Gene expression profiles of cytosolic heat shock proteins Hsp70 and Hsp90 from symbiotic dinoflagellates in response to thermal stress: possible implications for coral bleaching

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Abstract Unicellular photosynthetic dinoflagellates of the genus *Symbiodinium* are the most common endosymbionts of reef-building scleractinian corals, living in a symbiotic partnership known to be highly susceptible to environmental changes such as hyperthermic stress. In this study, we identified members of two major heat shock proteins (HSPs) families, *Hsp70* and *Hsp90*, in *Symbiodinium* sp. (clade C) with full-length sequences that showed the highest similarity and evolutionary relationship with other known HSPs from dinoflagellate protists. Regulation of HSPs gene expression was examined in samples of the scleractinian coral *Acropora millepora* subjected to elevated temperatures progressively over 18 h (fast) and 120 h (gradual thermal stress). Moderate to severe heat stress at 26°C and 29°C (+3°C and +6°C above average sea temperature) resulted in an increase in algal *Hsp70* gene expression from 39% to 57%, while extreme heat stress (+9°C) reduced *Hsp70* transcript abundance by 60% (after 18 h) and 70% (after 120 h). Elevated temperatures

decreased an *Hsp90* expression under both rapid and gradual heat stress scenarios. Comparable *Hsp70* and *Hsp90* gene expression patterns were observed in *Symbiodinium* cultures and *in hospite*, indicating their independent regulation from the host. Differential gene expression profiles observed for *Hsp70* and *Hsp90* suggests diverse roles of these molecular chaperones during heat stress response. Reduced expression of the *Hsp90* gene under heat stress can indicate a reduced role in inhibiting the heat shock transcription factor which may lead to activation of heat-inducible genes and heat acclimation.

Keywords *Symbiodinium* · Dinoflagellate · HSP · Real-time RT-PCR · Heat stress · Coral bleaching

Introduction

Heat shock proteins (HSPs) are molecular chaperones which are involved in maintaining regular cellular functions with a crucial role in protein folding, unfolding, aggregation, degradation, and transport (Sorensen et al. 2003). In addition, these highly conserved proteins are involved in cell differentiation and morphogenesis (Gunter and Degnan 2007), cell signaling, and in the protection of cells against stress and apoptosis (Arya et al. 2007; Beissinger and Buchner 1998; Li and Srivastava 2004). HSPs are organized into several families based on their molecular mass (kDa), with *Hsp70* and *Hsp90* proteins belonging to the two major families (70 and 90 kDa, respectively). Some *Hsp70* family members are constitutively expressed such as seen with cognate *Hsc70* or with inducible expression as seen with cytosolic *Hsp70* (Sorensen et al. 2003). Recent genome sequencing projects revealed the presence of multiple *Hsp70* isoforms (Kabani and Martineau 2008), originating through

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the gene duplication process (Bettencourt and Feder 2001) and exhibiting differential gene expression profiles under various stress conditions (Lin et al. 2001). Hsp90 members are one of the most abundant proteins, accounting for 1–2% of all cell proteins under non-stressed conditions (Csermely et al. 1998). In eukaryotes, Hsp90 are usually represented with four major types, two cytosolic forms, inducible (Hsp90- α) and constitutive (Hsp90- β) forms, endoplasmic reticulum and mitochondrial homologues (Chen et al. 2006; Pearl et al. 2008). Hsp90 operates as a dimmer influencing development, epigenetic changes and morphological evolution (Pearl et al. 2008). Conserved structures of cytosolic Hsp70 and Hsp90 proteins are characterized by the N-terminal ATPase domain and the C-terminal polypeptide-binding domain (Young et al. 2001, 2004). Induced HSPs act as molecular chaperones preventing cellular damage under conditions of environmental stress, although it is important to realize that the definition of stress will depend ultimately on the organism and its environmental history. Generally, stress is defined as a condition leading to a disturbance of regular organism functionality followed by a decrease in the fitness of the organism (Hoffman and Parsons 1991; Bijlsma and Loeschcke 2005). As such, the stress required for the HSP induction will depend on the organism's natural environment and niche history (Feder and Hofmann 1999). Major environmental stressors that affect HSP expression level are temperature, ultraviolet radiation, exposure to heavy metals and pesticides, hypoxia, salinity, and bacterial and viral diseases infections (Sorensen et al. 2003). Consequently, the role of HSPs in cell protection and as stress markers has been well-recognized in many organisms (Wahid et al. 2007), although considerable variability in the HSPs stress response was also observed in some marine organisms depend on species, tissue, HSP family, developmental stage, and stressor (Clark and Peck 2009a; Iwama et al. 2004). In previous studies, Hsp70-4 expression increased following heavy metal exposure in the zebrafish (Krone et al. 2003), low-molecular weight HSPs were induced by toxins and temperature in *Crustacea* (Timofeev et al. 2008). HSPs were up-regulated after exposure to elevated temperatures in reef-building corals *Montastraea faveolata* (Downs et al. 2000), *Goniopora djiboutiensis* (Sharp et al. 1997) and others (Van Oppen and Gates 2006). Also, host Hsp70 protein level increased in two species of Indo-Pacific corals, *Porites cylindrica* and *Stylophora pistillata* after exposure to short-term thermal stress (Fitt et al. 2009). Recent microarray data confirmed that thermal stress induces up-regulation of host *Hsp90* gene in the Caribbean coral *M. faveolata* (Voolstra et al. 2009), as well as of *Hsp70* and *Hsp90* in *Acropora millepora* larvae (Rodriguez-Lanetty et al. 2009). Thermal stress-induced

