Response of the symbiotic cnidarian *Anthopleura elegantissima* transcriptome to temperature and UV increase

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**Abstract**

Elevated temperature and solar radiation, including ultraviolet radiation, are now recognized as the primary environmental stresses that lead to mass cnidarian bleaching. This study takes a functional genomics approach to identifying genes that change expression soon after exposure to these stressors in the temperate sea anemone *Anthopleura elegantissima* that harbors *Symbiodinium*, the same genus of symbionts found in reef-building corals. Symbiotic anemones were subjected to elevated temperature or UV over a 24 h period. cDNA from these animals was hybridized to a 10,000-feature cDNA microarray of *A. elegantissima*. Overall, 2.7% of the 10,000 features were found to be differentially expressed as a function of temperature or UV stress. Of the 86 features sequenced, 45% displayed significant homology to sequences in GenBank. There are 27 features that were differentially expressed in both stress conditions. Gene ontology analysis placed the differentially expressed genes in a wide range of categories including cytoskeleton organization and biogenesis, protein biosynthesis, cell proliferation, apoptosis and transport. This suggests that the early stress response to elevated temperature and UV involves essentially all aspects of host cellular regulation and machinery and that downstream cnidarian bleaching is a complex cellular response in host tissues.

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1. Introduction

Symbioses between cnidarians and dinoflagellates are common in the marine environment; they form the foundation of the coral reef ecosystem (Dubinsky, 1990) and are a key component in some temperate communities (Muller-Parker and Davy, 2001). Despite their prevalence, these mutualisms appear to be delicately balanced on the edge of dysfunction and surprisingly intolerant of stress. There is now ample evidence that elevated sea surface temperature, resulting from global warming, causes stress in these mutualisms that leads to symbiosis dysfunction and breakdown, a phenomenon called cnidarian bleaching (Hoegh-Guldberg, 1999). Bleaching is named for the whitening of animal tissue most often due to the loss of pigmented dinoflagellates from the host cells (Hoegh-Guldberg, 1999; Douglas, 2003). In addition to temperature, other environmental factors, such as high light, high UV radiation and pollution can act synergistically to exacerbate stress (Stone et al., 1999; Hoegh-Guldberg, 2004; Lesser et al., 1992; Dunn et al., 2002, 2004). Coral bleaching leads to decreased growth and reproduction and increased disease and mortality (Brown, 1997; Hoegh-Guldberg, 1999). It is a clear threat to the overall health of reefs and is contributing to the decline of reefs globally.

Large-scale coral bleaching appears to be initiated by photosynthetic dysfunction in the symbiont, leading to oxidative stress in both symbiont and host, which starts a cascade of destruction that ultimately leads to the loss of symbionts from host tissue (Hoegh-Guldberg, 1999; Weis, in press). High temperature and UV, alone or in combination, cause symbiont photoinhibition via several mechanisms (reviewed in Hoegh-Guldberg, 1999; Venn et al., 2008). This leads to the generation of high concentrations of reactive oxygen species (ROS) in the symbiont that can cause cellular damage and further photoinhibition (Lesser and Shick, 1989; Lesser, 1996, 1997). ROS can then migrate into host tissues and cause DNA damage (Lesser and Freeman, 2004), protein carbonylation (Richier et al., 2006, 2008), and lipid peroxidation (Richier et al., 2005) and may trigger cellular signaling events that lead to symbiosis breakdown (Perez and Weis, 2006). Symbionts are ultimately lost from host cells via one or more mechanisms including host cell detachment, exocytosis, and host cell apoptosis or necrosis (Gates et al., 1992; Dunn et al., 2002, 2004). A comprehensive understanding of these processes, however, is far from complete.

The complexity of cnidarian bleaching makes it an ideal problem to tackle with functional genomics. The genomics era in biology is
offering new opportunities to examine non-model organisms, such as cnidarians and dinoflagellates in new complex and comprehensive ways. The placement of cnidarians at the base of the metazoan tree makes them of considerable interest to evolutionary biologists and therefore there is considerable recent genomic information available on cnidarians, including the *Nematostella vectensis* genome (*Putnam et al., 2007*), numerous EST databases and a soon-to-be-released *Hydra* genome. In addition there are some early genomic studies of cnidarian symbiosis (*Kortschak et al., 2003; Kuo et al., 2004; Schwarz et al., 2006; Rodriguez-Lanetty et al., 2006; deBoer et al., 2007*) and cnidarian symbiosis during stress (*Edge et al., 2005*).

