non-redundant DNA sequence collection using BLASTN program online [http://www.ncbi.nlm.nih.gov/] in order to eliminate various ribosomal RNA tags. A match was considered as significant when the possibility value (*E* value) was less than *E-10*. The remaining groups each were used as the queries in homologous searching against database protein amino acid sequences with BLASTX program online [http://www.ncbi.nlm.nih.gov/; 1]. A match was considered to be significant when the possibility value was less than 0.01.

RESULTS

Construction of P. donghaiense *cDNA library*

According to the growth curve of *P. donghaiense* (Fig. 1), cells of *P. donghaiense* were harvested on day 20 for isolation of total RNA. A cDNA library was constructed using about 1 μg total RNA. In order to obtain cells synchronous in growth and integrate molecular biological analysis with physiological study which needs to treat cells as less as possible and as even as possible, it is desirable to use total RNA in library construction in combination with PCR amplification ahead of ligation with vector. Ten milligrams of cells yielded about 5 μg total RNA with one-fifth used to synthesize cDNA. Reverse transcription was initiated by priming with oligodeoxythymidine, which should avoid the use of prokaryotic transcripts if any as templates. After amplification, about 0.25 μg cDNA >400 bp in length was obtained, which meets the requirement of further manipulations. About 50 ng cDNA was ligated with 50 ng pMD18-T vector and electroporated into *E. coli* JM109. Approximately, 6,000 clones were generated from a single ligation, and about 90% of them were recombinants as were determined by insert amplification of about 700 randomly picked clones using primer combinations of adaptor corresponding one and two sequencing primers each on vector. In this way, the orientation of insert is determined as well. About 600 clones with inserts were subjected to single pass sequencing from the 5' end of the strand corresponding to mRNAs, yielding 565 usable reads.

EST analysis

After eliminating those reads corresponding to rRNAs, the remaining were assembled into 272 singletons and 36 contigs, each was called an EST group. The consensus sequences of all groups have been submitted to GenBank with accession numbers DQ336341, DQ336342 and DW520905 – DW521210. Group 80 and 40 covered 60 and 18 reads, respectively, representing the two most prevalent mRNA transcripts. The GC content of these consensus sequences ranged from 29% to 84%, with an average of 55%. Homologous searching online using BlastX program found significant matches for 23 EST groups and assigned putative or known functions to 22 of them which represented 22 genes (Table 1) involved in metabolism (52%), cell

Table 1
EST groups showing significant GenBank match and their functional classification

EST group	Putative/ known function (no. of ESTs)	Significant match	e value
	Metabolism (12)		
4	chloroplast ATPH isoform 5	AAW80675	8.00E-25
41	methionine adenosyltransferase II	XP_424874	2.00E-6
267	phosphoribulokinase	AAW79321	3.00E-65
284	oxygen evolving enhancer 1 precursor	AAW77465	3.00E-18
293	amylopullulanase	AAA19800	1.00E-8
296	cytochrome oxidase subunit 1	AAM97696	7.00E-102
279	cysteine protease-like	XP_468427	2.00E-16
310	peroxidase	EAM25377	4.00E-30
311	phosphoglycerate/ bisphosphoglycerate mutase	ZP_00776870	1.00E-18
312	dual specificity protein phosphatase	CAJ02188	3.00E-4
313	sulfatase family protein	AAK23156	3.00E-25
314	cytochrome b	AAK67258	6.00E-74
	Cell structure/motility (2)		
177	alpha tubulin	AAM69359	8.00E-51
309	actin	AAM02969	2.00E-17
	Gene/protein expression (3)		
48	major basic nuclear protein	AAL61534	2.00E-27
211	major basic nuclear protein	AAL61533	2.00E-45
299	proliferating cell nuclear antigen	AAO14679	2.00E-93
	Cell signaling/communication (3)		
190	adult brain protein 239	XP_416454	2.00E-8
302	pre-B-cell colony enhancing factor 1	XP_782393	4.00E-48
315	calmodulin	CAA69660	1.00E-33
	Ribosomal protein (1)		
236	40S ribosomal protein S8	Q9FIF3	9.00E-53
	Others (2)		
278	hypothetical protein AdehDRAFT_0869	ZP_00401633	4.00E-4
287	CG4525-PA	XP_584727	4.00E-58

