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# Insights into possible cell-death markers in the diatom *Skeletonema marinoi* in response to senescence and silica starvation

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ABSTRACT

Diatoms are a hugely diverse microalgal class, which possesses unique biological features and complex metabolic pathways and may activate sophisticated mechanisms to respond to environmental changes. Abiotic stress factors may limit growth rate of diatoms, but may also trigger intracellular signaling pathways that cause cells to undergo programmed cell death (PCD).

Here we investigate the gene expression of different target genes related to cell death, namely programmed cell death 4 (PDCD4), tumor susceptibility gene 101 (TSG101), developmental and cell death (DCD) domain, death specific protein (DSP) and metacaspase (MC), using RT-qPCR in the cosmopolitan coastal centric diatom species *Skeletonema marinoi*, which contributes significantly to phytoplankton blooms in temperate waters. To this end, we undertook a detailed study of the best reference genes to analyze gene expression in *S. marinoi* under different experimental conditions (i.e. in different growth phases or under silica starvation).

Results showed that DSP gene expression had a clear and constant increase along the *S. marinoi* growth curve reaching its maximum during the senescent phase. On the contrary, PDCD4, DCD, TSG101 and MC did not show any significant variation. These findings indicate that the DSP gene is a possible PCD marker induced by aging in this diatom species. In contrast, levels of DSP transcripts induced by silica starvation were relatively low compared to those induced by cell aging suggesting differential activation and/or regulation of the PCD machinery in response to different stressful conditions. Our study also expands the list of reference genes available for the diatom *S. marinoi* for normalization of RT-qPCR data of cells cultivated under different growth phases or under silica starvation.



## 1. Introduction

Diatoms are key ecological players in the contemporary ocean, the ecological success of which is still poorly understood. This hugely diverse microalgal class possesses unique biological features and complex metabolic pathways (Bowler et al., 2010), in part obtained from their ancestors via secondary endosymbiosis (Armbrust, 2009; Bowler et al., 2008). Recent results suggest that diatoms use sophisticated mechanisms to respond to environmental changes. Vardi et al. (2006), for example, demonstrated that diatoms can sense the presence of polyunsaturated aldehydes in the environment and employ them as signaling molecules to control diatom population size. When stress conditions induce cell lysis rates to increase, aldehyde concentrations could exceed a certain threshold, and may function as a diffusible bloom-termination signal that triggers cell death in the population. A number of studies have shown that cell age, nutrient limitation and other abiotic stress factors, such as intense light, excessive salt concentration or oxidative stress, may not only limit growth rate of diatoms, but also trigger intracellular signaling pathways that cause cells to undergo programmed cell death (PCD, Bidle, 2015; Bidle and Falkowski, 2004). Phytoplankton PCD is a form of autocatalytic cell suicide in which an endogenous biochemical pathway leads to apoptotic-like morphological changes, including chromatin condensation, DNA fragmentation, cell shrinkage, and compartmentalization of cell contents into apoptotic bodies, and, ultimately, cellular dissolution (Parker et al., 2008; Bidle and Falkowski, 2004). However the molecular mechanisms behind the death signaling pathway are largely unknown. To better understand this process, we investigated the gene expression of different target genes related to cell death, namely programmed cell death 4 (PDCD4), tumor susceptibility gene 101 (TSG101), developmental and cell death (DCD) domain, death specific protein (DSP) and metacaspase (MC), using RT-qPCR in the cosmopolitan coastal centric diatom species *Skeletonema marinoi*. This diatom contributes significantly to phytoplankton blooms in temperate waters, including the Northern Adriatic Sea (Mediterranean Sea), where it forms dense, almost monospecific blooms in late winter and may reach concentrations of millions of cells per liter (Miralto et al., 1999, 2003).

