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RESEARCH ARTICLE

The cell specificity of gene expression in the response to heat stress in corals

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ABSTRACT

Previous transcriptional studies in heat-stressed corals have shown that many genes are responsive to generalized heat stress whereas the expression patterns of specific gene networks after heat stress show strong correlations with variation in bleaching outcomes. However, where these specific genes are expressed is unknown. In this study, we employed *in situ* hybridization to identify patterns of spatial gene expression of genes previously predicted to be involved in general stress response and bleaching. We found that tumor necrosis factor receptors (TNFRs), known to be strong responders to heat stress, were not expressed in gastrodermal symbiont-containing cells but were widely expressed in specific cells of the epidermal layer. The transcription factors AP-1 and FosB, implicated as early signals of heat stress, were widely expressed throughout the oral gastrodermis and epidermis. By contrast, a G protein-coupled receptor gene (GPCR) and a fructose bisphosphate aldolase C gene (aldolase), previously implicated in bleaching, were expressed in symbiont-containing gastrodermal cells and in the epidermal tissue. Finally, chordin-like/kielin (chordin-like), a gene highly correlated to bleaching, was expressed solely in the oral gastrodermis. From this study, we confirm that heat-responsive genes occur widely in coral tissues outside of symbiont-containing cells. Joint information about expression patterns in response to heat and cell specificity will allow greater dissection of the regulatory pathways and specific cell reactions that lead to coral bleaching.

KEY WORDS: Bleaching, Cnidarians, Cnidocytes, Coral reef, Heat stress, Tumor necrosis factor receptor

INTRODUCTION

The warming of ocean temperatures poses a threat to many ocean-dwelling organisms, especially reef-building corals, which are historically sensitive to changes in their local environment (Halpern et al., 2012; Hoegh-Guldberg and Bruno, 2010; Hughes et al., 2007; Pandolfi et al., 2003). One of the most characterized and noticeable outcomes of these local warming events is coral bleaching. During these bleaching events, the symbiotic algae, *Symbiodinium* spp., are predominately released from the gastrodermal cells of the coral. Severe bleaching leaves corals covered with translucent tissue, allowing the white skeleton to show through and, without their algal partners, corals can become more vulnerable to other threats and stressors, such as starvation and disease (Gates et al., 1992). Although a large number of studies have documented strong gene expression changes associated with bleaching conditions, little is known about whether these patterns are part of the bleaching reaction itself or part of the heat stress response that is seen in most animals, plants and fungi (Barshis et al., 2013; Bay and Palumbi, 2015; Dixon et al., 2015; Leggat et al., 2011; Meyer et al., 2009; Palumbi et al., 2014; Pinzon et al., 2015; Rose et al., 2015; Ruiz-Jones and Palumbi, 2014, 2015; Seneca and Palumbi, 2015). Most transcriptomics projects are based on slurries of tissue from corals and so information concerning specificity in cell expression is lost. Additionally, these studies rarely confirm their findings using cell biological techniques. As a first step, spatial gene expression visualization using histological techniques can be very revealing because it can provide visual evidence of where specific mRNAs are expressed within a cell or tissue (McFadden, 1995). Within cnidarians, this method has been primarily used in the model systems *Hydra* and *Nematostella vectensis* (Finnerty and Martindale, 1999; Kurz et al., 1991; Wolenski et al., 2013a,b).

In addition to cell biological techniques, transcriptomic techniques can be used to identify physiological states associated with gene expression such as generalized heat responses and those involved in bleaching. Rose and colleagues took advantage of the fact that different coral individuals within a species often bleach differently even when exposed to the same standard stress and searched for gene expression patterns predictive of bleaching results (Rose et al., 2015). They described a set of 277 genes (described in module 12 in their study) with expression that correlated with bleaching outcomes within the coral *Acropora hyacinthus*. Additionally, module 1 was identified as being highly enriched for a large number of general stress response-like genes, including tumor necrosis factor receptor (TNFR) genes (Rose et al., 2015). The TNFR gene family has recently been identified as having significant upregulation in corals exposed to heat stress but the actual function and localization of this gene response within the coral animal is still not understood (Barshis et al., 2013; Palumbi et al., 2014; Quistad and Traylor-Knowles, 2016; Quistad et al., 2014). Throughout the continuation of this article, we will refer to module 1 as the 'stress response module' and module 12 as the 'bleaching module'.

To begin to address the cellular specificity of heat response genes, we compared the local spatial expression patterns of stress response module genes and bleaching module genes in *A. hyacinthus* using *in situ* hybridization. Corals are diploblastic animals with only two cell layers. Algal symbionts (*Symbiodinium* spp.) are contained strictly within specialized gastrodermal cells whereas the epidermal layer has other sets of distinctive cells such as different cnidocytes, including nematocytes and spirocytes, and mucous cells called mucocytes (Peters, 2016). In this study, we tested the hypothesis that stress response module genes are general heat stress response

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List of abbreviations

aldolase	fructose bisphosphate aldolase C gene
anti-DIG	digoxigenin-labeled antisense
AP	alakaline phosphatase
chordin-like	chordin-like/kielin
GPCR	G protein-coupled receptor gene
LB	lysogeny broth
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SSC	saline sodium citrate solution
TNFR	tumor necrosis factor receptor

genes, and would therefore be expressed by the corals' cells within the epidermis and oral gastrodermis. In parallel, we tested genes from the bleaching module with the hypothesis that expression of these genes would be restricted to symbiont-containing cells. Also, two additional genes not found in the stress response module or bleaching module were tested. Using *in situ* hybridization in branches from adult corals, we found widespread expression of stress response module genes across tissue layers and more restricted expression in bleaching module genes to primarily the oral gastrodermis.

