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Endosymbiosis drives transcriptomic adjustments and genomic adaptations in cnidarians

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Abstract. To decipher inter-partner signaling within the cnidarian-dinoflagellate endosymbiosis, we developed genomic resources (cDNA library and microarrays) for the symbiotic sea anemone *Anemonia viridis*. Differential gene expression was quantified during thermal stress, with and without UV radiation, between symbiotic *vs* aposymbiotic specimens and gastroderm *vs* epidermis tissues. During stress time-course experiments, each stress showed a specific gene expression profile with very little overlap. We show that the major response to thermal stress is rapid (24 hours) but returns to the baseline levels after 2 days. UVR alone has little effect but potentiates thermal stress, as expression of a second set of genes becomes differentially expressed at day 5. Analysis of genes differentially expressed between symbiotic *vs* bleached and symbiotic *vs* stressed specimens defined a restricted subset of genes (Kern). Tissue specific expression mapping of Kern genes showed that many were specifically enhanced in the symbiotic cells (gastroderm). Altogether, these data define the Kern genes as major molecular components of the symbiotic interaction. Functional annotations highlighted several pathways including collagen fibrillogenesis, vesicular trafficking, lipid metabolism, calcium signaling, inorganic carbon transfer and cell death, that were modified by stress. Phylogenomic investigations of several Kern genes (calumenin, NPC2, SYM32, dermatopontin, and Rhbg) demonstrate that these issued from cnidarian specific duplication events, with the Kern member being preferentially expressed in the gastroderm and specifically responding to stress. Such host specific genes subfunctionalizations suggest both genomic and transcriptomic adaptations driven by the physiological constraints of endosymbiosis.

Key words: symbiosis, bleaching, functional genomics, genomic adaptation, thermal stress.

Introduction

Cnidarian–dinoflagellate endosymbioses are very sensitive to environmental stresses such as increased temperature and light, but also pathogens, pollutions and changes in salinity (Hoegh-Guldberg et al. 2007), of which the main consequence is the symbiosis breakdown, ultimately leading to the loss of zooxanthellae, or so-called bleaching. The establishment and maintenance of this partnership must therefore be dependent on intimate molecular communications between the partners, including recognition and tolerance of symbionts, as well as adaptations for mutual transport and exchange of nutritional resources (Weis and Allemand, 2009). Large-scale gene expression studies started to decipher this molecular dialogue and highlighted a modulation of the host transcriptome, in particular genes involved in cell adhesion, lipid metabolism, cell cycle regulation, or cell death (Rodriguez-Lanetty et al. 2006; Voolstra et al. 2009).

The symbiotic sea anemone *Anemonia viridis* is a large single polyp from which the two animal tissue layers (epidermis and gastroderm) can be easily separated with minimal cross contamination. In addition, as a non-calcifying anthozoan, it is therefore an amenable biological model to study symbiotic interactions between the cnidarian host and its dinoflagellate symbionts aside from the mineralization cross-regulatory pathways found in corals. To decipher the molecular dialogue caused by the presence of the dinoflagellate symbionts (*Symbiodinium* clade temperate A) within the sea anemone *A. viridis*, we compared transcriptomes of symbiotic and aposymbiotic specimens, using a symbiosis-dedicated microarray. This oligonucleotide microarray (2,000 features) was developed from the *A. viridis* 40,000 EST collection (Sabourault et al. 2009) and is dedicated to genes potentially involved in symbiosis regulatory pathways. We next investigated the early (1–5 days) changes in gene expression profiles in response to elevated temperature or UVR exposure and a combination of the two. For these two sets of experiments (symbiotic/aposymbiotic comparisons and stress time-course experiments), we also followed tissue-specific gene expression changes (gastroderm or epidermis). Here we describe a set of

genes involved in maintenance and disruption of symbiosis. Most of them show clear preferential tissue expression (essentially gastroderm) as well as marked enhanced expression in the symbiotic state and/or rapid decreased expression in response to stress leading to bleaching.

Material and Methods

Collection and maintenance of sea anemones
Mediterranean sea anemones, *Anemonia viridis* (Forskål, 1775), were collected on the French Riviera (Antibes, Villefranche-sur-Mer, Monaco and Menton, France) and maintained in closed-circuit seawater aquaria at 17.0 ± 0.5 °C with weekly water renewal. A complete description of specimens is available in Ganot et al. (2011) for symbiotic and aposymbiotic sea anemones, and in Moya et al. (2012) for the specimens used in the stress experiments.