changes in gene expression profile of coral *Acropora palmate* affected chaperone activity (DeSalvo et al. 2010b), while transcriptomic profiles of thermally stressed coral *M. faveolata* were found to be grouped according to *Symbiodinium* genotype (DeSalvo et al. 2010a). Also, in reef-building coral *A. millepora*, *Symbiodinium* genotype strongly influenced coral holobiont fitness as evaluated by growth, survival, and thermal tolerance (Mieog et al. 2009) with superior potential of algal symbionts for thermal-stress adaptation compared to their coral host (Császár et al. 2010). At the protein level, small algal HSPs isolated from thermally stressed coral host were up-regulated indicating a potential use as stress biomarkers (Downs et al. 2000). There is still no comprehensive information about the changes in the level of HSP genes from coral endosymbionts in the response to global environmental stressors, particularly to heat stress.

The unicellular photosynthetic dinoflagellates of genus *Symbiodinium* (also known as zooxanthellae) are endosymbionts of reef-building scleractinian corals. They are divided into eight major clades (A–H) and additional subclades (Coffroth and Santos 2005; Pochon et al. 2006). The stability of coral–algal partnership has been found to be susceptible to changes to the physical and chemical characteristics of the environment leading to breakdown of the symbiosis, subsequent loss of the symbionts from coral tissue and coral bleaching (Hoegh-Guldberg 1999; Hoegh-Guldberg et al. 2007; Lesser 2007). On the global scale, mass coral bleaching is mainly triggered by increases in sea surface temperatures (SSTs) above the long-term summer maxima of as little as 1°C (Hoegh-Guldberg 1999). The natural bleaching conditions on the Great Barrier Reef were reported to be between 31°C and 32°C after a 5-day period (Berkelmans and Willis 1999; Berkelmans 2002). Recent data report that since the mid-1970s SSTs across the tropical region increased by 0.4–1°C (Cantin et al. 2010), while global temperatures are predicted to rise at least 2°C by 2050–2100 due to rising carbon dioxide levels (IPCC 2007). Therefore, elevated sea temperatures are recognized as a major factor in the destabilization of the cnidarian–dinoflagellates symbioses and consequently the well-being of coral reef ecosystems globally (Hoegh-Guldberg 1999; Weis 2008).

Coral reefs, particularly in the reef flat, are characterized by huge daily variations in temperatures due to tidal and light conditions (Middlebrook et al. 2010). Differences in heating rates of reef-building corals were shown to influence symbiont photosynthetic productivity, density, and biochemistry (Middlebrook et al. 2010), while thermal history was observed as an important factor influencing the response of coral holobiont to thermal stress (Middlebrook et al. 2008). To explore initial molecular changes following the exposure of coral to thermal stress conditions, we monitored the alterations in the transcription profiles of

algal molecular chaperones HSPs, which are expected to be involved in the prevention of cellular damage during stress. Partial sequences of HSP genes of the dinoflagellate symbionts were previously reported from expressed sequence tag (EST) library proposing at least two isoforms of Hsp90 and one of Hsp70 (Leggat et al. 2007). Here, we report the full-length of coral dinoflagellate genes from the two major HSP families, *Hsp70* and *Hsp90*, and examine the changes in their expression profiles under various heat stress scenarios. Furthermore, we explore the impact of algal symbiotic status (*in hospite* and cultured *in vitro*) on the pattern of expression of these two HSP genes. Finally, we discuss the potential of using both of these genes as biomarkers for thermal stress in symbiotic dinoflagellates.