MATERIALS AND METHODS

Sample preparation

The samples used in this study were part of two larger genomic studies on thermal tolerance (Seneca and Palumbi, 2015; Rose et al., 2016) (Table S1). In short, small branches (approximately 25 mm in length) of *Acropora hyacinthus* (Dana 1846) were collected from six colonies and exposed to a heat stress or to control conditions as detailed in Seneca and Palumbi (2015). Branches were sampled 5 h from the start of the experiment (Seneca and Palumbi, 2015). Replicate branches were scored for bleaching extent (1=none, 5=total) at 20 h. Gene expression was measured using RNASeq as described in Seneca and Palumbi (2015).

Ten heat-stressed branches and two control branches from these six colonies were preserved in 4% paraformaldehyde in filtered seawater and then washed in phosphate buffered saline (PBS) and stored in methanol at −20°C for later processing (Wolenski et al., 2013a,b) (colony information in Table S1). Slide preparations of coral branches were prepared in RNase-free conditions by IDEXX Laboratories Inc. (Columbus, MO, USA). Samples were decalcified using Morse's solution (25% formic acid, 10% sodium citrate). Once calcium was removed, samples were processed for paraffin infiltration and embedded into tissue blocks. Prior to sectioning, microtomy equipment was cleaned and treated to be nuclease free (RNase Zap; Ambion, Inc., Houston, TX, USA) and the water bath was filled with DEPC-treated water (Sigma-Aldrich, St Louis, MO, USA). A new microtomy knife was used to prepare 5 µm sections from each sample. Sections were mounted onto charged microscope slides (Leica Biosystems, Inc., Buffalo Grove, IL, USA). One section from each sample was processed for hematoxylin and eosin (H&E) staining (Fischer et al., 2008).

In situ hybridization probe preparation

The protocol for designing anti-digoxigenin (DIG) mRNA probes was modified from Wolenski et al. (2013a,b). In short, the DIG-labeled antisense, single-stranded mRNA probes were designed for TNFR4, TNFR39, TNFR41, AP-1, aldolase, G protein-coupled receptor gene (GPCR), chordin-like and FosB. To begin, primers were designed to replicate amplicons approximately 400 bp in length for the genes of interest (Table S2). Each gene was then replicated using polymerase chain reaction (PCR) and then ligated into a pGemT easy vector system (Promega, Madison, WI, USA). JM109 High Efficiency Competent Cells (Promega) were transformed using a 2 min heat shock at 42°C and diluted in 900 µl of super optimal broth with catabolite repression medium and incubated for 60 min with shaking at 225 rpm at 37°C. Fifty microliters of each transformant was pipetted onto lysogeny broth (LB)/ampicillin/IPTG/X-Gal plates and grown overnight at 37°C. White colonies were picked and grown overnight in 3–4 ml of LB at 37°C while shaking at 150 rpm. Minipreps were preformed using a Qiagen Miniprep kit (Qiagen, Hilden, Germany) to purify the plasmids, and commercially available M13 primers were used in a PCR reaction for 29 cycles to further concentrate each sample. Samples were then sequenced using Sanger sequencing to verify that the correct product was cloned. Once verified using BLASTx, these products were then used to generate anti-DIG mRNA probes using a T7 or Sp6 Megascript kit (Ambion, ThermoFisher Scientific, Waltham, MA, USA).

In situ hybridization

In situ hybridization was performed on unstained coral tissue sections following a modified protocol previously published (Ragone Calvo et al., 2001). The paraffin was dissolved with two washes of 100% xylene for 5 min, then gradually rehydrated using ethanol dilutions of 95%, 75% and 50%. Samples were then pre-treated in 10 mg μl^{-1} of Proteinase K in 1× PBS at 37°C for 10 min. The reaction was stopped with 2% glycine in PBS wash for 5 min at room temperature. Samples were then incubated in 2× saline sodium citrate (SSC) solution for 10 min at room temperature.

To prepare the anti-DIG mRNA probes for hybridization, each anti-DIG mRNA probe was denatured while in hybridization buffer for 10 min at 90°C. Tissue on each slide was enclosed using a PAP pen (Sigma-Aldrich), and 50 µl of diluted anti-DIG mRNA probe was pipetted onto each slide. Hybridization of each probe was done at 50°C overnight. The next morning, slides were removed from the incubator and washed in 2× SSC and 1× SSC, both at room temperature for 10 min, and 0.05× SSC for 10 min at 42°C. The slides were then washed in alkaline phosphatase (AP) buffer for 1 min and incubated in Boehringer–Mannheim blocking buffer (Roche, Sigma-Aldrich) for 1 h at room temperature. Slides were then incubated with AP-Fab fragments (Roche, Sigma-Aldrich) and Blocking buffer (Roche, Sigma-Aldrich) overnight at 4°C. Anti-DIG mRNA probes were visualized using an AP substrate, BM purple (Roche, Sigma-Aldrich) for 1 h at room temperature in the dark. To stop the colorimetric reaction slides were then washed in Tris-EDTA buffer for 5 min, then washed with water for 1 min and mounted with glycerol. Slides were imaged using an Olympus BX43 microscope with objectives ranging from ×10 to ×60 and photographed using an Olympus DP21 camera attachment (Olympus, Center Valley, PA, USA).

