FAST TRACK

The host transcriptome remains unaltered during the establishment of coral–algal symbioses

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Abstract

Coral reefs are based on the symbiotic relationship between corals and photosynthetic dino-flagellates of the genus *Symbiodinium*. We followed gene expression of coral larvae of *Acropora palmata* and *Montastraea faveolata* after exposure to *Symbiodinium* strains that differed in their ability to establish symbioses. We show that the coral host transcriptome remains almost unchanged during infection by competent symbionts, but is massively altered by symbionts that fail to establish symbioses. Our data suggest that successful coral–algal symbioses depend mainly on the symbionts' ability to enter the host in a stealth manner rather than a more active response from the coral host.

Keywords: algae, coral, host, symbiont, symbiosis, transcriptome

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Introduction

The symbiosis between corals (Cnidaria: Hexacorallia: Scleractinia) and unicellular photosynthetic dinoflagellate symbionts (Alveolata: Dinophycea: *Symbiodinium*), also known as zooxanthellae, provides the foundation of the coral reef ecosystem. Whereas the algal symbionts contribute to their hosts' nutrition by providing photosynthetically fixed carbon, the coral host provides a sheltered and light-rich environment in addition to inorganic nutrients (Muscatine & Cernichiari 1969; Falkowski *et al*. 1984; Muscatine *et al*. 1984). In most cases this obligate symbiotic relationship is re-established each host generation (Harrison & Wallace 1990). While there is a high level of specificity within many mature coral–algal symbioses (Lajeunesse 2002; Coffroth & Santos 2005), field studies have shown that initially the developing coral will acquire a wide assortment of *Symbiodinium* strains (Coffroth *et al*. 2001; Little *et al*. 2004; Gómez-Cabrera *et al*. 2008). These will eventually be winnowed over time (hours to years), so that only one to a few of the strains establish the long-term symbioses characteristically found in adults (Goulet 2006; Rodriguez-Lanetty *et al*. 2006b). The underlying processes for initially accepting multiple strains

and then narrowing the assemblage to a single or a few strains are not known. Physiological performance among symbionts over the course of host ontogeny may explain the dynamics seen in the ontogeny of the symbiosis.

To examine molecular mechanisms during initiation and establishment of coral–algae symbioses, we followed gene expression profiles of coral larvae of *Acropora palmata* and *Montastraea faveolata* after exposure to *Symbiodinium* strains that differed in their ability to establish symbioses. We found that the coral host transcriptome remains almost unchanged during infection by symbionts that are able to establish symbioses. In contrast, it is massively altered during infection by symbionts that are not able to establish symbioses. Our data suggest that successful coral–algal symbioses depend mainly on the symbionts' ability to enter the host in a stealth manner, and that regulation of apoptosis, proteolysis and the immune system are central processes in the regulation of early coral–algae symbioses.

Materials and methods

Coral larvae

Egg and sperm of *Acropora palmata* colonies for the early time point of the experiment (i.e. 30 min after inoculation) were collected during spawning in Puerto Morelos, Mexico,

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on 1 September 2007 from one reef site (La Bocana Reef). Egg and sperm of *A. palmata* for the late time point of the experiment (i.e. 6 days after inoculation) were collected during spawning in Key Largo, Florida on 4 August 2004 from three reef sites (Horseshoe Reef, Little Grecian Reef, Dry Rocks). Egg and sperm of *Montastraea faveolata* colonies for both time points of the experiment (i.e. 30 min and 6 days after inoculation) were collected during spawning in Puerto Morelos, Mexico, on 3 September 2007 from one reef site (La Bocana Reef). For the collection of spawned bundles containing eggs and sperm, nets with collection tubes were placed over coral colonies on the night of spawning. For fertilization, the egg/sperm bundles of all colonies from a species were immediately combined upon collection in a bucket on the boat to fertilize as described in Miller & Szmant (2006; Szmant & Miller 2005). After fertilization, embryos were raised in 5 μм filtered seawater (FSW) in 100 L cooler bins to the competent planula stage. Some of the planulae had developed into unattached polyps ('cauliflower' stage) by the time the inoculation experiment began. Such polyps had developed mouths, tentacles, early mesenteries, and took up zooxanthellae by ingestion with phagocytotic cells in the mouth region. The two morphs are referred to as 'larvae' henceforth. Approximately 1500 *A. palmata* and 3000 *M. faveolata* larvae were put into 1 L plastic containers containing 500 mL 5 μм filtered seawater for each replicate. Zooxanthellae were added to a final concentration of 1000 cells/mL. For *A. palmata*, three replicates of untreated control larvae, *Symbiodinium* sp. CassKB8-inoculated (symbiosis competent), and *Symbiodinium* sp. Cass EL1-inoculated larvae (symbiosis incompetent) were sampled 30 min after inoculation and 6 days after inoculation. For *M. faveolata*, three replicates of untreated control larvae, *Symbiodinium* sp. Mf1.05b-inoculated (symbiosis competent), and *Symbiodinium* sp. EL1-inoculated larvae (symbiosis incompetent) were sampled 30 min and 6 days after inoculation. *Acropora palmata* larvae were inoculated several times with zooxanthellae. *Montastraea faveolata* larvae were inoculated once. Approximately 500 larvae for *A. palmata* and 1000 larvae for *M. faveolata* were sampled per triplicate at each time point. All samples were transferred to RNAlater (Ambion) and stored at –80 °C until processed further. *Symbiodinium* sp. CassKB8 (clade A1) and EL1 (clade A3) were originally isolated from *Cassiopea* sp., Kaneohe Bay, Hawaii by R.A. Kinzie III. *Symbiodinium* sp. Mf1.05b (clade B1) was isolated from *M. faveolata*, Florida Keys by M.A. Coffroth. All zooxanthellae were kept in F/2 medium (Guillard & Ryther 1962) with antibiotics at 24 °C under a 12/12 h light : dark cycle before they were used for inoculation of coral larvae.