Materials and methods

Sequence identity and phylogenetic analysis

The sequences of *Hsp40*, *Hsp70*, and *Hsp90* genes were identified from the EST library of *Symbiodinium* (genotype C3) isolated from the coral host *Acropora aspera* (Leggat et al. 2007). From Basic Local Alignment Search Tool (BLAST) search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) the representative sequences corresponding to analyzed *Hsp40*, *Hsp70*, and *Hsp90* genes and their accession numbers are shown in Table 1. Sequence analysis was performed using the BLAST search options tblastx and blastx, while translated sequences were analyzed using tblastn option and blastp. The highest scoring hits (E value $< 10^{-5}$) were used for the further sequences analysis and phylogenetic tree generation. Sequence analysis was performed using web based BioManager-ANGIS services (<http://bioman5.angis.org.au>). Alignments of selected sequences were utilized in ClustalW (Thompson et al. 1994), while manual adjustment of the multiple sequence alignments was done using GeneDoc software. The conserved regions from multiple sequence alignments was used in the phylogenetic analysis (Leignel et al. 2007) and excluded the variable C-terminal region. Sequences used in phylogenetic analyses for building a tree are shown in bold (Figs. 2 and 3). Phylogenetic trees were reconstructed using Maximum Likelihood estimates based on the Dayhoff PAM matrix. Bootstrap analysis (1,000 replicates) was then performed to test the robustness of each topology (Felsenstein 1989).

Experimental design

Nubbins (4–5 cm long) of *A. millepora* harboring *Symbiodinium* C3 genotype (LaJeunesse et al. 2003) were collected from the reef flat at Heron Island, Great Barrier

Reef, Australia (23°25'S; 152°07'E). The collected fragments were immediately transferred to a flow-through aquaria, then attached to nylon threads and acclimatize in nine independent aquarium tanks (60 L; three tank replicates per treatment) at 23–24°C (mean ambient temperature during winter) for 48 h. The ambient light levels were controlled throughout the experiment using shade cloths to mimic natural reef flat conditions. To examine the affect of thermal stress, nubbins were randomly distributed between the tanks at the time of acclimation and then exposed to two experimental temperature regimes: gradual ($\sim 0.1^\circ\text{C}$ increase per hour, 23–32 $\pm 1^\circ\text{C}$), fast thermal stress ($\sim 0.4^\circ\text{C}$ increase per hour, 24–32 $\pm 1^\circ\text{C}$), and a control group (stable at 23–24 $\pm 1^\circ\text{C}$; ambient temperature) over an 18-h period for the fast heat stress and a 120-h period for the control and medium temperature regimes (Fig. 1). The thermal stress conditions applied here are representative of different warming events seen on the Great Barrier Reef (GBR) which have resulted in coral bleaching (Berkelmans and Willis 1999; Berkelmans 2002; Dove 2004), with temperatures between 31 and 32°C over a 5-day period (gradual thermal stress) and between 25°C and 32°C over a 24-h period (fast thermal stress). Also, the long-term thermal thresholds for corals from the reef flat at Heron Island during 1980s was roughly 29.5–30°C (Potts and Swart 1984). Mean summer SSTs reported in the GBR were in the range of ~ 28.7 –29.5°C (Császár et al. 2010), while winter ambient temperature when this experiment was conducted was approximately 23–24°C. Water temperature for each replicate aquarium was measured every 2 min using StowAway TidbiT Loggers (Onset Computer Corporation, Bourne, MA, USA). A subset of coral branches ($n=4$) was removed from the treatment and control tanks at T0, T18, T72, and T120 h. Corals nubbins

Fig. 1 Coral nubbins were subjected to the two temperature regimes: gradual thermal stress (1°C increase per approximate 10 h, gray square); fast thermal stress (1°C increase per approximate 2.5 h, open circle), and a control group (stable at 23–24 $\pm 1^\circ\text{C}$; ambient temperature, filled triangle) as indicated by a daily temperature average. The nubbins were exposed to their experimental temperature regimes over an 18-h period for the fast heat stress and 120 h for the control and gradual thermal stress conditions

Table 1 GenBank accession numbers, gene name, and primer sequences of *Hsp* genes used for real-time RT-PCR analysis, while the sequences of reference genes (*Tub*, *Rp-S4*, and *SAM*) were adopted from Rosic et al. (2010b)

Symbol	Gene name	Forward primer sequence (5'-3') reverse primer sequence (5'-3')	GeneBank accession number
<i>Hsp40</i>	Dna J-like protein/ <i>Heat shock proteins 40</i>	GCGAAAATTTCACTGCGAGACT GGTCGGACAACCTTCATTAGTGGTA	EH035912.1
<i>Hsp70</i>	<i>Heat shock proteins 70</i>	CAGATGAGGCCGTGGCTTAT GGGAGTCACATCCAACAGCAA	EH038080.1
<i>Hsp90</i>	<i>Heat shock proteins 90</i>	GAGGATCTGCCACTGAACATCTC GCGAACATCTCCAAGCACTTC	EH038163.1
<i>Tub</i>	<i>Beta-Tubulin</i>	TGACGCAGCAGATGTTTGATG CGACATACGTCCACGGAAGAG	EH037669.1
<i>Rp-S4</i>	<i>Ribosomal protein S4</i>	CCGCACAAACTGCGTGAGT CGCTGCATGACGATCATCTT	EH036413.1
<i>SAM</i>	<i>S-adenosyl-L-methionine synthetase</i>	GCCTACATTTGCCGACAGATG AATGGCTTGGCAACACCAAT	EH036622.1

were immediately snap frozen in liquid nitrogen and stored at -80°C prior to processing.