RESULTS

Heat stress causes severe damage to all of the coral tissue layers

To examine the cellular architecture of coral tissues under heat stress, H&E staining was employed. The tissue and cellular architecture was drastically different between heat-stressed and control samples (Fig. 1). In the control samples, tissue epithelia and mesoglea were within normal limits. Nuclear and cytoplasmic contents were well preserved. Mucocytes were scattered and not

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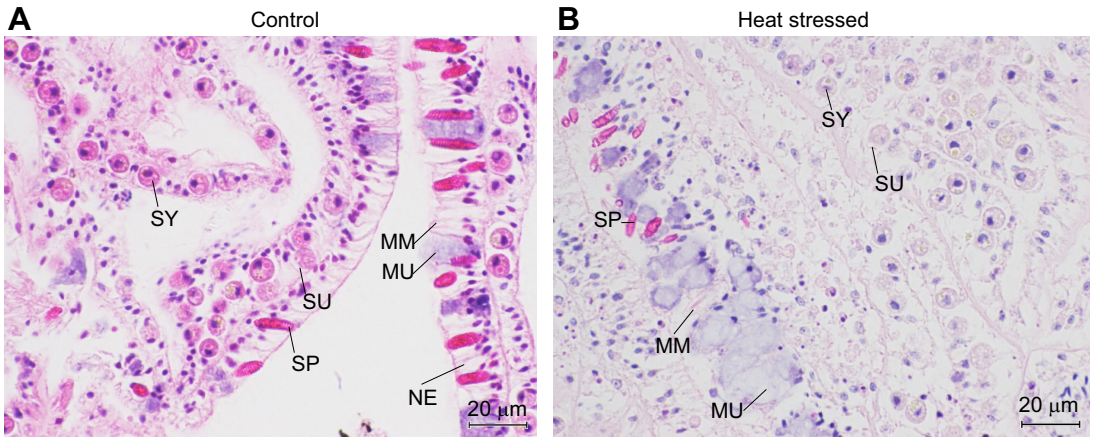

Fig. 1. H&E stained histological cross-sections of control and heat-stressed *Acropora hyacinthus*. (A) In the control samples, tissue epithelia and mesoglea were intact and normal, with well-preserved nuclear and cytoplasmic contents. Mucocytes were scattered and not hypertrophied. Spirocysts also in the epidermis stained brightly with eosin whereas nematocysts were pale. Gastrodermal cells contained the symbiotic algae *Symbiodinium* spp., which was in good condition with pink, slightly vacuolated cytoplasm and a normal nucleus, and pyrenoid body often visible. No signs of bleaching were present. (B) Heat-stressed samples had abnormal tissue architecture and loss of staining quality. Within the epidermis, mucocytes increased and both vacuolated epithelial cells and mucocytes lysed. Cnidocytes were present but were misshaped or stained weakly. Additionally, epithelium was disrupted, releasing spirocysts and microbasic mastigophores from lysed cells. *Symbiodinium* spp. were irregularly shaped, fewer in number, possessed shrunken or lysed nuclei with enlargement of the vacuoles in which they reside in gastrodermal cells (symbiosome) and more prominent green lipid vacuoles. SY, *Symbiodinium* spp.; SP, spirocyte; SU, symbiont-containing gastrodermal cell; MM, microbasic mastigophores; MU, mucocytes; NE, nematocytes.

hypertrophied, with pale, frothy, basophilic secretions present. Spirocysts also present in the epidermis stained brightly with eosin whereas nematocysts were pale. Gastrodermal cells contained the symbiotic algae *Symbiodinium* spp., most of which were in good condition with pink, slightly vacuolated cytoplasm and a normal dinokaryon (nucleus), and pyrenoid body often visible. No signs of bleaching were present (Fig. 1A).

In comparison, the heat-stressed samples had abnormal tissue architecture and loss of staining quality (Fig. 1B). Mucocytes had increased in number in the epidermis and were lysing, as were the other vacuolated epithelial cells. The organelles of cnidocytes were present but not as well formed or as stained as in the control corals; the epithelium was disrupted, releasing spirocysts and microbasic mastigophores from lysed cells. *Symbiodinium* spp. were misshapen and fewer in number in gastrodermal cells in comparison with the control samples, with pyknotic (shrunken) or lysing nuclei, enlargement of the vacuoles in which they reside in gastrodermal cells (symbiosome) and more prominent green lipid vacuoles. Marked-to-severe necrosis and lysing of cells of both the coral and algae were present. Overall, tissue staining was much lighter in the heat-stressed samples and clear delineation of the tissue layers was not as easily identified as it was in the controls. These observations confirm that our heated samples were experiencing cellular effects of heat stress, in contrast to the lack of signs of cellular heat stress in the control samples. At the time of sampling (5 h), these corals did not look visibly paler in color; however, the cellular signs of heat stress were present and this is an important factor to consider when examining heat-stressed corals (Rose et al., 2015; Seneca and Palumbi, 2015). In addition, this method confirmed that during this particular heat stress experiment, all of the tissues and cell layers in the heat-stressed samples displayed signs of stress. This included not only the gastrodermal cells containing *Symbiodinium* spp. but also the cnidocytes and mucocytes. This finding is crucial for our understanding of the heat stress response and helps to frame the gene expression work we previously conducted (Rose et al., 2015; Palumbi et al., 2014; Barshis et al., 2013; Seneca et al., 2015).