RNA, hybridizations, microarrays

Total RNA of coral larvae was isolated using QIAzol (QIAGEN) according to manufacturer's instructions. Larvae were homogenized for 2 min using a Mini-Beadbeater (Biospec) with 0.1 mm and 0.55 mm silica beads to disrupt cellular structures. RNA pellets were cleaned further with RNeasy Mini columns (QIAGEN). RNA quantity and integrity were assessed with a NanoDrop ND-1000 spectrophotometer and electrophoretic profiling with an Agilent 2100 Bioanalyser, respectively. For all experiments, 1 μg of total RNA was amplified using the MessageAmp II aRNA kit (Ambion) according to manufacturer's instructions. Microarray protocols followed those established by the Center for Advanced Technology at the University of California, San Francisco (http://cat.ucsf.edu/). For cDNA synthesis, 3 μg of aRNA per sample was primed with 3.5 nmol of random pentadecamers for 10 min at 70 °C. Reverse transcription (RT) lasted for 2 h at 42 °C using a master mix containing a 3:2 ratio of aminoallyl-dUTP to TTP. Following RT, single-stranded RNA was hydrolysed by incubating RT reactions in 10 μL 0.5 м EDTA and 10 μL 1 м NaOH for 15 min at 65 °C. After hydrolysis, RT reactions were cleaned using the MinElute Cleanup kit (QIAGEN). Cy3 and Cy5 dyes (GE Healthcare) were dissolved in 17 μL dimethyl sulphoxide (DMSO), and the coupling reactions lasted for 2 h at room temperature in the dark. Dye-coupled cDNAs were cleaned using the Min-Elute Cleanup kit (QIAGEN), and appropriate Cy3- and Cy5- labelled cDNAs were mixed together in a hybridization buffer containing 0.25% SDS, 25 mм HEPES, and 3× SSC. The hybridization mixtures were boiled for 2 min at 99 °C then allowed to cool at room temperature for 5 min. The cooled hybridization mixtures were pipetted under an mSeries Lifterslip (Erie Scientific), and hybridization took place in Corning hybridization chambers overnight at 63 °C. Microarrays were washed twice in 0.6× SSC and 0.01% SDS followed by a rinse in 0.06× SSC and dried via centrifugation. Slides were immediately scanned using an Axon 4000B scanner. Before hybridization, microarrays were postprocessed by (i) ultraviolet crosslinking at 60 mJ; (ii) a 'shampoo' treatment (3× SSC, 0.2% SDS at 65 °C); (iii) blocking with 5.5 g succinic anhydride dissolved in 335 mL 1-methyl-2-pyrrilidinone and 15 mL sodium borate; and (iv) drying via centrifugation. Three biological replicates of pooled larvae from each species and condition (i.e. untreated control, inoculated with competent *Symbiodinium* strain, inoculated with incompetent *Symbiodinium* strain) for both time points were hybridized against a pooled reference (Fig. S1, Supporting information). Pooled references were constructed by combining equal amounts of aRNA from all control samples from *A. palmata* or *M. faveolata*, respectively. References were labelled with Cy3, samples with Cy5. Microarrays consisted of polymerase chain reaction (PCR)-amplified cDNAs from either *A. palmata* or *M. faveolata*. For *A. palmata*, 2055 PCR-amplified cDNAs were spotted in duplicate on poly-lysine-coated slides yielding a microarray with 4110 total features. For *M. faveolata*, 1314 PCR-amplified cDNAs were spotted in duplicate on poly-lysine-coated

	A. palmata		*M. faveolata*	
	CassKB8	EL1	Mf1.05b	EL1
	(Competent)	(Incompetent)	(Competent)	(Incompetent)
Early	24	27	11	18
Late	42	656	17	215

Table 1 Number of differentially expressed genes between pairwise comparisons among untreated and inoculated coral larvae of *Acropora palmata* and *Montastraea faveolata* for a given time point and symbiont species. Time points were shortly after inoculation (early, 30 min), and after 6 days (late). Total number of genes assayed: 2049 for *A. palmata*, 1275 for *M. faveolata*.

CassKB8: larvae inoculated with *Symbiodinium* sp. CassKB8; EL1: larvae inoculated with *Symbiodinium* sp. EL1; Mf1.05b: larvae inoculated with *Symbiodinium* sp. Mf1.05b.

slides yielding a microarray with 2628 total features. Complementary DNAs were chosen from expressed sequence tag (EST) libraries described in Schwarz *et al.* (2008). To annotate the cDNAs, we performed a BLASTX analysis (E-value cut-off 1e-5) against the GenBank non-redundant DNA and protein database (nr). 65% of the *A. palmata* genes and 43% of the *M. faveolata* genes had functional annotations as determined by homology to known genes. The overlap of spotted genes between both arrays was small (~10%) as determined by reciprocal TBLASTX.