Culturing conditions

Populations of cultured *Symbiodinium* sp. were obtained from Professor Roberto Iglesias-Prieto (RSU Puerto Morelos, UNAM, Mexico) and identified as clade C ([Internal Transcribed Spacer]-type C1). Cultures were grown in f/2 medium (Guillard and Ryther 1962), at 25°C , 12:12-h day–night period, with an irradiance of $10\ \mu\text{mol quanta s}^{-1}\text{m}^{-2}$ (measured using a Li-Cor flat quantum sensor), at 25°C . The irradiance of $10\ \mu\text{mol quanta s}^{-1}\text{m}^{-2}$ was used for maintenance of cultured *Symbiodinium* prior experimentation and represented normal light conditions. During the exponential phase (cell number $\sim 10^{6-7}$ per ml), cultures of *Symbiodinium* were exposed to elevated temperatures of 29°C and 32°C that were generated simultaneously. To

identify early changes in gene expression in cultured *Symbiodinium* the experimental treatments lasted 24 h, which consisted of 12 h of darkness followed by 12 h of light ($n=3$). At the end of the treatment, the cells were centrifuged and the resulting pellet was snap frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

Total RNA extraction

RNA was extracted from coral branches using small fragments (0.5–1 cm long) of coral nubbins that were cut with a bone cutter and ground directly in liquid nitrogen. The resulting powder was put in 700 ml of RLT buffer with betamercaptoethanol (RNeasy kit, Invitrogen, Australia), homogenized with a hand homogenizer (Tissue-Tearor, Biospec products, Inc.) and centrifuged for 3 min at $13,000\times g$ at 4°C . The aqueous phase was then used for

Table 2 The best BLAST (blastp) hits ($E < 1.0 \times 10^{-5}$) for encoding sequences of *Symbiodinium Hsp70* and *Hsp90* genes including GenBank accession numbers, query coverage, and E value

Symbol	GeneBank accession number	Annotation	Species with the closest similarity (GeneBank accession number)	Query coverage (%)	E value
<i>Hsp70</i>	EH038080.1	Heat shock protein 70	<i>Cryptocodinium cohnii</i> (AAM02973.2)	94	0.0
			<i>Prorocentrum minimum</i> (ABI14407.1)	100	0.0
			<i>Perkinsus marinus</i> (EER12208.1)	100	0.0
			<i>Eimeria acervulina</i> (CAA81135.1)	100	0.0
			<i>Toxoplasma gondii</i> (XP_002365977.1)	94	0.0
<i>Hsp90</i>	EH038163.1	Heat shock protein 90	<i>Cryptocodinium cohnii</i> (AAM02974.1)	100	0.0
			<i>Karlodinium micrum</i> (ABI14419.1)	100	0.0
			<i>Amphidinium carterae</i> (ACF95812.1)	95	0.0
			<i>Perkinsus marinus</i> (EER07401.1)	97	0.0
			<i>Plasmodium vivax</i> (XP_002365977.1)	99	0.0

Fig. 2 The sequence and phylogenetic analyses of *Symbiodinium* Hsp70 gene. **a** Nucleotide and deduced amino acid sequences of Hsp70, where the amino acid sequence is presented below the nucleotide sequence. All three the Hsp70 family conserved motifs are shaded in *light gray* and *numbered* (I–III), while the C-terminal consensus sequence motif EEVD is shaded in *dark gray*. The Hsp70 protein family signatures were: GIDLGTYY, IYDMGGGTFD, and VVLVGGSTRIPKVQ. The residues used for building the tree are shown in bold (from 193 to 385 amino acids). **b** Phylogenetic analyses of the Hsp70 protein sequences of *Symbiodinium* and other organisms. Gene accession numbers for all used sequences were obtained from the NCBI database (<http://blast.ncbi.nlm.nih.gov/>) and are shown in the parentheses. The phylogenetic tree was tested using a 1,000 replicated bootstrap analysis (Felsenstein 1989) and the results higher than 50% are indicated at each node. A distance method using maximum likelihood estimates was based on the Dayhoff PAM matrix. The scale for the branch length (0.1 substitutions per site) is presented under the tree. Out-group used is indicated on the figure. Several entries for one species correspond to multiple HspP70 isoforms, while *asterisks* correspond to the cytosolic Hsp70 (*) and endoplasmic reticulum (ER) Hsp70 forms (**)