In this study we were interested in identifying genes from the temperate host anemone *Anthopleura elegantissima* that are differentially expressed soon after exposure to elevated temperature and UV, in order to elucidate the cellular cascade leading to symbiosis breakdown and bleaching. We conducted a transcriptome analysis using a 10,368 features cDNA microarray. This first molecular analysis of a cnidarian response to stresses known to cause bleaching could lend insight into the mechanisms driving bleaching in the host and provide information on cellular pathways to target for further study.

### 2. Materials and methods

#### 2.1. Collection and maintenance of organisms and experimental design

Specimens of *A. elegantissima* (Brandt) were collected in two locations: Neptune Beach and Boiler Bay on the central Oregon coast. Symbiotic animals were collected in the intertidal zone on sun-exposed rocks and transported back to Oregon State University (OSU) in plastic bags. To standardize the physical condition to which the animals were acclimated, the animals were maintained in a 400 L seawater tank in the laboratory for four weeks at 12 °C, under a light intensity of 130 μmol quanta m⁻² s⁻¹, on a 12 h light/12 h dark cycle. Animals were fed brine shrimp once a week. Animals were then transferred into 40 L experimental tanks, placed in a 12 °C cold room for a week and then subjected to one of three different conditions for 24 h: 1) control, 2) high temperature (+8 °C above ambient) or 3) UVB (12 W m⁻², λmax=368 nm, light emission range = 310–420 nm). The elevated temperature value of 20 °C was chosen based on previous studies on thermal stress of *A. elegantissima* (*Buchsbaum, 1968; Pearse, 1974; Schwarz, 2002*). Water in the high temperature tanks was heated using aquarium heaters. The temperature in each tank was monitored throughout the experiment to ensure that temperatures remained stable.

The elevated UV value was chosen based on UVB studies of corals that detected DNA damage following short-term exposure to UVB (*Baruch et al., 2005*). UVB (Q-Panel UVB 313, Cleveland, OH) was alternated with full spectrum vita light bulbs (Duro-test, which included both host and algal partners) using Trizol. cDNA probe synthesis was performed from 1 μg total RNA using SuperScript II (Invitrogen) and a Genisphere 3 DNA-50 microarray kit according to the manufacturer instructions. Slides for hybridization were chosen randomly from the batch of high quality printed arrays. Before hybridization, the slides were incubated for 10 min at 50 °C. cDNA from control and stressed organisms were combined and hybridized to arrays in a formamide-based hybridization buffer under LifterSips overnight in an oven at 50 °C. Following post-hybridization washes, Cy3 and Cy5 Capture Reagents were hybridized to the array in the formamide-based buffer under LifterSips for 4 h at 50 °C. Following post-hybridization washes, slides were scanned using a GenePix® 4200 scanner (Axon Instruments) and image acquisition and quality control was performed using the software GenePix® Pro 5.

#### 2.2. Construction of cDNA arrays

A previously constructed 10,368-feature *A. elegantissima* cDNA microarray from libraries of symbiotic and aposymbiotic animals was used for this study (*Rodriguez-Lanetty et al., 2006*). The approximately 5000 spots from the symbiotic library contained primarily host and not symbiont cDNA (see *Rodriguez-Lanetty et al., 2006* for details). cDNA inserts were PCR reamplified from frozen stocks using M13 forward and reverse vector primers. The size of cDNA inserts ranged between 0.4 and 2.5 kb. The CDNs, buffered in 3X SSC and 1.5 M betaine, were spotted on UltraGAPS™ coated slides (Corning) without duplication. Two serial dilutions of commercial alien cDNAs (Lucida Scorecard, Amersham; SpotReport® Alien Oligo Array validation system, Stratagene) were included in the spotting for setting scanner parameters and to homogenize the hybridization results for the all sets of arrays. After printing, the arrays were dried for 48 h in vacuum desiccators, and UV cross-linked at 300 mJ. The slides were subjected to quality control by hybridizing a Cy3 random 9-mer on spotted cDNA. An absent-spot threshold was fixed at 5% and 1% of features missing per pin block and per entire array respectively and stored in desiccators until hybridization.