Induction of expression for genes chosen for *in situ* analysis

The coral samples we examined were part of a large study of gene expression as a function of heat stress (Rose et al., 2015; Seneca and Palumbi, 2015). In these experiments, all of the genes that we chose for *in situ* hybridization showed induction 5 h after heating compared with controls (Fig. 2; 1.5- to 6-fold, $P<0.0001$). Stress response module genes (*AP-1*, *FosB* and *TNFR41*) showed no differential expression between heavily bleached versus less bleached colonies, and by 20 h the expression had dropped substantially back towards control levels (Fig. 2A). Alternatively, bleaching module genes (*chordin-like*, *aldolase* and *GPCR*) showed marked increases of gene expression in more heavily bleached colonies, and maintained high levels of expression at the 20 h time point when bleaching became apparent (Fig. 2B). By contrast, and *TNFR4* increased strongly at 5 h and returned to baseline by 20 h (Fig. 2C,D). Based on these results, we hypothesized that *AP-1*, *FosB* and *TNFR41* functioned in the early response of the coral to heat stress and would be expressed widely in coral cells but that the bleaching-sensitive *chordin-like*, *aldolase* and *GPCR* genes might be expressed in the cells that are undergoing bleaching in the coral oral gastrodermis.

Stress response module genes: *AP-1*, *FosB* and *TNFR41*

AP-1 is a transcription factor that is activated by stress and other factors, including cytokines and growth factors (Shaulian and Karin, 2002). This transcription factor is activated when JUN and FosB proteins dimerize (Shaulian and Karin, 2002). Additionally, FosB can be active on its own and has been linked to functions in the extracellular matrix control of smooth muscle cells (Ramachandran et al., 2011). *TNFR41* is part of the TNFR family, which was previously found to be highly upregulated in response to heat in corals (Barshis et al., 2013; Palumbi et al., 2014; Quistad et al., 2013, 2014; Quistad and Traylor-Knowles, 2016; Traylor-Knowles and Palumbi, 2014), and its role in other organisms includes activating signaling pathways leading to apoptosis or to proliferation of cells (Aggarwal, 2003; Cabal-Hierro and Lazo, 2012; Gaur and Aggarwal, 2003; MacEwan, 2002).

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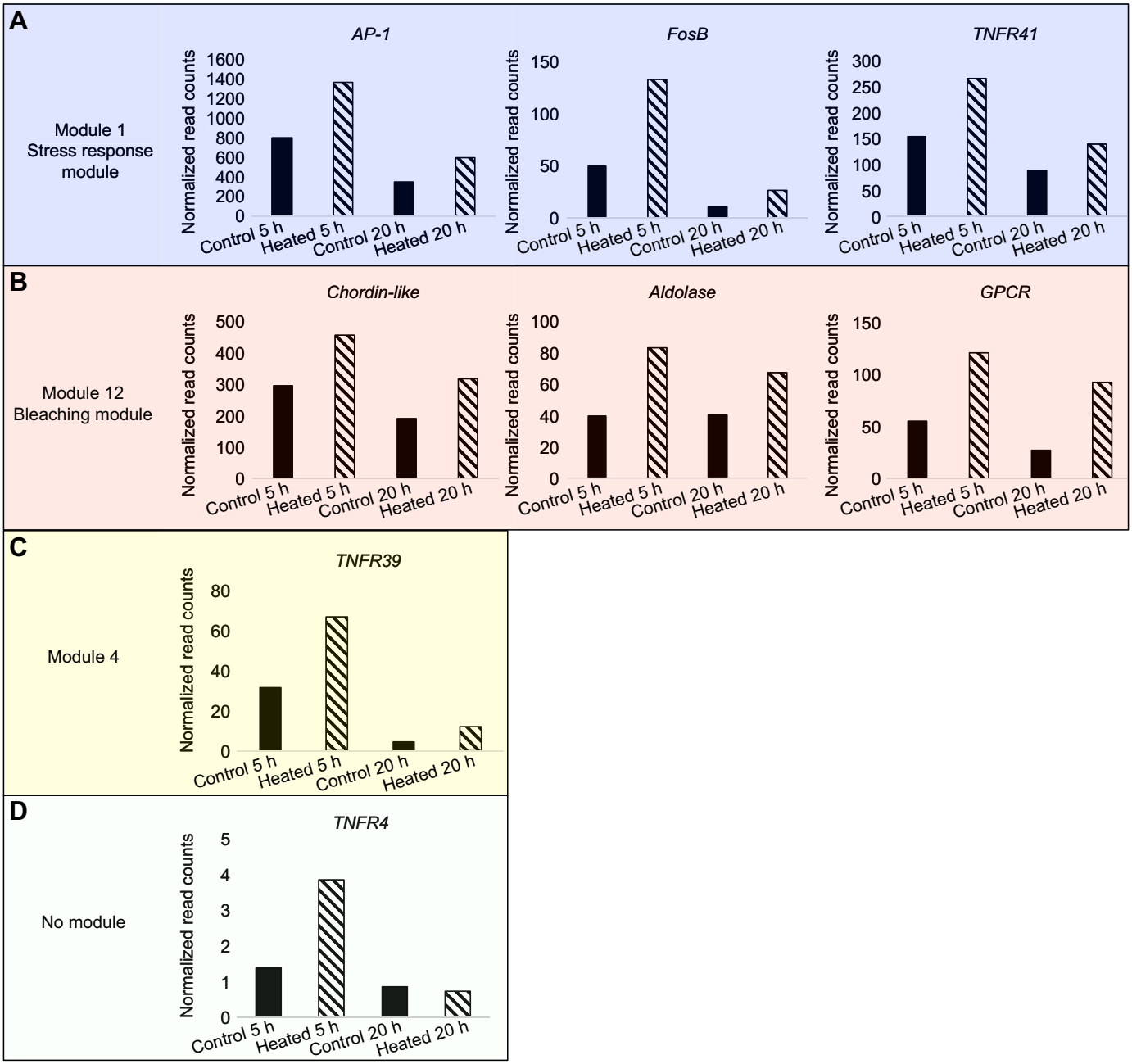