Fig. 3 The sequence and phylogenetic analyses of *Hsp90* gene from *Symbiodinium*. **a** Nucleotide and deduced amino acid sequences of *Hsp90*, where the amino-acid sequence is presented below the nucleotide sequence. All five highly conserved motifs defining *Hsp90* family are shaded in *light gray* and *numbered* (I–V), while the C-terminal consensus sequence motif MEEVD is shaded in *dark gray*. The conserved LXXLL sequence involved in binding to nuclear receptor is underlined. The residues used for building the tree are shown in bold (from 3 to 158 amino acids). **b** Phylogenetic analysis of the *Hsp90*-deduced amino acid sequences of *Symbiodinium* and other organisms. Gene accession numbers for all used sequences were obtained from the NCBI database (<http://blast.ncbi.nlm.nih.gov/>) and are shown in the parentheses. The phylogenetic tree was tested using a 1,000 replicated bootstrap analysis (Felsenstein 1989) and the results higher than 50% are indicated at each node. A distance method using maximum likelihood estimates was based on the Dayhoff PAM matrix. The scale for the branch length (0.1 substitutions per site) is presented under the tree. Out-group used is indicated on the figure. Several entries for one species correspond to multiple *Hsp90* isoforms, while *asterisks* correspond to the cytosolic *Hsp90* form (*)

the extraction of total RNA with RNeasy kit (Invitrogen) following the manufacturer's instructions. The RNA quantity and integrity was analyzed on an Agilent 2100 Bioanalyzer and 500 ng of high-quality total RNA (integrity number >7) was subjected to reverse transcription for cDNA synthesis.

RNA sample was isolated from *A. millepora* sperm (free of algal symbionts) and *Symbiodinium* cultures using specialized method combining Trizol and Qiagen RNeasy kits (Rosic and Hoegh-Guldberg 2010).

Synthesis of cDNA for qPCR

Reverse transcription was done using QuantiTect® Reverse Transcription Procedure (Qiagen, Australia). In brief, 0.5 µg of purified total RNA was used per reaction and incubated in gDNA Wipeout Buffer at 42°C for 2 min to eliminate traces of genomic DNA, followed by reverse transcription at the same temperature for 30 min. The obtained cDNA was used as a template in the quantitative polymerase chain reaction (qPCR) analysis and diluted 1:10 prior to use.

Primer design

Sequencing primers were designed to *Hsp70* and *Hsp90* from *Symbiodinium* (C3) EST database as outlined in Table 1 using Primer Express® Software v2.0 (Applied Biosystems, USA). The genes used for real-time reverse transcription (RT)-PCR analysis, GenBank accession numbers and primers sequences are listed in Table 1. To confirm the reproducibility of the primers within *Symbiodinium* cultures and the absence of coral RNA, a standard PCR amplification was carried out using a selection of primers (Table 1) as previously described by Rosic et al. (2010b). PCR conditions were: initial step at 94°C for 1 min, followed by 35 cycles of 94°C 20 s, 60°C 20 s and 72°C 1.5 min, with a final extension phase at 72°C for 10 min, followed by samples storage at 4°C.

Quantitative PCR and gene expression analysis

The quantitative PCR assays were performed by an Eppendorf 5075 (Applied Biosystems, USA) robot, using SYBR Green PCR master mix (Applied Biosystems, Warrington, Cheshire, UK) in 384-well plates in 7900HT Fast Real-time PCR System (Applied Biosystems, USA). PCR conditions were: initial denaturation of 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. At the end, a dissociation step was included: 95°C for 2 min, 60°C for 15 s and 95°C for 15 s. The final reaction volume was 10 µl and included 300 nM of primers. All reactions were carried out in three technical

replicates. The expression levels of targeted *Hsp70* and *Hsp90* genes were quantified according to geNorm directions (Vandesompele et al. 2002). The relative quantification method was applied for the relative abundance estimation of analyzed HSP genes using the best reference genes that showed the most stable expression patterns and specificity for *Symbiodinium* cultures (Table 1), as well as absence of coral origin (Rosic et al. 2010a, b). The expression of each gene was determined from C_T (cycles threshold) value that corresponds to a number of cycles required for the PCR amplification to reach a fixed threshold in the exponential phase (Walker 2002). C_T values were obtained for a specific threshold of 0.1 and transformed into quantities using maximal PCR efficiency for each gene ($E=2$). The real-time dissociation curve was used to check out for the presence of a unique PCR product. Following the normalization strategy outlined in Vandesompele et al. (2002) and using geNorm software (<http://medgen.ugent.be/~jvdesomp/genorm/>) the stability of House Keeping Genes expression was tested during exposure to thermal stress. The relative *Hsp* genes quantities were normalized to the reference genes with the most stable expression pattern as defined by the GeNorm analysis (Vandesompele et al. 2002).