Experimental design
Sampling protocol and experimental design are described in Ganot et al. (2011) for the comparison symbiotic/aposymbiotic, and in Moya et al. (2012) for the stress experiments (temperature with or without UVR). Another stress time-course experiment was performed in order to follow Rhbg gene expression: specimens named III_C, III_E and III_G were subjected to a temperature increase of +10.0°C (± 0.6°C, without UVR) for 7 days, then an additional +5°C for 2 days. Specimens were sampled at T=0, 1.5, 7, and 9 days.

RNA extraction, cDNA labeling and oligoarray hybridizations
Total RNA was extracted either from the whole tentacle (epidermis plus gastroderm and zooxanthellae) or from separated tissues using Trizol Reagent (Invitrogen), as described in Ganot et al. (2011). RQ1-DNase (Promega) treatment was carried out to avoid genomic DNA contamination. RNA quality was evaluated using the Agilent Bioanalyzer 2100 and quantified on a ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA).

cDNA synthesis and labeling were performed from total RNA using the ChipShotTM Direct labeling and Clean-up system (Promega), according to the manufacturer's instructions.

Five nanograms of a Lux mRNA exogenous standard was added to each mRNA sample before labeling. Hybridizations of *Anemonia viridis* Oligo2K oligoarray (GEO platform record GPL10546) and data analyses were performed as described by Ganot et al. (2011).

Semi-quantitative RT-PCR
Total RNA was reverse transcribed using oligodT primer and Superscript II reverse transcriptase (Invitrogen). PCR amplifications of Rhbg1 and Rhbg2 transcripts were performed using 10 nanograms of cDNA and the following primers: Rhgb1_F (5'AACGTCAATCGAAGCCACTCT-3'), Rhgb2_F (5'-ACATCCGAGAAGGCAAATCG-3'), and the same reverse primer Rhgb_R (5'-GGTCATAAGGAAGCCGAAGC-3').

Protein name	Symb.	Stress	G/E
Calcium binding			
Calumenin (CALUa)	**7,3**	**x,z**	**G**
Calumenin (CALUb)	1,2		
Calumenin (CALUc)	-1,3		
Calponin (Calp1)	1,1	X	
Calponin (Calp2)	-1,2		
Calponin (Calp3-D)	-1,0	z	G
Sterol transporter			
NPC2-D	**4,4**	**x,z**	**G**
NPC2a	-1,1	x	
pH regulation			
Carbonic anhydrase 2 (CA2-mb)	**2,9**	**x,z**	**G**
Transporter			
Rhbg-1*	4,4	x*	G*
Rhbg-2	1,1		E
SLC35aB3	1,0	x,y	E
Cell-cell interaction			
Sym32	**3,4**	**x,z**	**G**
MERP-1	-1,6		
MERP-1	-1,1	x,z	G
MERP-1	-1,4	X	G
C3 complement (C3-1)	1,5		G
C3 complement (C3-2)	**-2,3**	**X,Z**	
Dermatopontin (DPTa)	-1,1		
Dermatopontin (DPTb)	-1,4		
Dermatopontin (DPT-D)	-1,2	y,z	G
Collagen alpha-1(III)	-1,2	x	G
Collagen alpha-1(V)	-1,2	x	G
Collagen alpha-2(V)	-1,3	x	G
Collagen like	-1,3	x	G
Receptor signaling			
Tyrosine kinase receptor	**2,4**	**z**	**E**
Tyrosine-protein kinase SRK2	-1,2	x	G
Vesicle			
Mitochondrial fission 1 (FIS1)	**2,0**	**x,Y,z**	**G**

Table 1: Genes showing tissue preferential expression and differential expression in symbiotic *vs* aposymbiotic state or in response to thermal stress with or without UVR. Kern genes are in bold. Symb.: fold of differential expression (Symb/Apo). Stress: genes responding to thermal stress (x), to UVR (y) and to Temperature + UVR (z); upper and lower case letters correspond to enhance and decrease expression, respectively, relative to control. G/E: gastrodermal (G) or epidermal (E) preferential expression.
* this study.