Data analysis

Spot intensities were extracted and background subtracted using the TIGR Spotfinder software version 2.2.4 (Saeed *et al.* 2003). Duplicated spots were averaged over the median of intensities. Experimental data are accessible at the National Center for Biotechnology Information's (NCBI) GEO under GSE14923 for *A. palmata* and *M. favolata*. Only genes that were called in two out of three arrays for every experimental condition were subjected to further analysis. This yielded 2049 genes for *A. palmata*, and 1275 genes for *M. faveolata*. The ratio between the fluorescence intensity of the two channels was then used as input for Bayesian analysis of gene expression levels (BAGEL) (Townsend & Hartl 2002). The BAGEL software uses Bayesian probability to infer a relative expression level of each gene and statistical significance of differentially expressed genes. BAGEL computes an estimated mean and 95% credible interval of the relative level of expression of each gene in each treatment and time point for each species. We used the conservative gene-by-gene criterion of non-overlapping 95% credible intervals to regard a gene as significantly differentially expressed. Fold-changes were calculated as the ratio of the higher expression level to the lower expression level for the conditions to be compared. Trees were constructed by hierarchical clustering of arrays by the average linkage algorithm on the log2 of the relative expression level as estimated by BAGEL. Fold-changes for *Symbiodinium*-specific, competent-specific, and incompetent-specific genes were calculated by using the mean expression level of the two conditions that were not allowed to be significantly different from each other.

Chi-squared tests were conducted on numbers of differentially expressed genes as depicted in Table 1. The Yates correction for continuity was used in calculating the tests. Genes were functionally annotated and manually assorted into groups according to GO (Camon *et al.* 2003) and UniProtKB (Consortium 2008). All clone sequences are accessible via our EST database at http://sequoia.ucmerced.edu/SymBioSys/index.php.

Results

Patterns of differential gene expression

We inoculated unexposed planula larvae and unattached primary polyps of the Caribbean corals *Acropora palmata* and *Montastraea faveolata* with different strains of *Symbiodinium* that we had previously determined were able to establish symbioses in coral larvae (competent) or failed to do so (incompetent). While we chose different competent strains for each coral species (for *A. palmata*: *Symbiodinium* sp. CassKB8; for *M. faveolata*: *Symbiodinium* sp. Mf1.05b), the same incompetent strain was used (*Symbiodinium* sp. EL1). We followed the success of infection by fluorescent microscopy over 6 days, a time frame that is usually sufficient for coral larvae to accumulate and integrate competent zooxanthellae (Coffroth *et al.* 2001). Exposure of coral larvae to competent strains resulted in accumulation and integration of *Symbiodinium* into gastrodermal host cells, while larvae exposed to the incompetent strain did not appear to have any *Symbiodinium* within their tissues after 6 days.

Competitive hybridizations with cDNA microarrays (Desalvo *et al.* 2008; Schwarz *et al.* 2008) were performed at an initial stage of exposure to the symbionts (30 min after inoculation) and 6 days later (Fig. S1). Only a few genes were differentially expressed 30 min after exposure to different strains of *Symbiodinium* in both coral species (Table 1). After 6 days, the number of differentially expressed genes in coral larvae that were exposed to competent *Symbiodinium* strains (*Symbiodinium* sp. CassKB8/Mf1.05b) was still low. In contrast, we found a high number of differentially expressed genes in coral larvae inoculated with incompetent

Symbiodinium sp. EL1 (Table 1). The difference in the distribution of early and late differentially expressed genes depending on the symbiont strain is highly significant for both coral species (chi-squared test $P < 0.001$). Thus, coral larvae respond drastically different when exposed to competent and incompetent strains of *Symbiodinium* after they were given time to establish symbioses.

We constructed unrooted trees based on hierarchical clustering of all assayed genes by their $\log_2$ expression levels (Fig. 1). Both trees show that coral larvae inoculated with competent *Symbiodinium* strains cluster together with untreated control larvae, after the former were given time to establish symbioses, and are clearly separated from larvae that were inoculated with incompetent zooxanthellae. In *A. palmata*, the branch leading to larvae that were continuously inoculated over 6 days with incompetent *Symbiodinium* sp. EL1 shows the largest distance to all other nodes. In *M. faveolata* the branch between both time points shows the largest distance. This is most likely attributable to the fact that, in contrast to *M. faveolata*, larvae of *A. palmata* were repeatedly inoculated with zooxanthellae. Both trees also indicate that coral larvae behaved similarly in their early response to inoculation with any *Symbiodinium* strain. Inoculated coral larvae cluster together and are separated from untreated control larvae. While this is not obvious from looking at the numbers of differentially expressed genes, it would support the hypothesis that the same genes and processes are modified during phagocytotic uptake of symbionts by coral larvae, irrespective of the *Symbiodinium* species. Only in later stages do coral larvae react in a species-/competent-specific manner.