Statistical analysis

Statistical analysis was carried out using Statistica 7.0 (Statsoft Inc., Tulsa, OK, USA). Normality and homoscedasticity of the data was first confirmed using the Kolmogorov–Smirnov test and Levene's tests, respectively, while the influence of time and treatment on gene expression was tested by a two-way analysis of variance (ANOVA). Relative expression of the algal *Hsp70* and *Hsp90* genes isolated from thermally stressed corals was compared to the control at each sampling time (coral nubbins not exposed to elevated temperatures) using a *t*-test ($n=4$). The significance of differences was indicated as where $P<0.05$. Throughout the paper, values given are mean±standard deviation (SD).

Results

Molecular characterization of *Hsp70* and *Hsp90* genes

Contigs corresponding to HSP genes *Hsp40*, *Hsp70* and *Hsp90* with representative sequences were identified from the *Symbiodinium* EST database (<http://www.ncbi.nlm.nih.gov/>; Table 1). The best BLAST (blastp) hits (E value $<1.0\times10^{-5}$) of the deduced protein sequences are given in Table 2. Sequence analyses showed the closest similarity of *Hsp70* encoding sequence with dinoflagellate (*Cryptheco-*

dinium cohnii, *Prorocentrum minimum*, and *Perkinsus marinus*) and members of the Phylum Apicomplexa (*Eimeria acervulina* and *Toxoplasma gondii*). For the identified Hsp90 protein sequence, the greatest similarity was found to be with other dinoflagellates (for example *Gymnodinium chlorophorum*, *Lessardia elongate*, and *Alexandrium tamare*). Since RNA isolated from the coral host *A. millepora* contained both the algal and coral transcriptomes, the presence of selected algal HSP genes was tested in the coral host tissues using cDNA obtained from dinoflagellate-free *A. millepora* egg–sperm sample. Our results confirmed the lack of coral genes in *Hsp70* and *Hsp90*, while coral origin was identified by RT-PCR for *Hsp40* and therefore excluded from further analysis.

An open reading frame (ORF) of *Hsp70* was 2037 bp, encoding 647 amino acids with molecular mass of 70.58 kDa and the theoretical isoelectric point (pI) of 4.99 (Fig. 2a). Within the predicted *Symbiodinium* Hsp70 protein sequence three Hsp70 protein family signatures were identified: GIDLGTTY, IYDMGGGTFD and VVLVGSTRIPKVQ, including the cytosolic EEVD signal at the C-terminus. The *Hsp90* gene had an ORF of 2078 bp, an encoding protein of 691 amino acids, with a predicted size of approximately 80 kDa, and pI of 5.07 (Fig. 3a). All five conserved amino acid motifs characteristic of Hsp90 protein family have been recognized in *Symbiodinium* Hsp90 including a C-terminal MEEVD motif found in the cytosolic Hsp90 members.

Phylogenetic analysis of Hsp70 and Hsp90

Alignments of deduced amino acid sequences of algal HSP genes and a range of highly homologous HSP genes from other organisms (selected from BLAST analysis) were used for the construction of phylogenetic trees (Figs. 2b and 3b). The phylogenetic analysis shows evolutionary relationship of *Symbiodinium* Hsp70 with other dinoflagellates cytosolic Hsp70 counterparts forming a monophyletic group with their orthologous genes. According to phylogeny, encoding sequence of *Symbiodinium* Hsp90, also clustered with Hsp90 from other dinoflagellates and with parasitic protozoan from Phylum Apicomplexa supporting that within alveolates, dinoflagellates, and apicomplexa are sister groups (Fast et al. 2002).

Effect of thermal stress on the *Hsp70* and *Hsp90* gene expression

The level of HSP gene expression was examined using real-time RT-PCR and the relative quantification method applying the Genorm analysis for data normalization and reference genes for symbiotic dinoflagellates (Rosic et al. 2010b). Specific PCR products were confirmed in all

amplification reactions using the real-time dissociation curve.