Fig. 2. Gene expression of stress response module genes (*AP-1*, *FosB*, *TNFR41*), bleaching module genes (*chordin-like*, *aldolase*, *GPCR*), module 4 (*TNFR39*) and no module (*TNFR4*). The coral samples in this study were previously part of a large study on the gene expression induced by heat stress (Rose et al., 2015; Seneca and Palumbi, 2015). (A) Stress response module genes (*AP-1*, *FosB* and *TNFR41*) showed no differential expression between heavily bleached versus less bleached colonies and by 20 h the expression had dropped substantially back towards control levels. (B) Bleaching module genes (*chordin-like*, *aldolase* and *GPCR*) showed gene expression induction 5 h after heating with marked increases of gene expression in more heavily bleached colonies, and maintained high levels of expression at the 20 h timepoint when bleaching became apparent. (C,D) *TNFR39*, part of module 4, and *TNFR4*, part of no module, increased strongly at 5 h and returned to baseline by 20 h.

In situ localization of *AP-1* and *FosB* was found throughout the epidermis and the oral gastrodermis, including the gastrodermal cells containing *Symbiodinium* spp. (Fig. 3A,B, Table 1). *AP-1* expression was found in the spirocytes of the epidermis whereas expression of FosB was found in both the spirocytes and nematocytes (Fig. 3A,B). *TNFR41* showed a slightly different pattern, where expression was primarily segregated to the cnidocytes within the cnidoglandular bands of the mesenterial filaments in the oral gastrodermis, as well as within the spirocytes and the nematocytes within the epidermis (Fig. 3C).

Bleaching module genes: *chordin-like*, *aldolase* and *GPCR*

Aldolase is an important enzyme that functions in glycolysis (Dandekar et al., 1999). GPCRs are seven transmembrane-spanning receptors that have a wide range of roles in mammals and are one of the most abundant groups of receptors in eukaryotes (Pitcher et al., 1998). These functions include immune system regulation and cell density sensing (Pitcher et al., 1998). Lastly, *chordin-like* is an important developmental gene involved in dorso-ventral patterning in vertebrates (Abreu et al., 2002; Matsui et al., 2000), as well as in renal function (Lin et al., 2005). *Chordin-like* was expressed only within

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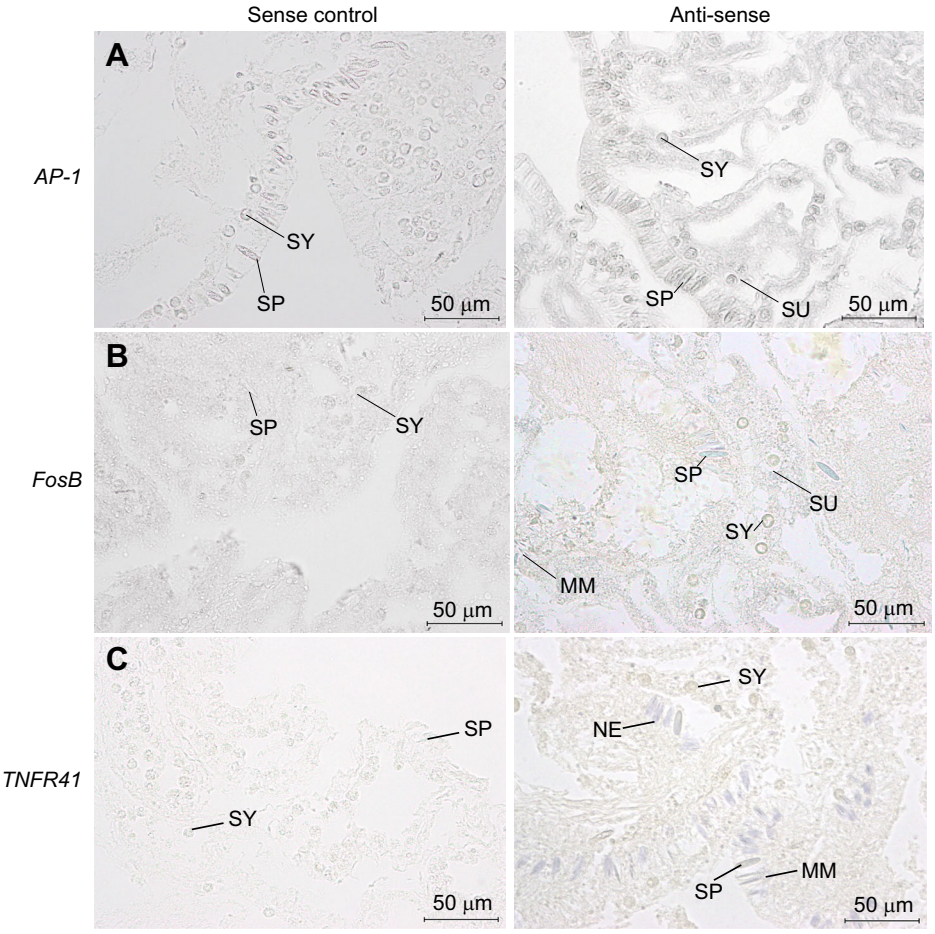