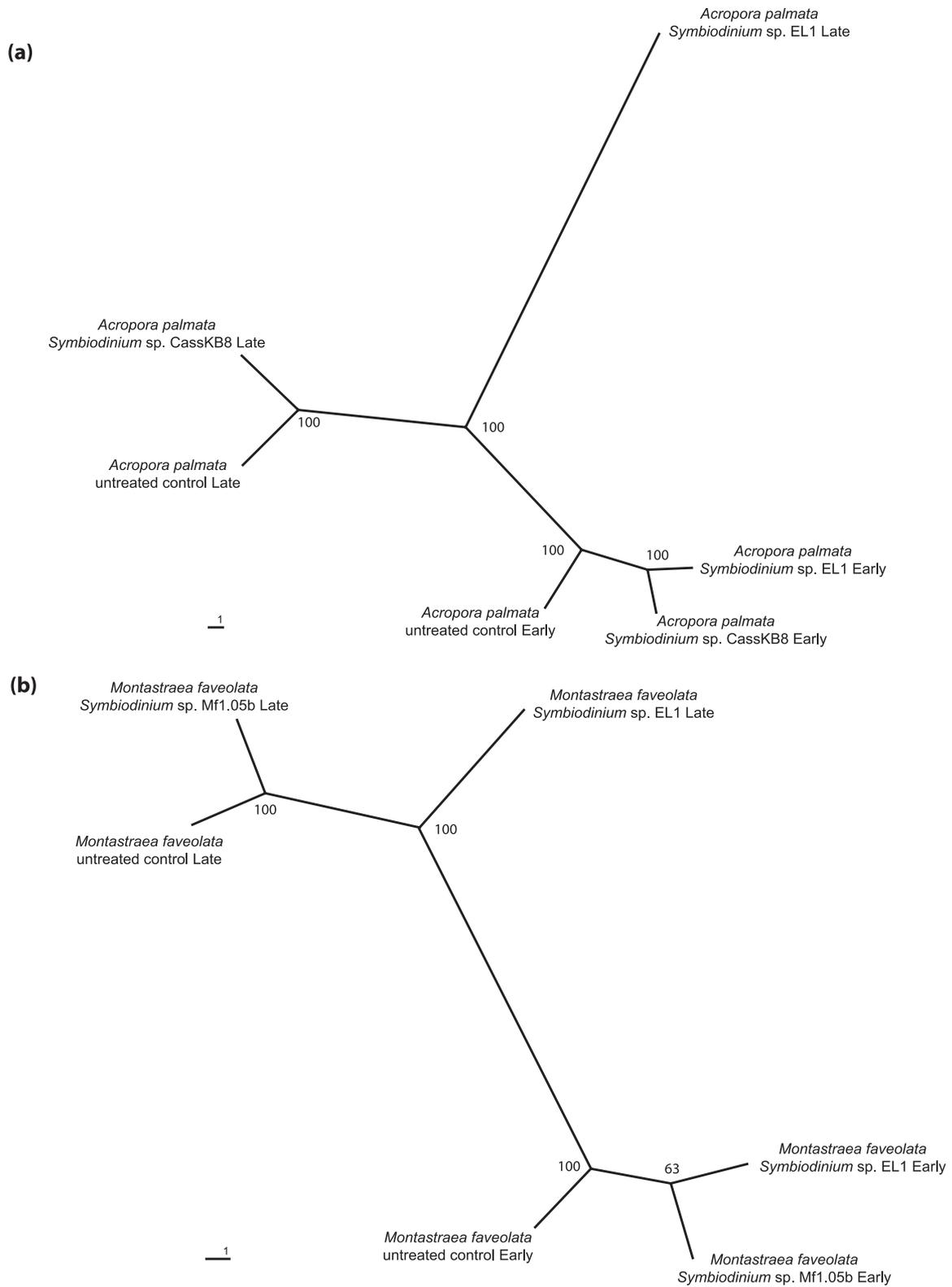### Regulation of specific genes and response pathways

To analyse differentially expressed genes more specifically, genes were classified into three main classes according to their expression pattern: *Symbiodinium* specific, competent specific, and incompetent specific. As a gene can be expressed either at higher or lower levels in one of the classes, a total of six categories emerge (Fig. 2). *Symbiodinium*-specific genes were defined as genes that were not significantly different between larvae that were exposed to competent and incompetent strains of *Symbiodinium*, but were significantly different from the expression of that gene in untreated control larvae (Fig. 2a). Competent-specific genes were significantly differentially expressed between larvae exposed to competent strains and larvae exposed to the incompetent strain or untreated control larvae. The latter two were not significantly different from each other (Fig. 2b). Incompetent-specific genes were differentially expressed between larvae exposed to *Symbiodinium* sp. EL1 and larvae exposed to competent strains or untreated control larvae. The latter two were not significantly different from each other (Fig. 2c).