The samples of scleractinian coral *A. millepora* were exposed to elevated temperatures reaching a maximum of 32°C after 18 h (fast thermal stress) and a 120 h-period (gradual thermal stress). A significant effect of the interaction between time and temperature increase was noted for both *Hsp70* and *Hsp90* expression levels (two-way ANOVA $P < 0.001$). Conversely, heat stress influenced the transcript abundance of *Symbiodinium* *Hsp70* and *Hsp90* genes compared to the control at each time point. Gradual thermal stress with temperature of 3°C above ambient (at 26°C, after 18 h) led to a 39% increase in *Hsp70* gene expression ($P < 0.05$), while a further increase in temperature reaching 29°C (after 72 h) enlarged transcript abundance for 57% ($P > 0.05$). At temperature of 32°C (9°C above mean ambient) *Hsp70* expression levels significantly dropped in both temperature treatments by 59% after 18 h ($P < 0.01$) and 69% ($P < 0.01$) after 120 h. The *Hsp90* transcript level decreased after 18 h to 57% ($P < 0.01$) at 26°C and 43% ($P < 0.05$) at 32°C. Prolonged exposure to elevated temperatures led to a significant drop of 23% ($P < 0.05$) after 72 h at 29°C and 22% ($P < 0.01$) after 120 h at 32°C. In aseptic cultures of *Symbiodinium* sp. (type C1), *Hsp70* gene expression increased after 24 h for 25% ($P > 0.05$) at 29°C, while a significant drop of 87% ($P < 0.05$) was reported at 32°C. A decrease in an *Hsp90* mRNA abundance of 20% ($P > 0.05$) and 25% ($P < 0.05$) was observed in cultured *Symbiodinium* at 29°C and 32°C, respectively.

Discussion

Rapid changes to ocean temperature as a result of global climate change are bringing reef-building communities closer to their thermal tolerance limits. Comprehensive alterations occur within the cells and tissues of reef-building corals and their dinoflagellate endosymbionts when exposed to elevated temperatures. These changes ultimately affect the functionality and stability of the coral–algal symbioses (Hoegh-Guldberg 1999; Weis 2008). Understanding how corals respond to these challenges is crucially important in terms of understanding how they may respond to future changes in sea temperature. HSPs represent a class of molecular chaperones that are well-known for their quick responses to environmental stresses (Sorensen et al. 2003) and therefore provide potentially potent biological markers of stress levels experienced by corals. This study focused on changes to the gene expression of coral dinoflagellate HSPs in response to both rapid and gradual thermal stress conditions. Here, we also report the full-length of an ORF and the encoding sequences for members of two major HSP families, genes *Hsp70* and *Hsp90*. The amino acid

Fig. 4 The relative expression of *Hsp*-genes for *Symbiodinium* within the coral host *Acorpora millepora* exposed to two heat stress scenarios: fast and gradual thermal stress reaching a maximum of 32°C. For data normalization of the relative quantities were used two the most stable reference genes as defined by the GeNorm analysis: *Tub* and *SAM* (with average expression stability *M* value of 0.35 and pairwise variations for *V2/3* of 0.14). The ratio of *Hsp* gene expression for treatment and control at the given time point (T/CTRL) is presented for *Hsp70* (a) and *Hsp90* (b). All data are given as the means of values obtained from three independent biological replicates $\pm$ SD. The statistical difference between means is indicated as * $P < 0.05$ or ** $P < 0.01$

sequences of the identified HSPs revealed the presence of three conserved Hsp70 protein family signatures and a cytosolic EEVD C-terminal domain (Fig. 2), which is thought to be involved in peptide-binding activity (Ramya et al. 2006). In protein sequence of putative algal Hsp90 was confirmed a presence of five conserved motifs and the C-terminal MEEVD found in cytosolic Hsp90 members (Fig. 3). This MEEVD domain has been found to be involved in inter-domain communication and peptide-binding (Gupta 1995). Generally, two Hsp90 cytosolic isoforms are recognized in vertebrates, Hsp90 α and Hsp90 β (Lees-Miller and Anderson 1989; Theodoraki and Mintzas 2006), while invertebrates usually have only one Hsp90 gene (Gao et al. 2008). Phylogenetic analysis on protein sequences of *Symbiodinium* *Hsp70* and *Hsp90* genes demonstrates the closest evolutionary relations for both Hsp70 and Hsp90 with other dinoflagellates (Figs. 2 and 3). BLAST analysis revealed the highest sequence homology with red algae *C. cohnii* that is also photosynthetic dinoflagellate (Table 2).

Fig. 5 Relative quantification of the *Hsp* genes expression level in *Symbiodinium* cultures exposed to heat stress of 29°C and 32°C for a 24-h period. Data normalization was done using the most stable reference genes as defined by the GeNorm analysis: *Rp-S4* and *SAM* with *M* value of 0.43 and pairwise variations for *V2/3* below recommended cut-off of 0.15 (Vandesompele et al. 2002). Results are given as the mean of three biological replicates $\pm$ SD. The statistical difference between means is indicated as * $P < 0.05$ or ** $P < 0.01$