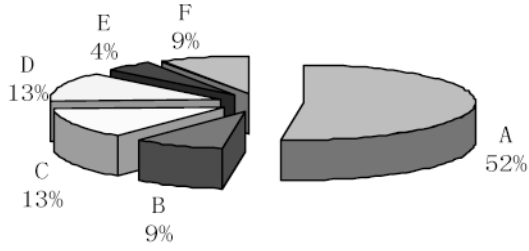

Fig. 2. Percentage of ESTs assigned to different functional groups. A, metabolism; B, cell structure/motility; C, gene/protein expression; D, cell signaling/communication; E, ribosomal protein; F, other

structure/motility (9%), gene/protein expression (13%), cell signaling/communication (13%) and ribosomal protein (4%) (Fig. 2) categorized according to the methods described previously [8]. Special attention should be paid to 4 protein homologues including cysteine protease (caspase), proliferating cell nuclear antigen (PCNA), calmodulin and protein phosphatase which may evidence the induction of PCD in aging *P. donghaiense*. The tags of caspase and PCNA were aligned with the corresponding regions of their most similar homologues as showed in Figs 3 and 4.

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                    10        20        30        40        50        60        70        80
                    ----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-
O. sativa           NGEWGDHVTLQAAADKYGVKIFVMTSFKDTCYIEIQPKWQKSNKVVLLSFWAEVHYNSIYPQNDAPRSQTTRKRRWWPFSHN
A. thaliana         NGEWGDHVTLQAAADLPGVRMFVITSFKDTCYIEILPHFQKSNRLICLSFWAEVHYNSIYPEGELPIPEGKKKKKYWVF---
P. donghaiense      HGEWGDNVSLQAIADAFGVHTVVVTSFPRACIIEIQPRKVKSEQVIWLSFWAELHYSSLRPRGGAPQSGVVSGAAEAGCNIS
                    ***** * *** **  **   * ***   * *** *   **     ****** ** *  *    *
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Fig. 3. Alignment of amino acid sequence deduced from EST group 279 with those of *Oryza sativa* (XP_468427) and *Arabidopsis thaliana* (NP_568136). Conservative amino acids were marked with asterisks

DISCUSSION

A small scale EST analysis was carried out for *P. donghaiense* at late exponential growth phase. It is difficult to profile the transcripts based on our limiting data set, however, 22 functional genes have been identified. Their identification will facilitate our understanding of the important physiological processes of *P. donghaiense*. In addition, they will also provide the starting points for the isolation of corresponding full-length cDNA and genomic fragments. In this way, the molecular biological study of *P. donghaiense* will be promoted to a certain extent.