#### 2.3. Hybridization of arrays

For probe construction, total RNA was extracted from anemones (which included both host and algal partners) using Trizol. cDNA probe synthesis was performed from 1 μg total RNA using SuperScript II (Invitrogen) and a Genisphere 3 DNA-50 microarray kit according to the manufacturer instructions. Slides for hybridization were chosen randomly from the batch of high quality printed arrays. Before hybridization, the slides were incubated for 10 min at 50 °C. cDNA from control and stressed organisms were combined and hybridized to arrays in a formamide-based hybridization buffer under LifterSips overnight in an oven at 50 °C. Following post-hybridization washes, Cy3 and Cy5 Capture Reagents were hybridized to the array in the formamide-based buffer under LifterSips for 4 h at 50 °C. Following post-hybridization washes, slides were scanned using a GenePix® scanner (Axon Instruments) and image acquisition and quality control was performed using the software GenePix® Pro 5.

#### 2.4. Experimental design and statistical analysis of microarray data

Four biological replicates per condition were used for the two-comparison design (*Wu et al., 2003*) and these were dye swapped (*Kerr, 2003*). Ratio-intensity plots were constructed for each array to determine whether or not intensity dependence of log ratios, which appears as curvature, was present. An rLowess curve-fitting transformation (*Yang et al., 2002*) was applied to the data. The transformation was applied to all the arrays to maintain consistency in the whole data set as suggested by *Cui et al. (2003)*. To detect differentially expressed genes between the two conditions, the following 2-stage ANOVA mixed model (*Wu et al., 2003*) was fitted to the log transformed intensity data: \[ Y=A (Array) +D (Dye) +S (Sample=Individual) +C (Condition) +E (Error). \]

In this mixed model, the arrays and samples were treated as random factors and dye and condition as fixed factors. The F statistics, a shrinkage estimator for gene-specific variance components that makes no assumption about the distribution of variances across genes was estimated (*Cui et al., 2005*). After the statistics were estimated, p values were calculated from random permutations (N permutations = 100 with sample reshuffling), and adjusted to correct for Type I error derived from multiple testing using the false discovery rate (FDR) approach (*Wu et al., 2003*). All the analyses described above were conducted using the software R/Maanova (*Wu et al., 2003*).
2.5. Sequencing and gene identity

PCR products generated from cDNA inserts isolated from clones were sequenced directly and all sequencing was performed from the 5' end using the T3 primer specific to the Lambda Zap II phage vector (Stratagene). Vector and low quality sequences were masked manually. A BlastX search of NCBI non-redundant proteins was performed and those with Expect values (E values) of less than $1 \times 10^{-4}$ were considered to be homologues to the matched known protein. For each gene, we report the hit with the lowest expect values that are to a known genes. The Gene Ontology (Harris, 2004) classifications were assigned to all sequenced clones blasted through AMIGO.

3. Results and discussion

The analysis of 10,368-feature cDNA array revealed a total of 284 (2.7%) features that were differentially expressed (Bayesian t test, $P<0.05$) under either elevated temperature or UV. Of these, 216 were differentially expressed due to elevated temperature with 84 and 132 being up- and down-regulated respectively, while 68 were sensitive to UV with 18 and 50 being up- and down-regulated respectively (Fig. 1). Twenty-seven features were regulated by both stressors and in all cases, direction of regulation, up or down, was the same for both stressors. Ten were up- and 17 were down-regulated respectively (Fig. 1).