We found *Symbiodinium*-specific genes for *A. palmata* and *M. faveolata* for the early and late time point (Table 2;

Class (by gene expression)	Early Number	Late Number
A. palmata		
Symbiodinium specific	**12**	**18**
Higher expression in control	5	6
Higher expression in CassKB8-/EL1-exposed	7	12
Competent specific	**0**	**15**
Higher expression in CassKB8-exposed	0	7
Lower expression in CassKB8-exposed	0	8
Incompetent specific	**0**	**524**
Higher expression in EL1-exposed	0	217
Higher expression in control and CassKB8-exposed	0	307
M. faveolata		
Symbiodinium specific	**1**	**6**
Higher expression in control	1	2
Higher expression in Mf1.05b-/EL1-exposed	0	4
Competent specific	**4**	**5**
Higher expression in Mf1.05b-exposed	2	0
Lower expression in Mf1.05b-exposed	2	5
Incompetent specific	**1**	**123**
Higher expression in EL1-exposed	1	44
Higher expression in control and Mf1.05b-exposed	0	79

CassKB8: *Symbiodinium* sp. CassKB8; EL1: *Symbiodinium* sp. EL1; Mf1.05b: *Symbiodinium* sp. Mf1.05b.

Table 2 Number of differentially expressed genes for the three main expression classes: *Symbiodinium* specific, competent specific, and incompetent specific. A gene can be preferentially expressed either higher or lower in one of the classes, which leads to six categories (Fig. 1). Time points were shortly after inoculation (early, 30 min), and after 6 days (late). Total number of genes assayed: 2049 for *Acropora palmata*, 1275 for *Montastraea faveolata*

Fig. 1 Radial trees of hierarchically clustered transcriptomes for *Acropora palmata* and *Montastraea faveolata* as calculated by the average linkage algorithm on the $\log_2$ ratio of the expression levels estimated by BAGEL (Townsend & Hartl 2002). Support values are based on 1000 bootstrap replicates. Time points were shortly after inoculation (30 min), and after 6 days (late). Scale bar at lower left corner displays overall Euclidean distance between expression vectors. (a) *A. palmata*, (b) *M. faveolata*.

(a) *Symbiodinium*-specific

(b) Competent-specific

(c) Incompetent-specific
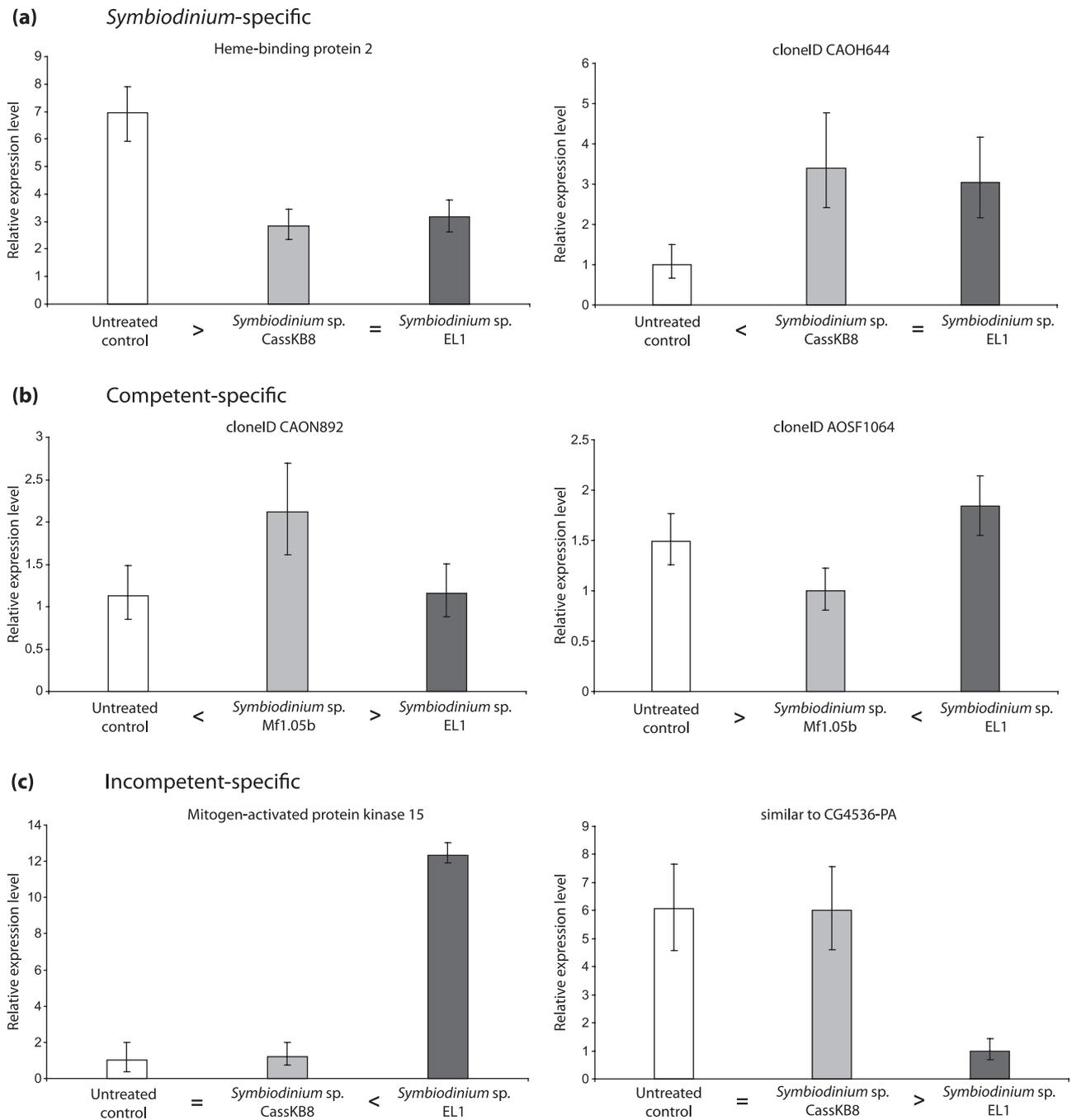
Fig. 2 Illustrative examples of gene classification according to relative levels of expression. The logical operators (=, <, >) denote the relationship among the 95% credible intervals of the mean expression levels obtained by Bayesian computation (Townsend & Hartl 2002) for the three main classes. Inequality signs denote statistically significant differences. Depicted are the relative expression levels and their 95% credible intervals relative to the node with the lowest level of expression set to 1. Denoted are the *Symbiodinium* strains with which coral larvae were inoculated. (a) *Symbiodinium*-specific genes; (b) competent-specific genes; (c) incompetent-specific genes.

