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The transcriptomic response of the coral *Acropora digitifera* to a competent *Symbiodinium* strain: the symbiosome as an arrested early phagosome

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Abstract

Despite the ecological significance of the relationship between reef-building corals and intracellular photosynthetic dinoflagellates of the genus *Symbiodinium*, very little is known about the molecular mechanisms involved in its establishment. Indeed, microarray-based analyses point to the conclusion that host gene expression is largely or completely unresponsive during the establishment of symbiosis with a competent strain of *Symbiodinium*. In this study, the use of Illumina RNA-Seq technology allowed detection of a transient period of differential expression involving a small number of genes (1073 transcripts; <3% of the transcriptome) 4 h after the exposure of *Acropora digitifera* planulae to a competent strain of *Symbiodinium* (a clade B strain). This phenomenon has not previously been detected as a consequence of both the lower sensitivity of the microarray approaches used and the sampling times used. The results indicate that complex changes occur, including transient suppression of mitochondrial metabolism and protein synthesis, but are also consistent with the hypothesis that the symbiosome is a phagosome that has undergone early arrest, raising the possibility of common mechanisms in the symbiotic interactions of corals and symbiotic sea anemones with their endosymbionts.

Keywords: *Acropora*, *Symbiodinium*, symbiosis, symbiosome, transcriptome

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Introduction

The relationship between scleractinian corals and photosynthetic dinoflagellates of the genus *Symbiodinium* (commonly known as zooxanthellae) is fundamental to the functioning of coral reef ecosystems. The photosynthetic activity of *Symbiodinium* enables the massive calcification that is characteristic of zooxanthellate corals; the endosymbiotic dinoflagellates provide up to 90% of host energy requirements in the form of photosynthetically fixed carbon and in return derive benefits from the host in the form of nutrients (Muscatine 1990). Although the relationship is obligate on the part of the

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coral, it is only meta-stable. Under stress, *Symbiodinium* cells are expelled, leave or are digested *in situ* and the coral host appears white or bleached. Unless symbionts are replaced relatively quickly, the coral host dies. Mass coral bleaching, often induced by abnormally high sea surface temperatures, is one of the major contributors to the global decline of coral reefs (Hoegh-Guldberg 1999; Weis 2008). Despite the importance of the relationship between coral and *Symbiodinium*, surprisingly little is known about the molecular events underlying its establishment, maintenance or collapse under stress. Although *Symbiodinium* occur within endodermal coral cells, they are separated from the host cytoplasm by a host-derived vacuole known as the 'symbiosome' (Davy *et al.* 2012). Symbiosome membranes are thought to originate from the plasma membrane of coral cells during symbiont uptake via phagocytosis (Hohman *et al.* 1982); the phagosome membrane surrounding the symbiont becomes the symbiosome membrane, which confers protection from host lysosomal degradation and controls nutrient transport (Fitt & Trench 1983; Davy *et al.* 2012). Approximately 15% of coral species maternally inherit their symbionts, while 85% acquire their symbionts from the environment at each generation, a process known as horizontal transmission (Baird *et al.* 2009). Although adult corals typically associate with specific strains of *Symbiodinium*, the larvae are relatively promiscuous (Cumbo *et al.* 2013). Nevertheless, a degree of specificity exists; for example, Voolstra *et al.* (2009) reported that the *Symbiodinium* strain EL1 is 'incompetent' to establish symbioses with *Acropora palmata* or *Orbicella* (formerly *Montastraea*) *faveolata* (note that here we use the term 'noncompetent', rather than 'incompetent', to describe strains that are unable to establish symbiosis). The establishment of an association via horizontal transmission therefore requires communication between the partners during the early stages of the interaction (Wood-Charlson *et al.* 2006), in addition to mechanisms for nutrient exchange in the symbiotic state (Meyer & Weis 2012). The few published studies that have addressed host gene expression during the initial contact between coral and *Symbiodinium* cells are summarized in Table 1 and discussed further below.