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Genes encoding for PDCD4 have been identified in animal and plant kingdoms and in many unicellular eukaryotic microorganisms including the diatom species *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Cheng et al., 2013). In animals, PDCD4 acts as a tumor-suppressing protein that inhibits protein synthesis during translation initiation (Ozpolat et al., 2007), while, in plants, PDCD4 has been implicated in ethylene signaling and abiotic stress responses (Cheng et al., 2013).

TSG101 is an eukaryotic gene related to ubiquitin-conjugating enzymes. Altered transcripts of this gene have been detected in many human malignancies. The TGS101 protein may play a role in cell growth and differentiation and acts as a negative growth regulator (Oh et al., 2002).

The DCD domain has been found in plant proteins, where it is quite well conserved at the amino acid sequence level and, as indicated by biological studies, mediates signaling development and PCD (Tenhaken et al., 2005). A distinct member of the DCD domain has also been found in a hypothetical protein of the diatom *T. pseudonana*, while it is absent in proteins of bacteria, fungi and animals (Tenhaken et al., 2005).

Death specific protein genes have been identified in the diatoms *Skeletonema costatum* and *T. pseudonana*, where they encode calcium-regulated and membrane associated proteins (Thamatrakoln et al., 2013; Chung et al., 2005, 2008). In metazoans, it is well known that an imbalance in the intracellular calcium homeostasis can be toxic for the cell, and results in the activation of different calcium-regulated proteins leading to cell death (Orrenius et al., 2003; Inbal et al., 2002; Nakagawa and Yuan, 2000; Robertson et al., 2000).

Metacaspases are a family of caspase-orthologous genes that are only found in plants, fungi, and protists, but not in animals. In plants, metacaspases have a key role in PCD, but very little is known about these enzymes in unicellular eukaryotes and even less about their roles in cell death (Choi and Berges, 2013).

In order to study these death-related genes, we undertook a detailed study of the best reference genes to analyze gene expression in *S. marinoi* under different experimental conditions (i.e. in different growth phases or under silica starvation). To date, studies on reference genes have been undertaken only for the diatoms *P. tricornutum* (Siaut et al., 2007), *T. pseudonana* (Alexander et al., 2012), *S. costatum* and *Chaetoceros affinis* (Kang et al., 2012), *Ditylum brightwellii* (Guo et al., 2013) and *Pseudo-nitzschia multistriata* and *Pseudo-nitzschia arenysensis* (Adelfi et al., 2014). Our study therefore expands on the list of reference genes available for diatoms and also provides new insights into the regulatory processes leading to cell death in diatoms.

## 2. Material and Methods

### 2.1. Cell culturing and experimental conditions

Batch cultures of *S. marinoi* (CCMP 2092, Sarno & Zingone in Sarno et al., 2005) were grown in two experimental conditions: in silicate-enriched Guillard's f/2 medium (nutrient replete condition, Guillard, 1975) or in a modified f/2 medium with a reduced $Si(OH)_4$ concentration, from 107 μM to 36 μM (Si-starved), both prepared with 0.2-μm-filtered and autoclaved seawater. Cultures were grown in two-liter polycarbonate bottles constantly bubbled with air filtered through 0.2 μm membrane filters (Sartorius, Goettingen, Germany). Each experiment was performed in triplicates. Cultures were kept in a climate chamber (RefCon, Napoli, Italy) at 20 °C on a 12 h:12 h light:dark cycle at 100 μmol photons $m^{-2}$ $s^{-1}$. Initial cell concentrations were about 5000 cells/mL upon inoculation and culture growth was monitored daily from samples fixed with one drop of Lugol (final concentration of about 2%) and counted in a Bürker counting chamber under an Axioskop 2 microscope (20×) (Carl Zeiss GmbH, Jena, Germany). Aliquots of 50 mL of *S. marinoi* culture were collected every day, from day 4 to day 9 in replete treatment (N = 18) and from day 5 to day 8 for Si-starvation (N = 12), always at the same time of day in order to avoid interference due to circadian variability, and were harvested by centrifugation at 3900 g for 30 min at 4 °C using a cooled centrifuge with a swing-out rotor (DR 15P, Braun Biotechnology International, Allentown, PA, USA). The pellet was re-suspended in 800 μL of TRIzol (Invitrogen, Carlsbad, CA, USA), incubated for 2–3 min at 60 °C until the pellet was completely dissolved. Samples were frozen in liquid nitrogen and kept at −80 °C until analysis.