Fig. 3. Spatial expression of stress response module genes. (A) *AP-1* expression is found throughout the epidermis and the oral gastrodermis, including the gastrodermal cells containing *Symbiodinium* spp. as well as the spirocytes of the epidermis. (B) *FosB* expression is also found throughout the epidermis and the oral gastrodermis, including the gastrodermal cells containing *Symbiodinium* spp. as well as in both spirocytes and nematocytes. (C) *TNFR41* expression was primarily segregated to the cnidocytes within the cnidoglandular bands of the mesenterial filaments in the oral gastrodermis, as well as within the spirocytes and the nematocytes within the epidermis. SY, *Symbiodinium* spp.; SP, spirocyte; SU, symbiont-containing gastrodermal cell; MM, microbasic mastigophores; NE, nematocytes.

the oral gastrodermis (Fig. 4A, Table 1), specifically within the gastrodermal cells containing *Symbiodinium* spp. and throughout the extracellular matrix in the oral gastrodermis. In contrast, both *aldolase* and *GPCR* were expressed in the gastrodermal cells containing *Symbiodinium* spp., as well as in epidermal spirocytes and nematocytes (*GPCR* only) (Fig. 4B,C, Table 1).

Non-responsive genes: *TNFR4* and *TNFR39*

Much like *TNFR41*, the spatial gene expression of *TNFR4* and *TNFR39* in heat-stressed corals included specialized cells primarily found in the epidermis, especially the nematocytes (Fig. 5). Both *TNFR4* and *TNFR39* were expressed within the cnidoglandular bands of the mesenterial filaments of the oral gastrodermis;

Table 1. Summary of target genes and expression patterns

Gene name	Contig number	Uniprot ID	Module number from Rose et al., 2015	Module phenotype correlation from Rose et al., 2015	Tissue	Cell
AP-1	contig211469_148901_120483	P05627	1	Stress response	Oral gastrodermis, epidermis	Gastrodermal cells, spirocytes
FosB	contig182044	P13346	1	Stress response	Oral gastrodermis, epidermis	Gastrodermal cells, spirocytes, nematocytes
TNFR41	contig151747	P08138	1	Stress response	Cnidoglandular band (oral gastrodermis)	Nematocytes, spirocytes
Chordin-like	contig78169	Q9IBG7	12	Bleaching	Oral gastrodermis	Gastrodermal cells
Fructose bisphosphate aldolase C	contig108694	P53448	12	Bleaching	Oral gastrodermis epidermis	Gastrodermal cells, spirocytes
GPCR	contig77727	Q8IZP9	12	Bleaching	Oral gastrodermis, epidermis	Gastrodermal cells, nematocytes, spirocytes
TNFR4	contig82602	P47741	n.a.	n.a.	Cnidoglandular band (oral gastrodermis)	Nematocytes
TNFR39	contig151755	P08138	4	n.a.	Cnidoglandular band (oral gastrodermis)	Nematocytes

n.a., not applicable.

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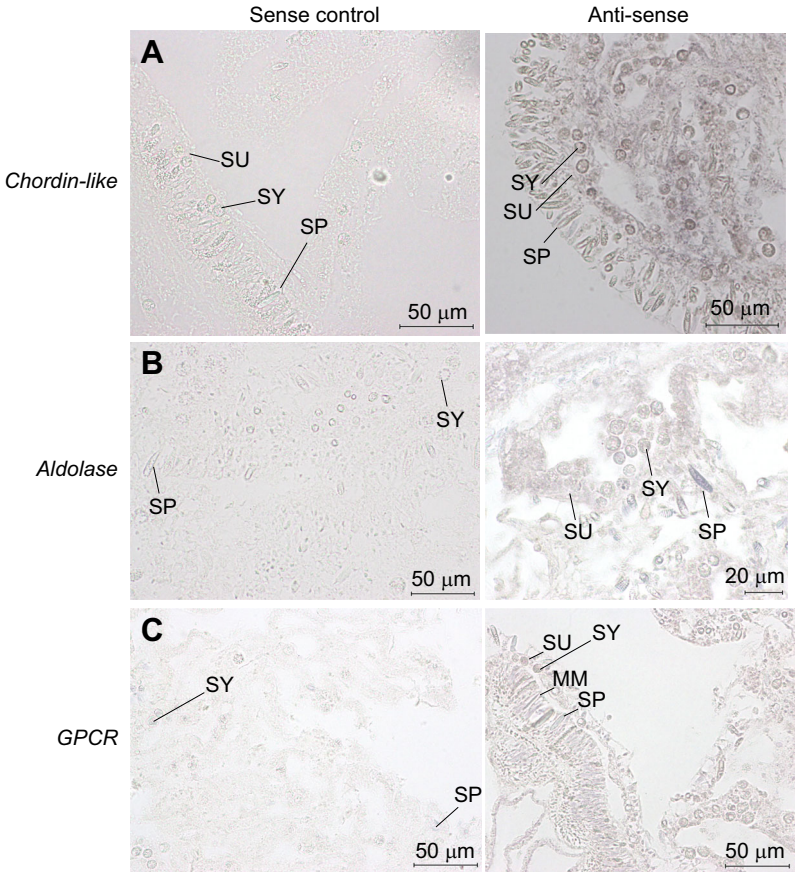