Two algal HSPs exhibited differential regulation upon exposure to heat stress. A positive effect of moderately elevated temperatures on *Hsp70* expression level was reported at 26°C and 29°C (+3°C and +6°C above average sea temperature), while negative regulation was seen at highly elevated temperature of 32°C (+9°C). Up-regulation of *Hsp70* expression under heat shock treatment has also been observed in shrimps (Luan et al. 2010), the Antarctic limpet (Clark and Peck 2009b), then in the embryos of the coral *M. faveolata* and *A. millepora* larvae (Rodriguez-Lanetty et al. 2009; Voolstra et al. 2009). The results of the short-term heat stress at highly elevated temperature of 32°C on *Hsp70* expression for *Symbiodinium* in hospite and culture (Figs. 4 and 5) cannot be directly compared due to differences in algal thermal history, heating rate, and symbiotic status. However, down-regulation of *Hsp70* mRNA abundance in cultures (87% reduction) and dinoflagellates that remain within the coral host (59% reduction) suggests a possible acclimation or a potential protective role of coral host during thermal stress as previously suggested by Richier et al. (2005). Similarly, in *Symbiodinium* cultures, heat stress above 30°C led to photosynthetic dysfunction (Iglesias-Prieto et al. 1992) and exceeding 32°C led to severe photoinhibition (Takahashi et al. 2008; Takahashi et al. 2009), with higher susceptibility of algal cells to thermal stress than the coral host (Sharp et al. 1997).

The gene expression profile of *Hsp90* was down-regulated by elevated temperatures in both, symbiotic and cultured environments (Figs. 4 and 5). The hyperthermic stress induced reduction in HSP expression has also been shown for cytosolic *Hsp90* in *Arabidopsis* (Yamada and Nishimura 2008) and *Drosophila* (Rutherford and Lindquist 1998). In humans, Hsp90 was found to negatively regulate a heat shock transcription factor (HSF) preventing HSF interaction with the transcriptional machinery (Zou et al. 1998). Consequently, upon heat stress, Hsp90 dissociates from

HSF, activating heat-inducible genes resulting in heat acclimation in *Arabidopsis* seedlings (Yamada and Nishimura 2008). In the same way, overproduction of Hsp90 led to down-regulation of HSF and reduced heat tolerance in yeast and in plants (Cheng et al. 1992; Yamada and Nishimura 2008). Additionally, an importance of Hsp90 in response to environmental changes is proposed to be due to its central role in translation of genetic differences into phenotypic variations (Sangster et al. 2008) and function “as a capacitor for morphological evolution” (Rutherford et al. 2007; Rutherford and Lindquist 1998).

Comparable changes in expression profiles of *Hsp70* and *Hsp90* genes were found for *Symbiodinium* in cultures and within coral host exposed to elevated temperatures. Consequently, we propose the self-regulating response of algal HSPs to thermal stress independently of symbiotic status. In the present study, we revealed a differential regulation of two inducible HSPs, cytosolic *Hsp70* and *Hsp90* by exposure to heat stress. Symbiotic status did not seem to control the expression pattern of HSPs, suggesting that the beginning of the response to thermal stress occurred within *Symbiodinium* independently of host. Previous studies also reported that after coral exposure to heat stress, initial changes occurred in symbiotic dinoflagellates (see reviews Venn et al. 2008; Weis 2008). Our results demonstrated *Hsp70* up-regulation by moderately elevated temperatures (26°C and 29°C) and down-regulation at 32°C, which may correlate to the thermal threshold of photosynthetic activity prior to photoinhibition (Iglesias-Prieto et al. 1992). Therefore, *Hsp70* seems to provide a good basis for a potential stress biomarker in symbiotic dinoflagellates. In contrast, down-regulation of *Hsp90* expression during either moderate (gradual heat stress) or severe (fast heat stress) thermal conditions suggests about its different role in response to elevated temperatures. Further experiments are required to precise the mechanisms driving these molecular regulations as well as their potential protective role for the prevention of the cell damage in *Symbiodinium* in response to thermal stress. Additional investigations should therefore focus on the relationship between the differential expression of HSP family members and the regulation of a HSF that is involved in activation of heat-inducible genes and heat acclimation. Also, because of the significant interaction between time and temperature increase, we were not able to differentiate whether the effect of the treatment was due to absolute temperature or the length of time that the organism has been exposed to heat stress. Future studies should include treatments with different temperatures in order to identify precisely the causes of this late change in the relative expression of HSPs.

The importance of HSPs in environmental stress resistance has been revealed from ecological and evolutionary aspects (Sorensen et al. 2003). In a similar fashion to plants,

reef-building corals and their algal endosymbionts cannot move and escape to cooler spots, and therefore they have to develop molecular mechanisms to acclimate and survive the heat-induced damage. As HSPs serve as molecular chaperones, they play an important role in organisms' heat acclimation and environmental plasticity (Hong and Vierling 2000). Consequently, our understanding of HSP regulation mechanisms and more particularly of its interaction with HSF in response to heat stress is crucial for understanding the capability of symbiotic dinoflagellates and their reef-building corals to tolerate future changes to the environment. Our study has provided a new insight into changes occurring in coral endosymbionts under heat stress and an important step in understanding the capacity of corals and their endosymbionts to tolerate changes in tropical oceans as result of rapid climate change.

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