### 2.2. RNA extraction and reverse transcription

The total RNA was extracted from the pellet following the procedure described in Barra et al. (2013). DNase treatment was carried out using Dnase I recombinant, Rnase-free (Roche, Basel, Switzerland) according to the manufacturer's protocol to eliminate potential genomic DNA contamination. Total RNA sample was purified and concentrated using RNeasy MinElute Cleanup Kit (Qiagen, Venlo, Netherlands) and eluted in 20 μL RNase-free water. Concentration of the resulting RNA samples was assessed by absorbance at 260 nm (ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). The integrity of total RNA was checked by agarose gel electrophoresis. From each RNA sample, 600 ng was retro-transcribed in complementary DNA (cDNA) with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions, using the T100 Thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA).

### 2.3. Primer design

Candidate reference genes and genes of interest (Table 1) were chosen considering the putative sequences reported in the *S. marinoi* transcriptome (Cod. MMETSP1039) deposited in the public database CAMERA (http://camera.crbs.ucsd.edu/mmetsp/list.php) and iMicrobes, interactive query tool for microbial data (http://data.imicrobe.us/sample/view/1867). Selected reference genes were histone 4 (H4), alpha and beta tubulin (TUB A and TUB B), TATA-binding protein (TBP), elongation factor 1α (EF1a), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cyclin dependent kinase (CDS), ribosomal protein small subunit 30S (RPS), actin (ACT) and ubiquitin (UB). Primers were designed using Primer3 program V. 0.4.0 (Untergrasser et al., 2012; Koressaar and Remm, 2007; Table 2) and each sequence was initially tested by standard PCR. Reaction was carried out in 20 μL volume with 2 μL of 10× PCR reaction buffer (Roche, Basel, Switzerland), 2 μL of 10 × 2 mM dNTP, 0.2 μL of 5 U/μL Taq (Roche, Basel, Switzerland), 1 μL of 20 pmol/μL for each oligo, 1 μL of cDNA templates and nuclease-free water to 20 μL. The PCR program consisted of a denaturation step at

**Table 1**
Camera ID numbers for candidate reference genes and genes of interest used for RT-qPCR.

| Gene symbol | Gene name | Transcript IDs |
|---|---|---|
| *Putative reference genes* | | |
| H4 | Histone H4 | MMETSP1039-20121108\|9473 |
| TUB B | Tubulin beta | MMETSP1039-20121108\|10653 |
| TUB A | Tubulin alpha | MMETSP1039-20121108\|11367 |
| TBP | TATA binding protein | MMETSP1039-20121108\|947 |
| EF1a | Translation elongation factor-1-alpha | MMETSP1039-20121108\|686 |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | MMETSP1039-20121108\|11983 |
| CDK | Cyclin dependent kinase | MMETSP1039-20121108\|8806 |
| RPS | 30S ribosomal protein | MMETSP1039-20121108\|3026 |
| ACT | Actin | MMETSP1039-20121108\|18642 |
| UB | Ubiquitin | MMETSP1039-20121108\|19467 |
| *Target genes* | | |
| PDCD4 | Programmed cell death 4 | MMETSP1039-20121108\|5969 |
| TSG101 | Tumor susceptibility gene | MMETSP1039-20121108\|10158 |
| DCD | Development and cell death domain | MMETSP1039-20121108\|11669 |
| DSP | Death specific protein | MMETSP1039-20121108\|13048 |
| MC | Metacaspase | MMETSP1039-20121108\|20858 |

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