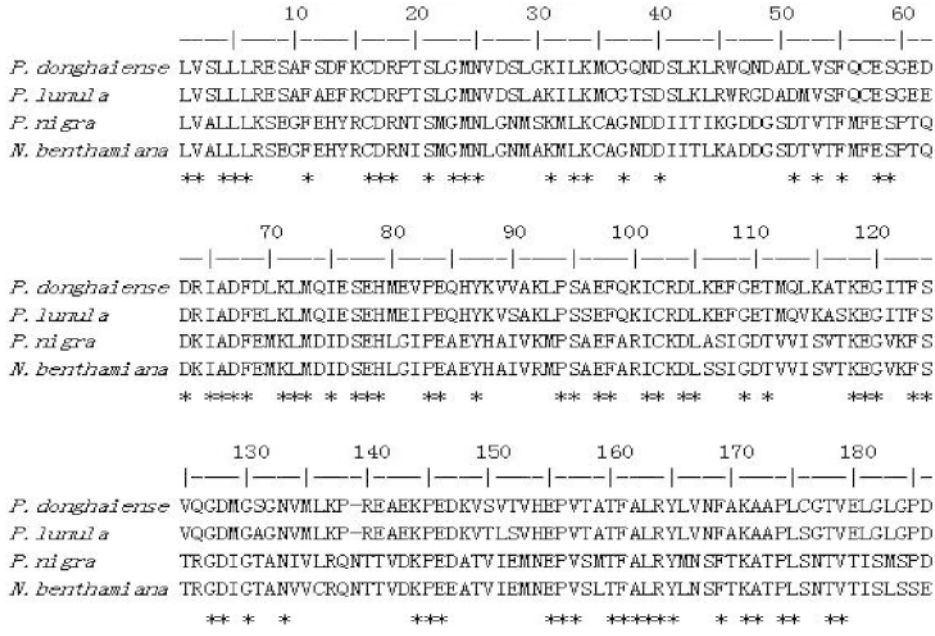

Fig. 4. Alignment of amino acid sequence deduced from EST group 299 with those of *Pyrocystis lunula* (AAO14679), *Populus nigra* (Q9MAY3) and *Nicotiana benthamiana* (AAG24908). Conservative amino acids were marked with asterisks

The proliferation and death of a red tide causing phytoplankton are component parts of breakout and extinction of red tide. Animal grazing and sedimentation were considered as the reasons of cell disappearance of phytoplankton at the beginning. Then it was believed that exogenous virus infection was the main reason of the death of phytoplankton. In recent years, programmed cell death (PCD) was suggested as one of causes of the death of phytoplankton. The only clue of PCD in phytoplankton at the level of gene expression is the identification of the transcripts of metacaspase orthologues in *E. huxleyi* [24]. Very interestingly, an EST group was found to be the transcripts of cysteine protease (caspase) encoding gene in this study. Its identification proved preliminarily the induction of PCD in aging *P. donghaiense*, and should facilitate our study on the death mechanism of this dinoflagellate. In addition, the expression of this gene may also be developed into a physiological marker potentially usable in the monitoring of *P. donghaiense* red tide. It was not clear if this tag represented the gene of metacaspase; the tag was too short to make phylogenetic analysis.

The identified EST groups contained also an orthologue of proliferating cell nuclear antigen (PCNA). Three PCNA proteins form a sliding DNA clamp which provides a sliding platform for the replication of lagging strand of DNA [6]. PCNA is involved in diverse cell processes including PCD. When it is absent or at a low

concentration, the cell will die in a programmed manner [16]. It has been used as a physiological indicator of vigorous cell proliferation in the diagnosis of cancer. Similarly, the identification of the PCNA homologue should facilitate our understanding of the mechanism of fast growth of this dinoflagellate and its death as well.

The important proteins identified included also calmodulin and protein phosphatase, which interact with diverse proteins to regulate their functions, and further the performance of cells as we have learned. In addition, actin and alpha tubulin hold the potentials of being used in phylogenetic studies of *P. donhanense*.

Total RNA was used for cDNA synthesis and the PCR amplification of cDNA was introduced ahead of library construction. These modifications allow us to approach the scientific problems performed by a very limited number of cells. Although direct use of total RNA in cDNA synthesis and cDNA library construction holds advantages in avoiding the risk of RNA digestion by acute RNase and overcoming the limitation of biomass, yet proportional distribution of natural mRNA profile may be biased by such modifications to some extent. However, the loss of representativeness is reimbursed by the gain of feasibility. In fact, the thermocycling number of PCR amplification of cDNA had been minimized to that of yielding operable amount of cDNA only. It should be noticed that almost one-fifth of usable sequencing reads correspond to different rRNAs, especially 28S rRNA. The reason for this may be the initiation of reverse transcription at the internal of rRNAs where adenine repeat locates, and the high proportion of rRNAs. Obviously, a strategy should be developed to avoid such interference in order to reduce the cost of sequencing, or alternatively, cDNA library should be normalized ahead of sequencing.

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