The majority of features (100 of 216) responding to elevated temperature displayed a positive or negative fold change between 1.5 and 5 (Fig. 2A). However there were 10 features that were down-regulated and 7 that were up-regulated more than 20 fold (Fig. 2A). Most features (35 of 68) responding to UV also had fold changes (positive and negative) between 1.5 and 5 (Fig. 2B). Most of the remaining features were down-regulated (Fig. 2B). Overall, fold change values were higher in features responding to UV compared to elevated temperature.

From the 284 significant features, 86 were chosen for sequencing. Of these, 27 displayed significant homology to sequences found in GenBank with induced expression higher than a fold change of 1.1 and a significant BlastX hit (E<$10^{-4}$). A functional classification of those sequences is suggested in the Table 1, and shows a wide range of biological processes e.g. cytoskeletal organization and biogenesis, protein biosynthesis, cell proliferation, apoptosis and transport. This suggests that there is a complex effect of environmental stressors on host gene expression.

3.1. Unknown sequences

Forty-seven of the 86 features sequenced (55%) displayed no significant homology in BlastX searches. This high proportion of unknown sequences is similar to other functional genomic studies of cnidarians including 55% in an EST study of Aiptasia pulchella (Kuo et al., 2004), 64% from an EST study of the coral Acropora millepora (Kortschak et al., 2003), and 65% from A. elegantissima using the same array as this study (Rodriguez-Lanetty et al., 2006).

3.2. Cnidarian host as the likely origin of transcripts

We did not specifically test for the origin, host or symbiont, of the differentially expressed features on the array, for example with confirmation by PCR of host-only or symbiont-only material. However, it is likely that the vast majority of features on the array are of host origin. Almost half (5000) of the array features were from a cDNA library from aposymbiotic host RNA and another 5000 were from a library from symbiotic host RNA that was extracted under conditions to virtually eliminate contaminating symbiont RNA (see Rodriguez-Lanetty et al., 2006 for details). Furthermore, of the 27 features that had significant BlastX hits, 16 were features from the aposymbiotic library (indicated by the APO prefix in the OSU-CGB ID, Table 1). Finally in all cases, top hits for the 27 features were to metazoan sequences, and not to apicomplexan or dinoflagellate sequences (data not shown).

3.3. Cellular pathways affected by thermal and UV stress

Over the last several decades, elevated temperature and UV have been repeatedly shown to induce, separately or simultaneously, cnidarian bleaching (Hoegh-Guldberg, 1999; Weis, in press). Thus, there is some information on the cellular pathways involved (see Introduction) but no complementary genomic approach has been performed. Our transcript analysis begins to identify some of the changes in gene expression that are likely involved in response to these stresses and that might cause bleaching. Bioinformatic identification and ontogenetic analysis of the type of genes that are differentially expressed (1) suggest effects of both stresses on host cells, and (2) provide clues to the pathways that lead to cnidarian bleaching.

3.4. Cytoskeleton organization and biogenesis

Our transcript analysis identified two actin homologs that were down-regulated during thermal stress. This result mirrors heat shock studies of animal cell lines, which show a similar down-regulation of genes responsible for cytoskeletal organization (Shyy et al., 1989; Goode et al., 2000). Like elevated temperature, UV caused a down-regulation of actin but also a 4.76 down-regulation of tropomyosin, an actin-binding protein important in cytoskeletal remodeling (O'Neill et al., 2008). Down-regulation of such important cytoskeletal elements suggests that this stressor causes cytoskeletal disorganization in host tissues and may have implications for changes in host cellular architecture as it relates to harboring symbionts within its tissues.

3.4.1. Protein biosynthesis

Among the candidates identified as significantly down-regulated by thermal stress were several transcripts related to ribosomal proteins (Table 1). This down-regulation under elevated temperature

![Fig. 1. Venn diagram of the 286 array features differentially expressed as a function of elevated temperature (grey) or UV (dashed lines). Size of circles is proportional to number of features (listed in each circle, with number of features in common listed in overlapping regions).](image-url)
has already been described in *Drosophila melanogaster* (Bell et al., 1988), the yeast *Saccharomyces cerevisiae* (Gasch et al., 2000) and in the tropical coral *Montastrea faveolata* (Edge et al., 2005). In the fish *Gillichthys mirabilis*, as has been widely observed across taxa, exposure to high temperature can inhibit general protein synthesis concomitant with the preferential production of specific stress response proteins (Buckley and Hofmann, 2002).