Results

In a first experiment, we compared gene expression in five symbiotic and six aposymbiotic sea anemones (with different bleaching causes), to describe the transcriptomic profiles of the stable symbiotic and

aposymbiotic phenotype. In a second experiment, we exposed sea anemones for 21 days thermal stress with or without ultraviolet radiation (UVR) treatment (conditions that resulted in 80% decrease of symbionts after 3 weeks) or UVR alone, and we followed changes in gene expression during the five initial days of stress. DNA microarrays and quantitative RT-PCR (qPCR) analyses outlined characteristic gene expression signatures for the symbiotic and aposymbiotic states (experiment 1). We defined a restricted subset of 39 common genes (called the 'Kern' genes), which shared the same regulation profile in most of our investigated specimens (≥8/11 sea anemones), and therefore contribute to the maintenance of symbiosis (Ganot et al. 2011). Functional annotation of this gene set revealed two categories of cellular regulation: up-regulated genes in the symbiotic state were mainly involved in cell adhesion, lipid metabolism and transport, calcium homeostasis and inorganic carbon conversion whereas repressed genes were mainly involved in vesicular trafficking, RNA processing, cell death and ion transport. We also demonstrated that many of the Kern genes with enhanced expression in the symbiotic state were preferentially expressed in the symbiotic cells (gastroderm). Taken together, these findings imply that the aposymbiotic and therefore heterotrophic state triggers vesicular trafficking, whereas the symbiotic and therefore autotrophic state favors metabolic exchanges between host and symbiont. To gain further insights into symbiosis disruption and examine the possible role of those Kern genes in the initial events leading to bleaching, we imposed to symbiotic specimens a + 10°C thermal stress with or without UVR (experiment 2) and monitored the early transcriptomic response. In addition to newly identified responding genes, expression of 19 of the 39 Kern genes previously described was affected (Moya et al. 2012), strengthening the view that these latter are involved in the molecular events leading to symbiosis breakdown and bleaching. Early responding genes include genes involved in lipid metabolism and transport, calcium homeostasis, inorganic carbon conversion, solute transport, cell adhesion, receptor signaling, oxidative stress, cell death and RNA processing. In addition, we established that the major response to thermal stress is rapid (24 h) but returns to the baseline gene expression profile after 2 days. UVR alone has little effect but potentiates thermal stress, as a second response at 5 days was observed when the two stresses were coupled (Moya et al. 2012). Furthermore, the expression of some Kern genes, including carbonic anhydrases and the Niemann-Pick C2-D (NPC2-D) isoform, was specifically repressed in the symbiotic compartment (gastroderm),

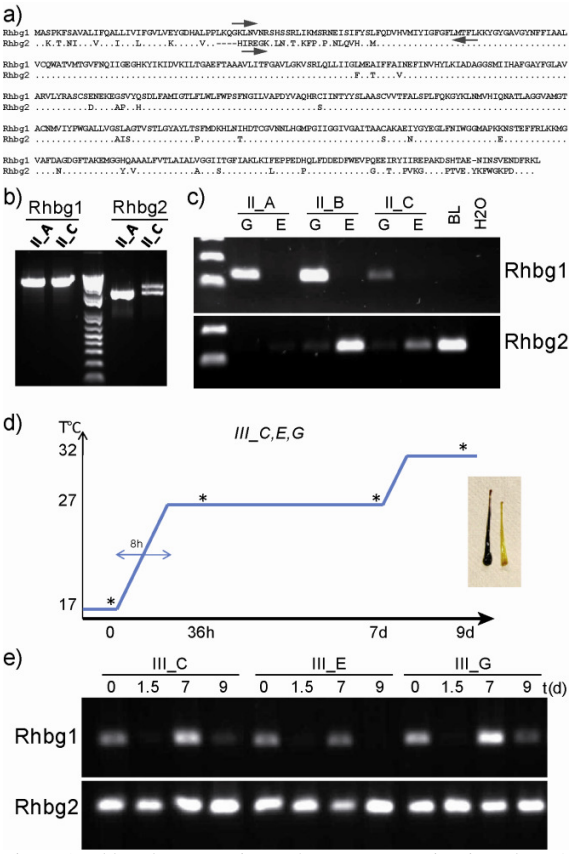

Figure 1: Rhbg, the *A. viridis* NH4 transporter, exists in at least 2 copies sharing 85% identities (90% similarities). Rhbg1 is symbiosis-associated as it is both endodermal specific and down-regulated after temperature stress or in bleached specimens. Rhbg2 is preferentially expressed in the ectoderm and not regulated. **a)** Alignment of the 2 proteins with arrows showing the position of primers used for PCR. **b)** PCR on genomic DNA from *A. viridis* specimens II_A and II_C. **c)** Semi-quantitative RT-PCR (Reverse Transcribed PCR) on RNA extracted from gastrodermal (G) and epidermal (E) tissue fractions of 3 anemones II_A, B and C, as well as from total tentacle of a bleached anemone (BL); H_2O, negative control. **d)** Specimens III_C, III_E and III_G were subjected to a temperature stress of +10°C at T=0, then a second increase of +5°C at T=7 days. Specimens were sampled at T=0, 1.5, 7, 9 days and RNA was extracted. Inserted photography shows tentacles at day 9 of a control and a stressed (bleached) specimen. **e)** Semi-quantitative RT-PCR of the sampled specimen showing that Rhbg1 is down-regulated after each temperature stress, albeit raised back after stress.