Table S1, Supporting information). Heme-binding protein 2 (AOKF1561) that plays a role in apoptosis and 6–4 photolyase (AOKF2211) that is involved in DNA repair were the most highly downregulated genes in early infected *A. palmata* larvae. Sorting Nexin 7 (CAOG407) that functions in cell communication and an unannotated gene (CAOH644) were the most highly upregulated genes in early infected *A. palmata* larvae. In the late time point, we identified arsenite-resistance protein (AOKF629) among the upregulated genes in infected larvae of *A. palmata*. Arsenite-resistance protein

shows almost complete homology with the fau gene, a tumor suppressor gene which contains a ubiquitin-like region fused to S30 ribosomal protein. This protein regulates the ERK-MAPK cascade and might be implicated in the macrophage response to lipopolysaccharides (Nakamura & Yamaguchi 2006). For *M. faveolata*, we identified only one *Symbiodinium*-specific gene in the early time point. This gene has no annotation (CAON592) and was higher expressed in untreated control larvae. In the late time point, we identified a gene that triggers apoptosis (AOSF1475: death-inducer obliterator 1) among the upregulated genes in infected larvae. Overall, gene expression differences in this class of genes were up to a factor of 3.22 (*A. palmata*, CAOH644: unannotated).

We did not identify any competent-specific genes in *A. palmata* larvae in the early time point, but a number of genes in the late time point for both species (Table 2; Table S2, Supporting information). These genes were associated with processes such as cytoskeleton/cell adhesion, metabolism, signal transduction, cell cycle/growth/differentiation, regulation of transcription, and protein degradation. Among them, GLUT8 a carbohydrate transporter (CAOG660) and Ezrin (AOKF1216) that functions as an actin-cytoskeleton linker protein. It has been shown that association of calmodulin (CaM) and ezrin/radixin/moesin (ERM) to L-selectin confers resistance to proteolysis (Killock *et al.* 2009). In *M. faveolata*, we identified a total of nine competent-specific genes, four in the early and five in the late time point. Among them, a cyan fluorescent GFP-like protein (AOSF1131) and a homologue of the cylindromatosis (CYLD) protein (AOSF626) that encodes a deubiquitylating enzyme. CYLD is a negative regulator of the NF-kappaB and JNK signaling, and controls a number of seemingly disparate cellular processes. It appears to act by regulating a specific type of polyubiquitination that does not result in recognition and degradation of proteins by the proteasome but instead controls their activity through diverse mechanisms (Courtois 2008). Gene expression differences in this class of genes were up to a factor of 2.49 (*A. palmata*, CAOI1114: unannotated).

We found only one incompetent-specific gene in the early time point. This gene was identified in larvae of *M. faveolata* (hypothetical protein LOC767953 from *Bos taurus*). In contrast, we identified a large number of incompetent-specific genes in both species in the late time point (Table 2; Table S3, Supporting information). In both coral species, we found differential expression of genes affecting cell adhesion/cytoskeleton, cell cycle/growth/differentiation, protein biosynthesis, protein degradation, response to stress, metabolism, regulation of transcription, immune response, and RNA modification. Most of the biological categories were represented in both categories of genes, that is, they were either up- or down-regulated in coral larvae inoculated with *Symbiodinium* sp. EL1. We identified cytochrome *b* (AOKF526, AOKF2089), MAPK15 (mitogen-activated protein kinase 15: AOKF1380), and CASC3 (cancer susceptibility candidate

3: AOKF783) among the most highly differentially expressed genes in *A. palmata*. Cytochrome *b*, besides its important role as a component of the mitochondrial respiratory chain complex, has been revealed to have a role as a mediator of FAS-induced apoptosis in recent functional genetic screens (Komarov *et al.* 2008). Mitogen-activated protein kinases (MAPK) integrate diverse extracellular signals, and regulate complex biological responses such as growth, differentiation and death. CASC3 is a core component of the exon junction protein complex that is deposited on spliced mRNAs at exon-exon junctions and plays important roles in post-splicing events but is supposed to have additional functions (Baguet *et al.* 2007). Furthermore, we found tubulin alpha chain and beta tubulin to be up-regulated in the class of incompetent-specific genes in *A. palmata*. These same genes were conversely down-regulated as a consequence of successful symbiosis in a recent screen for symbiosis genes in the sea anemone *Anthopleura elegantissima* (Rodriguez-Lanetty *et al.* 2006a). In late larvae of *M. faveolata*, we found LWamide neuropeptide (CAON1226) and the DNA mismatch repair protein Msh2 (CAON1055) among the most up-regulated genes. The LWamide family of neuropeptides have the ability to induce metamorphosis of *Hydractinia serrata* planula larvae into polyps (Takahashi *et al.* 2008). Overall, incompetent-specific up-regulated genes had consistently higher fold changes than incompetent-specific down-regulated genes. The class of incompetent-specific genes in the late time point held the largest number of differentially expressed genes and the highest differences in expression