### 3.5. Oxidative stress

We detected a 17.8 fold up-regulation of the ferritin in animals subjected to temperature increase. Ferritin is an important iron-storage protein with a cytoprotective role (Crichton, 1990) and plays a significant role in innate immunity (Beck et al., 2002). Most organisms possess proteins, including ferritin that can sequester transition metals, thereby reducing the ability of these metals to catalyze free-radical formation. In response to the production of damaging oxygen radicals, genes such as ferritin, superoxide dismutase, cytochrome c, peroxidase and thioredoxin are up-regulated to neutralize these reactive oxygen species (ROS) (Cairo et al., 1995). *In situ* high temperature and UV is known to induce ROS production, leading to cellular damage in symbiotic cnidarians (Lesser, 1996, 2006). We suggest here that ferritin is induced in response to increases in ROS following thermal stress. In recent work by Schwarz et al. (2008), ferritin was identified as a highly expressed gene in data set of two scleractinian corals (*Acropora palmata* and *A. millepora*) and appeared to be undergoing adaptive evolution.

Another class of genes, known to respond to oxidative stress, was differentially expressed in response to either thermal or UV stress. Transcripts related to CCAAT/enhancer binding proteins (C/EBPs) were significantly regulated in response to both thermal and UV stress. C/EBPs are a family of six transcription factors, including C/EBP-α and C/EBP-β, that regulate cellular proliferation and differentiation through a variety of mechanisms (Nevlov, 2007). C/EBP-α is anti-proliferative, and it acts as a general tumor suppressor (Lekstrom-Himes and Xanthopoulos, 1998). In contrast, C/EBP-β both inhibits and promotes cell cycle progression (Nevlov, 2007), including playing an anti-apoptotic role in cell proliferation (Shimizu et al., 2007). In vertebrates, C/EBPs proteins are involved in apoptotic cascades in response to ROS. Cell characteristics of apoptosis (nuclear condensation, membrane blebbing and caspases activation) were observed as a result of oxidative stress dependent induction of CCAAT/enhancer protein homologous protein (CHOP) (Tagawa et al., 2008; Yokouchi et al., 2008).

In this study, a C/EBP-α homolog was 5.42 fold down-regulated and a C/EBP-β homolog was 1.59 fold up-regulated as a function of elevated temperature. In response to UV, another transcript, corresponding to a C/EBP-β homolog was 6.27 fold down-regulated. In addition, a tissue factor pathway inhibitor homolog (TFPI), which induces apoptosis and inhibits angiogenesis in vertebrates (Sierko et al., 2007) was 6.51 fold down-regulated. Taken together, these data suggest that oxidative stress is a major actor in the *A. elegantissima* cellular response to thermal and/or UV stress. Furthermore, this corroborates evidence from hyperthermic stress-induced bleaching studies of other anemones and corals that implicate a role for host cell apoptosis in the loss of symbionts from host tissues (Dunn et al., 2002, 2004, 2007a; Richier et al., 2006, 2008).

### 3.6. Membrane trafficking

Finally, transcripts of a Rab7 homolog were greatly down-regulated (35 fold) under UV stress. Rab7 is a small GTPase that plays an important role in endosome/lysosome trafficking and maturation (Bucci et al., 2000). Lysosomal regulation and maturation is an important process in symbiotic cnidian host cells as the symbionts are contained within vacuoles that, instead of being digested, persist within the cell. Recent work in another symbiotic anemone has tied differential presence of several Rabs, including Rab7, to successful persistence of symbionts within host cell vacuoles.
Table 1
Known sequences showing significant expression differences between stressed and unstressed A. elegantissima

<table>
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<th>Functional category</th>
<th>OSL_CGRR ID</th>
<th>GenBank Accession ID</th>
<th>E values</th>
<th>Accession # of homologues</th>
<th>FC high temperature</th>
<th>FC UV</th>
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Identification was based on significant blast hits (E values <10−5) from GenBank. Ratios of expression (fold change: FC) are shown. Genes discussed in the text are indicated with (*).