suggesting a tissue-specific modulation of gene expression in response to stress. Table 1 presents a short list of genes that both display a tissue preferential expression (either epidermal or gastrodermal) and are differentially expressed in symbiotic *vs* aposymbiotic states or in response to thermal stress (with or without UVR). Furthermore, several of these "symbiosis-associated" genes were found as several genomic copies in *A. viridis* and other anthozoans. Phylogenetic analyses demonstrated that for Sym32, Calumenin, NPC2

(Ganot et al. 2011) and DPT (Moya et al. 2012) these copies resulted from cnidarian-specific gene duplications. Interestingly, at the transcriptional level, only one of the paralogs was up-regulated both in the gastroderm and in the presence of symbionts, and was differentially regulated in response to stress. The other paralogs were not differentially regulated.

Our symbiosis-dedicated microarray was developed using 60mer oligonucleotides specifically designed for each gene. However, such approach cannot discriminate differential expression of two genes highly similar in sequences (or splice variant). In *A. viridis*, we identified two closely related genes coding for the vertebrate homolog of the ammonium transporter "Rhesus blood group family type B glycoprotein" (Rhbg). Although the two genes are very similar in their nucleotide coding sequence, sequencing of their respective genomic sequences showed that they were present as two separate copies. The two proteins Rhbg1 and Rhbg2 are almost identical except for their N- and C-Term sequences (Fig. 1), however, their tissue regulation at the transcription level is strikingly different, as monitored using RT-PCR with specific primers: rhbg1 appears restricted to the gastrodem whereas rhbg2 is very preferentially expressed in the epidermis. In a bleached specimen, expression of rhbg1, but not rhbg2, was highly repressed. When subjected to a serial increase of temperature (+10°C for 7 days and +5°C for 2 more days), expression of rhbg1 was strongly repressed immediately after each temperature raise, as opposed to rhbg2. Search in *Nematostella vectensis* genome revealed several similar duplication for this ammonium transporter arguing for an actinarian-specific gene duplication (data not shown). Rhbg proteins have been described as gas channels for either NH_3 or CO_2, depending on organisms (microorganisms or vertebrates) (Peng and Huang, 2006; Musa-Aziz et al. 2009). In light of our expression result in symbiotic cnidarian, it would be of interest to investigate the transporter properties of the two Rhbg copies.

Discussion

In non-model organisms for which functional genetic tools are not yet available, as it is the case for most anthozoans, identification and characterization of genes involved in the process of symbiosis is only accessible via indirect evidence, such as correlation between symbiosis disruption and gene differential expression. However, correlation between an altered gene expression pathway and a modified phenotype does not necessarily discern which one is the cause or the consequence of the other. In our attempt to characterize symbiosis-associated genes, we compared gene expression profiles of *Anemonia viridis*, a symbiotic non-calcifying anthozoan, either in the stable states of symbiotic *vs* aposymbiotic or during a stress time-course experiment that induces symbiosis breakdown. Combining the results of these two screens with tissue preferential expression analyses gives strong indication on genes functional implication in regard to symbiosis. Sym32, Calumenin-a, NPC2-D, CA2-mb, Rhbg1 and FIS1 all share the common features of gastrodermal-specificity in gene expression, as well as the down-regulation of their expression in the aposymbiotic state or in response to stress. As a working hypothesis, the following involvements in the host-symbiont interaction can be speculated: Sym32 is involved in the host-symbiont cellular association, Calumenin is part of the vitamin K cycle regulating Sym32, NPC2 participates in the uptake and transport of sterols from the symbiont to the host, CA2 regulates the transport of CO_2 from the environment to the symbiont, Rhbg is involved in the exchange of CO_2/NH_3 and FIS1 regulates the control of mitochondrial proliferation.

However speculative these assigned functions may be, it is important to note that Sym32, Calumenin-a, NPC2-D and Rhbg1 are issued from specific anthozoan gene duplications, implying genomic adaptations to symbiosis, and they respond to environmental conditions at the transcriptomic level. They should therefore be top-listed as candidate symbiosis-associated genes.

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