Discussion

Patterns of differential gene expression

Our data imply that neither competent nor incompetent *Symbiodinium* strains induce a strong transcriptomic host response shortly after inoculation. Alternatively, only few host cells could be affected initially. After several days though, coral larvae inoculated with competent and incompetent strains of *Symbiodinium* behave remarkably different. Inoculation of coral larvae with incompetent symbionts results in a massive transcriptomic response by the coral host. By contrast, only few genes are differentially expressed when the host was inoculated with a competent strain. The number of differentially expressed genes and the hierarchically clustered transcriptome trees show that coral host transcriptomes remain almost unaltered upon infection with competent symbionts and strongly resemble those of untreated control larvae over time. Hence, competent symbionts do not seem to provoke a major change in gene expression in their hosts. As such, our data provide evidence for the theory that competent coral symbionts do not trigger recognition and rejection in the host system (Trench 1993). At this point,

however, we cannot elucidate whether the reason is because competent symbionts remain invisible to the host (Trench 1993), or because they manipulate the hosts' response accordingly by suppression or modification of host responses (Nguyen & Pieters 2005). Both mechanisms may play a role. In general, there are many different ways of how symbiotic protists enter and remain vital in a host cell (Nguyen & Pieters 2005; Saeij *et al.* 2007; Carlton *et al.* 2008). Our analysis of the different gene classes further substantiates this conclusion, as we identified only a relatively small number of *Symbiodinium* and competent specific genes, but very many incompetent specific genes. Hence, many genes were specifically up- or down-regulated in response to incompetent symbionts, but showed a similar expression pattern in untreated control and competent *Symbiodinium*-exposed coral larvae.

Regulation of specific genes and response pathways

The genes that belong to the *Symbiodinium* specific class are most likely genes that are either (i) important upon host-symbiont recognition and phagocytotic uptake of *Symbiodinium* (early time point), or (ii) play a role in unspecific long-term modification of the coral host transcriptome (late time point). We did not find any *Symbiodinium* specific gene in either coral species that was consistently up- or down-regulated between early and late time points. This is in accordance with the assumption that the molecular processes and genes that play a role during the initial onset are distinct from those employed during the establishment and maintenance of symbioses. In a recent time course study on ectomycorrhizal symbioses, it was shown that distinct symbiosis genes exist that are expressed during the different stages of setup and establishment of symbioses (Le Quere *et al.* 2005; Sébastien Duplessis 2005). The genes we identified in the *Symbiodinium* specific class point towards apoptosis, MAPK signalling, and the immune system as processes or pathways that play a role in host-algae interactions.

The genes that belong to the competent specific class are assumed to have a mandatory function in initiation and maintenance of successful symbioses as they were exclusively up- or down-regulated in coral larvae upon exposure to competent *Symbiodinium* strains. We found no competent specific genes that were consistently higher or lower expressed across time points in either species. From the genes we identified as differentially expressed, it seems that processes related to carbohydrate metabolism, antiproteolysis, and NF-kappaB signaling (i.e. regulation of immune system) play an important role. We also identified a cyan fluorescent protein as differentially expressed in the competent specific class of genes in the early time point of inoculated *Montastraea faveolata* larvae. Although the functional role of fluorescent proteins in coral are unclear at the moment, these proteins have been shown to be under positive selection in

corals indicating that they must have a dedicated, yet unidentified, role in coral physiology (Alieva *et al.* 2008). Overall, our results for the competent specific class of genes are in concordance with recent molecular studies in cnidarian-algal symbioses that identified only few differentially expressed genes with small fold-changes during symbiosis (Barneah *et al.* 2006; deboer *et al.* 2006; Rodriguez-Lanetty *et al.* 2006a). This is, however, in contrast to what has been found in symbiotic relationships in other systems (Abshire & Neidhardt 1993; Natera *et al.* 2000; Cullimore & Denarie 2003; Wernegreen 2004; Dale & Moran 2006; Moran 2006; Chun *et al.* 2008; Heller *et al.* 2008; Martin *et al.* 2008). Chun *et al.* (2008) identified hundreds of differentially expressed genes as a result of symbiosis initiation in a study on symbiosis between the squid and luminous bacteria. Moreover, they conducted a comparison with data from Rawls *et al.* (2004*)*. Rawls *et al.* (2004*)* looked for differentially regulated transcripts that are shared between zebrafish and mouse in studies of the response of gut epithelia to colonization by their normal microbiota. Using this data set, Chun *et al.* (2008*)* found that 45 of 59 transcripts were in a shared gene family, suggesting that these transcripts may represent a core set of conserved ancient host responses. Hence, one might expect a more active response from the coral host since setting up symbioses with algae is clearly going to be beneficial. Instead, it appears that successful coral–algal symbioses depend mainly on the symbionts' ability to enter the host in a stealth manner that either circumvents or suppresses a host response.