Voolstra *et al.* (2009) used cDNA microarrays to investigate expression differences in *A. palmata* and *Orbicella* (*Montastraea) faveolata* larvae after exposure to competent (i.e. a strain able to establish symbiosis) and noncompetent (i.e. a strain with which *Acropora* and *Orbicella* had previously failed to establish symbiosis) *Symbiodinium* strains. With the competent strain, few changes were detected at either 30 min or 6 days post-infection, whereas in both species, major transcriptional differences were observed at 6 d post-infection when larvae were exposed to the noncompetent *Symbiodinium* strain. From this, the authors concluded that competent *Symbiodinium* strains establish themselves by essentially avoiding host recognition, either circumventing or modulating the host immune responses that are normally triggered when noncompetent strains are encountered. cDNA microarrays were also used when studying the infection of *Fungia scutaria* larvae with competent *Symbiodinium* and the authors came to essentially the same conclusions (Schnitzler & Weis 2010), qualified by the possibility that the transcription signal may have been small or that a window of expression may have been missed.

In this study, next generation sequencing (NGS) (Illumina RNA-Seq) was used to investigate gene expression changes that occur in coral larvae during exposure to a competent strain of *Symbiodinium*. Transient expression changes affecting 1073 genes were detected at 4 h post-infection, but by 48 h post-infection the expression of these and all other host genes had returned to baseline levels. Analysis of the 4-h coral data suggests the involvement of both partners in the establishment of the symbiosis; there is an active response on the part of the host in recognizing the symbiotic partner, but an apparent suppression of host immune responses may be initiated by the symbionts. The gene expression data imply that translation and oxidative metabolism are suppressed in the coral host during the infection process, and altered expression of some apoptosis-related genes was also observed. Most significantly, some of the coral data are consistent with published hypotheses about the establishment of symbiosis in sea anemones, implying that, despite the deep evolutionary divergence between these two lineages (Shinzato *et al.* 2011), common molecular mechanisms may apply.

Materials and methods

Coral larvae and Symbiodinium culture

Gravid *Acropora digitifera* colonies were collected in front of the Sesoko Station of the Tropical Biosphere Research Center at the University of the Ryukyus in Sesoko Island, Okinawa, Japan, and maintained in flow-through aquaria until spawning occurred (19 June 2013). After fertilization, *A. digitifera* embryos were raised in 0.2 μm filtered sea water (FSW) under ambient conditions. *Symbiodinium* infection experiments were set up 6 days post-spawning, by which time the oral pore and coelenteron of the larvae were sufficiently well developed to enable symbiont uptake (Harii *et al.* 2009). *Symbiodinium* sp clade B1 (now known formally as *S. minutum*; culture ID= CCMP3345), originally isolated from the anemone *Aiptasia pallida,* was used in this experiment. Note that the strain of *Symbiodinium* used was selected on the basis that, in addition to

Table 1 Summary of published data on gene expression analysis of coral larvae during onset and establishment of coral–algal symbiosis

Authors	Coral host/species	Technique	Genes of Interest	Results
Grasso *et al.* (2008)	*Acropora millepora*	cDNA microarray	C-type lectin Carbonic anhydrase	Despite focusing on development, this study described some symbiosis-related transcripts
Schwarz *et al.* (2008)	*Orbicella faveolata*, *Acropora palmata*	EST analysis	Lectins Tachylectins Thrombospondin type I repeats Ferritin Scavenger receptors	Transcripts associated with innate immunity were identified as candidate genes of interest in symbiosis
Voolstra *et al.* (2009)	*O. faveolata*, *A. palmata*	cDNA microarray	NA	Larvae were inoculated with competent and noncompetent symbionts. This study found that there were few changes in larval transcriptome in the case of the competent strain however significant changes in the case of a noncompetent strain The authors suggested that initiation of the symbiosis depends on the ability of the symbionts to enter their host in a stealth manner without mounting an active response from the host
Schnitzler & Weis (2010)	*Fungia scutaria*	cDNA microarray	NA	Larval transcriptome showed very few changes during onset of symbiosis with competent symbionts. The study suggested suppression of host response during early colonization by symbionts
Mohamed *et al.* (present study)	*Acropora digitifera*	Illumina RNA-Seq	1073 genes	Discussed in the present paper

being compatible with *A. digitifera*, it was the strain for which the most comprehensive sequence data existed. The original intention was to simultaneously study gene expression changes in the coral host and the symbiont, but too few reads mapped to the *Symbiodinium* transcriptome to permit statistically valid analyses. Prior to inoculation, *Symbiodinium* cultures were maintained at 25 °C in Guillard's f/2 marine water enrichment solution (G0154; Sigma-Aldrich) under a 12-/12-h light/dark cycle.

