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Insights into the transcriptome of the marine copepod *Calanus helgolandicus* feeding on the oxylipin-producing diatom *Skeletonema marinoi*

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ABSTRACT

Diatoms dominate productive regions in the oceans and have traditionally been regarded as sustaining the marine food chain to top consumers and fisheries. However, many of these unicellular algae produce cytotoxic oxylipins that impair reproductive and developmental processes in their main grazers, crustacean copepods. The molecular mode of action of diatoms and diatom oxylipins on copepods is still unclear. In the present study we generated two Expressed Sequence Tags (ESTs) libraries of the copepod *Calanus helgolandicus* feeding on the oxylipin-producing diatom *Skeletonema marinoi* and the cryptophyte *Rhodomonas baltica* as a control, using suppression subtractive hybridization (SSH). Our aim was to investigate differences in the transcriptome between females fed toxic and non-toxic food and identify differentially expressed genes and biological processes targeted by this diatom. We produced 947 high quality ESTs from both libraries, 475 of which were functionally annotated and deposited in GenBank. Clustering and assembling of ESTs resulted in 376 unique transcripts, 200 of which were functionally annotated. Functional enrichment analysis between the two SSH libraries showed that ESTs belonging to biological processes such as response to stimuli, signal transduction, and protein folding were significantly over-expressed in the *S. marinoi*-fed *C. helgolandicus* compared to *R. baltica*-fed *C. helgolandicus* library. These findings were confirmed by RT-qPCR analysis. In summary, 2 days of feeding on *S. marinoi* activated a generalized Cellular Stress Response (CSR) in *C. helgolandicus*, by over-expressing genes of molecular chaperones and signal transduction pathways that protect the copepod from the immediate effects of the diatom diet. Our results provide insights into the response of copepods to a harmful diatom diet at the transcriptome level, supporting the hypothesis that diatom oxylipins elicit a stress response in the receiving organism. They also increase the genomic resources for this copepod species, whose importance could become ever more relevant for pelagic ecosystem functioning in European waters due to global warming.

1. Introduction

Diatoms are responsible for approximately 50% of marine primary production and play a major role in the transfer of energy through marine food chains (Mauchline, 1998). However, upon cell damage several species produce cytotoxic compounds such as poly-unsaturated aldehydes (PUAs) and other oxylipins deriving from the oxidative metabolism of fatty acids (d'Ippolito et al., 2004; Fontana et al., 2007; Pohnert, 2000). Oxylipins and PUAs in particular impair several reproductive and developmental processes in copepods, such as gametogenesis (Poulet et al., 2007a), embryogenesis (Ianora et al., 1995), egg viability (Ianora et al., 2003), larval development (Carotenuto et al., 2002) and sex differentiation (Carotenuto et al., 2011). They also induce malformations (teratogenesis) and apoptosis in newly hatched nauplii (Ianora et al., 2004). Reduced copepod fecundity, hatching success and larval fitness have been reported during major spring diatom blooms in the Mediterranean Sea (Northern Adriatic Sea) (Miralto et al., 1999), the Northern Eastern Pacific Ocean (Dabob Bay, Washington, USA) (Halsband-Lenk et al., 2005; Pierson et al., 2005), the Northern Eastern Atlantic Ocean (English Channel) (Poulet et al., 2006) and in upwelling areas of the South-Eastern Pacific Ocean (off the coast of Chile) (Poulet et al., 2007b; Vargas et al., 2006).

The diatom *Skeletonema marinoi* is an ubiquitous bloom-forming species found in both northern and southern temperate

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latitudes (Kooistra et al., 2008). The strain used in the present study originates from the Northern Adriatic Sea, where the species dominates the winter-spring phytoplankton bloom (Aubry et al., 2004). This *S. marinoi* strain is reported to produce up to 14 different oxylipins, including PUAs, oxoacids, hydroxyacid, epoxy alcohols, and fatty acid hydroperoxides, which reduce egg hatching success and cause malformations in the offspring of the copepod *Calanus helgolandicus* during in vitro incubation assays (Fontana et al., 2007).

Calanus helgolandicus is one of the most widely studied copepod species in terms of development, growth, reproduction and recruitment (Bonnet et al., 2009; Cook et al., 2007; Ianora and Miralto, 2010; Pond et al., 1996; Rey-Rassat et al., 2002). It is one of the dominant zooplankton species in European waters, living in open and coastal waters from the temperate Eastern Atlantic Ocean to the Mediterranean basin (Northern Adriatic Sea) (Bonnet et al., 2009). A recent survey on the distribution of *C. helgolandicus* in European waters has shown that the species is gradually expanding northwards, with peaks in abundance moving from Mediterranean coasts to the North Sea, due to an increase in water temperatures in the last decade (Bonnet et al., 2005). Considering the predicted further increase in water temperatures due to general global warming, *C. helgolandicus* could replace its congener *Calanus finmarchicus* and become the dominant *Calanus* species in the North Sea (Bonnet et al., 2009). Despite extensive studies on *C. helgolandicus*, the molecular mode of action of diatoms and diatom oxylipins on this species is still not clear. Only recently has it been shown, using RT-qPCR, that 2 days of feeding on *Skeletonema marinoi* inhibits a series of genes involved in aldehyde detoxification, apoptosis regulation and cytoskeleton structure (Lauritano et al., 2011a,b, 2012).