Fig. 4. Spatial expression of bleaching module genes. (A) *Chordin-like* was expressed only within the oral gastrodermis specifically within the gastrodermal cells containing *Symbiodinium* spp., and throughout the extracellular matrix of the oral gastrodermis. (B) *Aldolase* was expressed in the gastrodermal cells containing *Symbiodinium* spp., as well as in epidermal spirocytes. (C) *GPCR* was also expressed in the symbiont-containing gastrodermal cells, as well as in both nematocytes and spirocytes. SY, *Symbiodinium* spp.; SP, spirocyte; SU, symbiont-containing gastrodermal cell; MM, microbasic mastigophores.

however, symbiont-containing gastrodermal cells did not show localization of any TNFR gene after heat stress (Figs 4C, 5, Table 1).

DISCUSSION

Spatial expression of RNA is an important first step for understanding gene function

This study utilized *in situ* hybridization to localize the expression of different genes previously shown to be responsive to heat stress in corals. These data allowed us to test whether expression included the symbiont-containing gastrodermal cells that spark the bleaching response. Expression localization shows that TNFR expression is only in the epidermal nematocytes (*TNFR4*, *TNFR39* and *TNFR41*), in the spirocytes (*TNFR41* only) and within the cnidoglandular bands of the mesenterial filaments in the oral gastrodermis (*TNFR4* only). Staining within symbiont-containing cells was minimal. By contrast, *AP-1* and *FosB* both had expression throughout the epidermis and the oral gastrodermis. Lastly, *chordin-like* was mostly restricted to symbiont-containing cells.

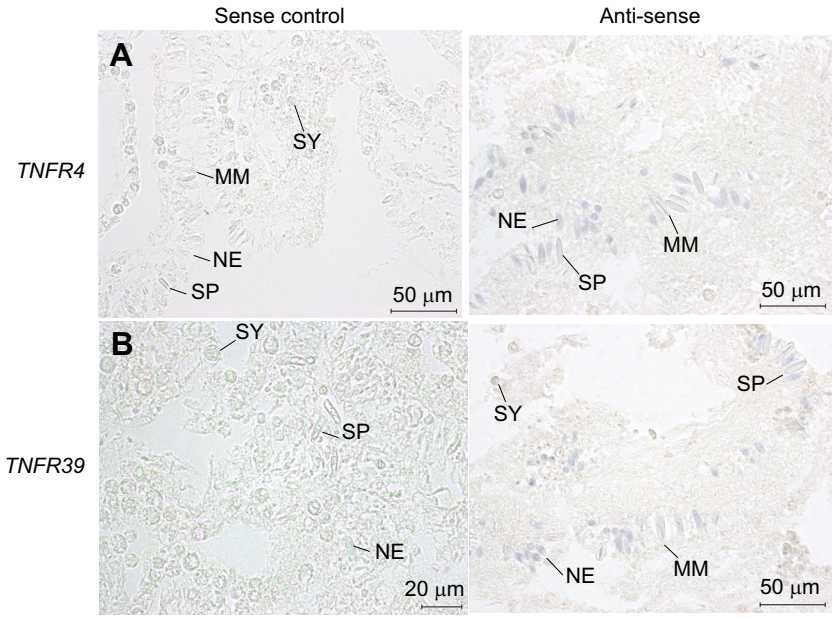

Fig. 5. Spatial expression of *TNFR39* (module 4) and *TNFR4* (no module). (A) *TNFR4* was expressed primarily in the nematocytes and within the cnidoglandular bands of the mesenterial filaments of the oral gastrodermis. (B) *TNFR39* was also expressed primarily in the nematocytes and within the cnidoglandular bands of the mesenterial filaments of the oral gastrodermis. SY, *Symbiodinium* spp.; SP, spirocyte; MM, microbasic mastigophores; NE, nematocytes.

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Our data add to the emerging characterization that the genetic response to heat in corals is a complex mix of responses from different types of cells. Most cell types, ranging from eubacteria to single-celled eukaryotes to metazoans, can mount a response to an acute heat increase (Feder and Hofmann, 1999). In fact, the heat shock promoter element is one of the classic mechanisms for cells to respond to changes in the external physical environment (Feder and Hofmann, 1999). These responses are not necessarily involved in the bleaching mechanism in corals, but because they are initiated by heat, they may appear to be. For example, Seneca and Palumbi (2015) found over 5000 genes were responsive to heat stress in corals; however, expression in only approximately 10% of these differed in corals that bleached after heating compared with conspecifics that did not (Rose et al., 2015).

Because coral gene expression studies derive from mixtures of all cell types and tissue layers, prior studies of the heat response have been unable to dissect the different cellular roles of different genes. Our initial *in situ* hybridization data suggest that there is a set of genes that is localized to the symbiont-containing cells. Some of these are also expressed in epidermal tissues but one of our target genes, *chordin-like*, is only upregulated in symbiont-containing cells.

The role of TNFRs in heat stress

Transcriptomic evidence shows that multiple TNFR genes are activated quickly during heat stress and expressed in a coordinated fashion (Barshis et al., 2013; Palumbi et al., 2014; Seneca and Palumbi, 2015). The evidence we report here suggests that these TNFRs, and by extension perhaps more genes of the stress response module (module 1) from Rose et al. (2015), are not directly involved in the bleaching mechanism but rather are part of the generalized stress response (Rose et al., 2015). The broad range of TNFR types, and signaling networks they can activate, make them likely candidates for roles in stress modulation (Traylor-Knowles and Palumbi, 2014). Untangling the TNFR signaling mechanisms in corals will be challenging. There are many different types (42 in total) and they possess rich sequence diversity (Quistad and Traylor-Knowles, 2016).