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(Chen et al., 2003, 2004, 2005). Furthermore, there is early evidence that autophagy, which involves changes in membrane trafficking and maturation of lysosomes, plays a role in cnidarian bleaching (Dunn et al., 2007b). In this context, differential expression of Rab7 with UV suggests that this parameter causes changes in membrane trafficking that could be involved in loss of symbionts during bleaching.

3.7. Unexpected failure to detect differences in well-known stress response genes

We have identified several genes operating in pathways that are known to function in the stress and bleaching responses in symbiotic cnidarians. However, it is notable that some well-known stress response genes, especially the heat shock proteins (HSPs), important mediators of cellular damage, were not shown to be differentially expressed in our study. HSPs have been widely shown to be up-regulated prior to and during bleaching in corals (reviewed in Coles and Brown, 2003). Two hypotheses could be proposed to explain this observation: 1) The fold change values of these genes were below the threshold chosen to proceed for sequencing or 2) the stress period of 24 h was inappropriate to observe a change in expression of such genes.

3.8. Comparing temperate and tropical cnidarian-dinoflagellate mutualisms

Cnidarian-dinoflagellate partnerships in temperate habitats face dramatically different environmental challenges than do their tropical counterparts. For example, A. elegantissima is found in the mid-intertidal of the North American Pacific coast in a very broad latitudinal range extending from Mexico to Alaska (Secord and Augustine, 2000). Its physiology is widely tolerant to a variety of parameters including temperature, salinity and desiccation, in comparison to the much more narrowly tolerant tropical cnidarians (Muller-Parker and Davy, 2001). These differences are also reflected in the maintenance and regulation of the symbiosis (reviewed in Muller-Parker and Davy, 2001) and there are likely to be differences in the stress response and symbiosis dysfunction as well. Temperature-induced bleaching in A. elegantissima has been observed in the laboratory (Buchbaum, 1968; Pearse, 1974) as a result of temperature elevations considerably more dramatic than the 1–4 °C stresses that are typical for corals. Furthermore, although apsymbiotic A. elegantissima are common in nature in low light environments (Buchbaum, 1968; Secord and Muller-Parker, 2005), there is no evidence (but also no studies) of temperature-induced bleaching in the field.

Despite these ecophysiological contrasts between temperate A. elegantissima and its tropical counterparts, this study suggests that there are shared functional pathways that drive cellular stability of the partnership. The differential expression of genes known to be involved in response to oxidative stress and in membrane trafficking point to two areas that have been shown to be critical to symbiosis stability and breakdown in tropical cnidarian-dinoflagellate symbioses. At the time of publication, although there are no functional genomic studies published on coral temperature stress and bleaching, we know of several studies on the verge of publication. Future work comparing this study with those of tropical, stenotolerant corals will prove very interesting.
4. Conclusions

There was considerable overlap in the expression profiles of anemones subjected to the two different stressors, even in the relatively small number of sequences that we investigated in this study. As other studies have suggested, this points towards oxidative stress, the secondary stressor arising from both elevated temperature and UV, as fundamental to driving the stress response. However, there were also major discrepancies in gene expression between the two stress responses, which suggests either differences in molecular targets or in the kinetics of induction by high temperature and UV. These results corroborate what has been previously reported by Hoegh-Guldberg and Smith (1989): that the effects on corals of elevated UV alone do not closely match those on corals sampled during mass bleaching events.

Finally, the functional classification of genes from this study is diverse. Clearly, no single process operates in response to elevated temperature and UV. This supports previous studies that suggest that bleaching is likely a result of multiple interactions of cellular pathways (Gates et al., 1992; Dunn et al., 2007b). The primary pathways highlighted by the present work, one of the first genomic approaches aimed at understanding the proximate events occurring during elevated temperature and UV stress, overlaps with functional studies of bleaching. The utility of cDNA microarrays, therefore, is to focus attention on those genes that are directly responsive to stress, with further molecular, biochemical and physiological analyses before and during bleaching being the next arena of experimental pursuit.

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