To enrich these studies and identify a wider set of differentially expressed genes and biological processes induced by a *Skeletonema marinoi* diet, we used suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) to generate two reciprocal Expressed Sequences Tags (ESTs) libraries of *Calanus helgolandicus* fed for 2 days on *S. marinoi* and the control algae *Rhodomonas baltica*. The obtained *C. helgolandicus* ESTs were further assembled into contiguous transcripts (contigs), which were functionally annotated and used for quantitative gene expression analysis using RT-qPCR.

Genomic and transcriptomic studies are still under-explored in copepods, being mainly limited to species such as the fish parasites *Lepeoptheirus salmonis* and *Caligus rogercresseyi*, the tidepool *Tigriopus californicus* and the planktonic congener *Calanus finmarchicus* (Bron et al., 2011). To date, there are only 196 nucleotide sequences for *Calanus helgolandicus* deposited in GenBank (October 2013), 26 cDNA/mRNA sequences of which correspond to those deposited by (Lauritano et al., 2011a,b, 2012).

Our study shows that the *Skeletonema marinoi*-fed *Calanus helgolandicus* library was significantly enriched in ESTs encoding for genes involved in protein folding, response to stimuli and cytoskeleton functioning, and that up-regulation of molecular chaperones and signal transduction genes was confirmed in *S. marinoi*-fed *C. helgolandicus* using RT-qPCR. These results suggest that 2 days of feeding on *S. marinoi* activates a generalized Cellular Stress Response (CSR) in *C. helgolandicus*, and that these genes help protect the copepod from the harmful effects of the diatom diet.

2. Materials and methods

2.1. Copepods collection and feeding experiments

Calanus helgolandicus females were collected in September 2009 from a 500-L re-circulating copepod cultivation system established at the Stazione Zoologica Anton Dohrn (Carotenuto et al., 2012) and originating from specimens collected in the North Adriatic Sea from March to April 2009. The copepod culture was fed a mixed food assemblage of *Rhodomonas baltica*, *Prorocentrum minimum* and *Isochrysis galbana* and kept at 18 °C and 38‰ salinity. Adult *C. helgolandicus* females sampled from the breeding tank (~1200 specimens) were sorted under a stereomicroscope (Leica) and transferred to several 10 L beakers filled with 0.22 μm filtered sea water enriched with either the diatom *Skeletonema marinoi* (SKE) (45,000 cells/ml, 1 mgC/L) or the control diet *R. baltica* (RHO) (8000 cells/ml, 1 mgC/L) known to support high egg hatching success and survival in this copepod species (Carotenuto et al., 2012). Both algae were grown as semi-continuous batch cultures to late-exponential phase of growth in 2-L glass jar containing K medium (Keller et al., 1987) or f/2 medium (Guillard and Lorenzen, 1972), for *R. baltica* or *S. marinoi*, respectively. Algal cultures were grown in a temperature-controlled chamber kept at 18 °C and on a 12:12 h light:dark cycle. After 48 h of feeding, *C. helgolandicus* females from each of the two groups were transferred to filtered seawater (FSW) for an additional 24 h, in order to allow gut evacuation. A total of 500 *R. baltica*-fed *C. helgolandicus* females and 500 *S. marinoi*-fed *C. helgolandicus* females were collected, washed in FSW and transferred to two 14-mL polypropylene tubes with 5 ml Trizol Reagent (Invitrogen, San Diego, CA, USA). Samples were frozen directly in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. RNA extraction and mRNA purification

Total RNA of *Calanus helgolandicus* was extracted from each group using Trizol Reagent and a phenol:chloroform separation according to the manufacturer's protocol. The procedure was the same as reported in (Lauritano et al., 2011b, 2013). RNA quantity and purity were checked by Nano-Drop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies Inc., Wilmington, DE, USA), and by gel electrophoresis. Poly A^+ RNA of each sample was purified from total RNA using the Dynabeads® mRNA Purification Kit (Dynal Biotech ASA, Smestad, Norway) and the concentration and purity were assessed by Nano-Drop.

2.3. Suppression subtractive hybridization libraries

Suppression subtractive hybridization (SSH) libraries were performed using the PCR-Select™ cDNA subtraction kit (Clontech, USA) following the manufacturer's instructions. Briefly, 2 μg of poly A^+ RNA from each total RNA *Calanus helgolandicus* sample were used for double strand (ds) cDNA synthesis, followed by *Rsa I* digestion, adaptors ligation and two hybridization steps. We performed two SSH: a forward subtraction where the cDNA from *C. helgolandicus* fed *Skeletonema marinoi* was used as tester and the cDNA from *C. helgolandicus* fed *Rhodomonas baltica* was used as driver, to obtain a library enriched for transcripts whose expression was induced during *S. marinoi* feeding; and a reverse subtraction, where the cDNA from *C. helgolandicus* fed *R. baltica* was used as tester and the cDNA from *C. helgolandicus* fed *S. marinoi* was used as driver, to obtain a library enriched for transcripts whose expression was induced during *R. baltica* feeding. The last two steps of suppressive PCR reactions were performed using the 50× Advantage® cDNA Polymerase Mix (Clonthech, USA).

The amplified subtracted cDNAs of *Calanus helgolandicus* were cloned into the pCR®II-TOPO® TA Cloning® vector (Invitrogen) and transferred into One Shot® TOP10F′ Competent Cells (Invitrogen). The chemically transformed cells were plated onto LB agar supplemented with 100 μg ml^{-1} of ampicillin, 40 mg ml^{-1} of X-Gal and 100 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubated at 37 °C overnight for the blue-white colony screening. All white and light blue clones were randomly picked and transferred into 2 ml LB-ampicillin (100 μg ml^{-1}) tube and