Incompetent specific genes are differentially expressed in coral larvae as a consequence of exposure to incompetent strains of *Symbiodinium*. Hence, we interpret expression changes in this class of genes to reflect the response of the host to incompetent symbionts, which fail to establish persisting symbioses. The differentially expressed genes belong to many different categories, and we find up- and down-regulated genes that assort to the same biological processes. Hence, incompetent *Symbiodinium* strains cause a broad transcriptomic response that is characterized by misexpression of genes from diverse biological processes.

Our analysis of differentially expressed genes suggests that regulation of apoptosis and the immune system, possibly via MAPK and NF-kappaB signaling, is likely to be of substantial significance in symbioses. We identified genes in all three classes (i.e. *Symbiodinium*, competent, incompetent specific) that are associated with these processes or pathways. Furthermore, genes involved in these processes showed among the highest differences in expression (e.g. CYLD, Heme-binding protein 2, Arsenite-resistance protein, cytochrome b, MAPK15). Apoptosis has been identified as a post-phagocytotic winnowing mechanism in a coral-dinoflagellate mutualism in a recent paper by Dunn & Weis (2009). The authors showed that exposure of symbionts that are not successful in colonizing larvae of the scleractinian coral *Fungia scutaria* cause a significant increase in caspase activation,

and hence apoptosis. We provide further evidence for this finding. Furthermore, the regulation of proteolysis, respectively antiproteolysis seems to be central as evidenced by the list of competent specific genes.

Evolutionary conserved transcriptional responses

Due to the little overlap of both array platforms, we found only few identical genes between both species. We identified, however, the same processes and very similar genes that were differentially expressed in both species in the list of incompetent specific genes. Of course, a detailed analysis of all differentially expressed genes must follow and should be the focus of future efforts. As corals are non-model organisms, we deal with many genes that lack functional annotation but are nevertheless promising candidates. These may very well include those yet unidentified genes that play important roles in symbiosis.

Our samples might not represent the entire genetic diversity of the two species under study as our analysis is based on sampling of coral genotypes from one (in the case of *M. faveolata*), or four reefs (in the case of *Acropora palmata*), respectively. However, genetic differentiation between regions (and inferred gene flow) varies markedly among coral species (Ayre & Hughes 2004). To this end, we are not able to assess the effect of genotype subsampling. In addition, different symbionts have different physiologies and such differences have been found within, as well as between, genetically defined groups of *Symbiodinium* (Kinzie *et al.* 2001; Iglesias-Prieto *et al.* 2004; Sachs & Wilcox 2006; Loram *et al.* 2007; Stat *et al.* 2008; Voolstra *et al.* 2008). However, we find biologically coherent patterns of transcriptomic responses in two evolutionary distant coral species upon exposure to competent and incompetent strains of *Symbiodinium. Acropora palmata* is a member of the Long/Complex clade of scleractinian corals, and *M. faveolata* is a member of the Short/Robust clade (Romano & Palumbi 1996; Medina *et al.* 2006). The two coral species are separated by 240–288 million years (Medina *et al.* 2006). Our data suggest that the onset and establishment of coral–algal symbioses is an evolutionarily conserved process that predates the split between the Robust and Complex clades of scleractinian corals. Overall, transcriptome differences mainly arose at later time points when incompetent zooxanthellae were not able to successfully establish symbiotic relationships. Symbiotic larvae, by contrast, remarkably resembled their untreated counterpart at this time point. Apoptosis and the regulation of the immune system likely via MAPK and NF-kappaB signaling seem to be central processes in the regulation of coral-algae symbioses during these stages. Our data imply that corals do not significantly alter their transcriptome in response to competent algae, and that successful coral–algal symbioses hence depend on the symbionts' ability to not trigger recognition, respectively evade rejection in the host system.

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CRV is interested in evolutionary genomics, adaptation and symbiosis. JAS is interested in coral symbiosis. JS is interested in ecology and evolution of marine organisms. SS works on the diversity of coral-associated microbes and how these communities change as a function of disease and bleaching. MDS works on the molecular basis of bleaching. AMS focuses on the physiological ecology of reef corals and on nutrient dynamics in tropical coastal systems. MAC examines the population dynamics and early ontogeny of the symbioses between the zooxanthellae and cnidarian hosts. MM is working on a wide range of topics dealing with the ecology and evolution of marine biodiversity.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Microarray hybridizations performed for *Acropora palmata* and *Montastraea faveolata*. Three replicates of untreated control larvae, larvae inoculated with competent, and larvae inoculated with incompetent strains of Symbiodinium were performed for two time points. Each arrow represents a microarray hybridization. Cy3-labelled samples are placed at the tail, Cy5-labelled samples are placed at the head of the arrow. (A) Hybridizations carried out for *A. palmata*. (B) Hybridizations carried out for *M. faveolata*.

Table S1 Grouping of *Symbiodinium*-specific genes as determined by functional annotation from GO and UniProtKB

Table S2 Grouping of competent-specific genes as determined by functional annotation from GO and UniProtKB

Table S3 Grouping of incompetent-specific genes as determined by functional annotation from GO and UniProtKB

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