AP-1 and FosB as heat stress regulators

The AP-1 transcription factor is an important transcriptional regulator for general stress response in many organisms, including humans and mice (Shaulian and Karin, 2002). In this study, we showed that both *AP-1* and its subunit *FosB* are expressed within the symbiont-containing cells as well as within spirocytes and nematocytes (*FosB* only), suggesting that these genes are important in the overall stress response in corals to heat stress (Fig. 4A,B). These genes are expressed within symbiont-containing cells, which could indicate a role in bleaching or symbiosis; however, they were not part of the bleaching module. This potential role would need to be further investigated.

The role of aldolase, chordin-like and GPCR in the mechanism of bleaching

The fundamental mechanisms of bleaching are still not well understood. Recently, Bieri and colleagues showed that of all of the proposed types of bleaching mechanisms during heat stress, exocytosis of the symbiont cell was the most prevalent (Bieri et al., 2016). Both GPCRs and aldolase are implicated in exocytosis of insulin through the insulin pathway (Kao et al., 1999; Madiraju and Poitout, 2007). In the insulin pathway, GPCRs act as modulators of insulin secretion and aldolase acts as a scaffolding protein for the glutamate receptor, which regulates glucose transport (Kao et al., 1999; Madiraju and Poitout, 2007). In addition, glucose has been reported as the dominant metabolite transferred between *Symbiodinium* spp. and the coral host cell (Burriesci et al., 2012). Glucose phosphorylation and the GPCR system are needed for the synthesis of cAMP (Rolland et al., 2001), an important component in cell proliferation of *Symbiodinium* spp. (Wang et al., 2008). Further studies are needed to elucidate the functional role of these genes, but these expression data are a first step in pinpointing the possible mechanisms of coral bleaching. In addition to this, the expression of these genes found within the spirocytes and in some cases the nematocytes could also indicate that these genes are involved in other stress-related processes and are important to the overall health of the coral.

Chordin-like had very specific staining to the oral gastrodermis and there was no staining found within the epidermis (Fig. 4A). In mammalian models, chordin-like acts to increase bone morphogenic protein signaling in a paracrine fashion, thus influencing renal health in adults and dorso-ventral patterning during development (Abreu et al., 2002; Lin et al., 2005; Matsui et al., 2000). However, the role of this gene in corals is not understood. The expression of this gene within the oral gastrodermis, and its previously found high correlation to the bleaching phenotype (Rose et al., 2015), leads us believe that this gene may have a role in the bleaching response, but further investigation would need to be done to verify this.

The role of cnidocytes in response to heat stress

One of the most surprising findings of this study was that the cnidocytes, specifically spirocytes and nematocytes, were expressing genes involved in stress response and bleaching. Spirocytes and nematocytes are a subgroup of specialized cells called cnidocytes, which are a characteristic cell type of cnidarians (Mariscal, 1984). Spirocytes are primarily activated by the stimulus of food and create a fibrous-like matrix on discharge of their solid tubules to increase surface area and the stickiness of the tentacle (Mariscal, 1984; Mariscal et al., 1976; Mariscal and McLean, 1976; Westfall et al., 1999) whereas nematocytes are stinging cells used during prey capture and inter-colony aggression (Hidaka, 1985). While further investigations on these cell types needs to be done, it is possible that they play an important role in generalized stress response.

Previous studies in the starlet sea anemone, *N. vectensis*, have found that nematostomes, structures that contain cnidocytes as well as other cell types, are part of the immune system of *N. vectensis* (Babonis et al., 2016). Also, Wolenski and co-authors found that NF-κB, a generalized stress response transcription factor, was required for cnidocyte development and was expressed in specific cnidocyte populations in adult anemones (Wolenski et al., 2013a,b). Together this evidence points to novel functions of these cell types and highlights that future investigations of cnidocytes, particularly in corals, are needed to truly understand their complex diversity and function.

Conclusions

Our study presents evidence that genes previously found to be correlated with the stress response and bleaching response are spatially localized in different coral cell types. This expression of particular genes in the areas surrounding the symbiont suggest that the bleaching response and the stress response may be two different but interacting mechanisms important to the coral's response to changes in their environment.

Using *in situ* hybridization techniques to localize expression patterns is a first step towards separating the roles of different genes

in coral bleaching. Utilizing this technique will help researchers have a better understanding of the functional significance of genes found in large-scale sequencing projects and will allow for better biomarker development. However, there are other tools that are being developed that show promise in more fine-scale dissection of cellular roles of genes in bleaching. For example, in the invertebrate *Botryllus schlosseri*, the use of FACS and RNAseq has enabled researchers to discover the cell types that are the vertebrate precursor of the hematopoietic stem cell (Rosental et al., 2016). This single cell or cell population analysis allows for fine-scale resolution of the stress response, with the focus on cellular interactions rather than on organismal reaction. Application of such tools to coral cells may provide a crucial boost to further disentangling the general heat response from the specific bleaching response in these animals.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
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Table S1: Information on the samples that we used in this study including symbiont type and treatment.

Click here to Download Table S1

Table S2: Primers used in this study to produce in situ hybridization probes.

Click here to Download Table S2