Symbiont acquisition experimental design

Acropora digitifera larvae were divided into 2 treatment groups, with 3 replicates per treatment. In each replicate, 700 larvae were placed into 1-L plastic containers containing 700 mL of 0.2 μm FSW. The first group of larvae was inoculated with *Symbiodinium* sp. clade B cells which were washed three times in 0.2 μm FSW before being added to each replicate to a final concentration of 5×10^3 cells/mL; note that this density is similar to that in sediment around coral reefs (Littman *et al.* 2008). The second group of larvae was not inoculated with *Symbiodinium* (control). Containers were held at 26 °C in a constant temperature room, under fluorescent lamps that provided light (86 ± 2 μmol photon/m^2/s at the surface) on a 12-/12-h light/dark cycle. At 4, 12 and 48 h post-*Symbiodinium* inoculation, 10 larvae from each replicate were collected and washed by pipetting in 0.2 μm FSW, ensuring that no algae were attached to the larval surface. Larvae were inspected under a fluorescence microscope for symbiont chlorophyll fluorescence to determine if successful infection with *Symbiodinium* had occurred.

Larval sampling and RNA isolation

At 4, 12 and 48 h post-infection, aliquots of 150 larvae from each replicate were washed in 0.2 μm FSW and, ensuring as little liquid carry-over as possible, snap frozen immediately in liquid nitrogen and stored at −80 °C until further treatment. Total RNA was isolated using TRIzol® reagent (Life Technologies) according to the manufacturer's instructions. Larvae were homogenized for 2 min in 2 mL TRIzol reagent using a Polytron (Kinematica AG, Lucerne, Switzerland) followed by phase separation with chloroform. The clear upper aqueous phase containing RNA was carefully transferred into a new RNase-free microtube and RNA precipitated by addition of an equal volume of isopropanol. After centrifugation, the RNA pellets were washed with 75% ethanol. Finally, RNA was dissolved in 40 μL of RNase-free water and stored at −80 °C. RNA quality and quantity were assessed using a NanoDrop ND-1000 spectrometer, and integrity checked by electrophoretic profiling with an Agilent 2100 Bioanalyzer.

High-throughput sequencing and data analysis

Messenger RNA (mRNA) was isolated from 1 μg of total RNA and 16 RNA-Seq libraries prepared using the TruSeq RNA Sample Preparation Kit (Illumina). Libraries were sequenced on an Illumina HiSeq 2000 platform at the Okinawa Institute of Science and Technology (OIST), Okinawa, Japan. Sequencing produced 333.2 million individual 100-bp paired-end reads.

The raw reads were mapped onto the *A. digitifera* transcriptome assembly v1.0 (Shinzato *et al.* 2011) (http://marinegenomics.oist.jp/genomes/) using BOWTIE version 0.12.7 (Langmead *et al.* 2009) (http://bowtie-bio.sourceforge.net/index.shtml) with default parameters. The read alignments were visualized using INTEGRATED GENOMICS VIEWER (IGV) version 2.3.34 (Thorvaldsdottir *et al.* 2013) (http://www.broadinstitute.org/igv/). Then RSEM version 1.1.17 (Li & Dewey 2011) (http://deweylab.biostat.wisc.edu/rsem/) was used to quantify the abundances of the transcripts for each library.

Differential gene expression was inferred based on the mapping counts using the edgeR package (Robinson *et al.* 2010) in the R statistical computing environment (http://www.r-project.org). The three *Symbiodinium*-infected replicates were compared to the corresponding control samples at each of the three time points (note that, at the 12-h time point only two replicates per condition were available), producing three expression profiles at 4, 12 and 48 h post-infection. *P*-values for differential gene expression were corrected for multiple testing using the Benjamini and Hochberg algorithm. For further analyses of differential expression between treatment and control groups, only genes with a false discovery rate (FDR) of ≤0.05 were considered.

To infer the functions of the differentially expressed genes (DEGs), for both up- and down-regulated genes with absolute $\log_2$ (fold change) >1, two stages of analysis were employed. Initial BLASTX analysis ($e \leq 10^{-3}$) was performed against the SWISS-PROT annotated protein database (http://www.uniprot.org) and the resulting hits were then filtered (at $e \leq 10^{-15}$) by searching for close matches in *Nematostella vectensis* and/or *Acropora digitifera*. GO enrichment analyses and the identification of enriched biological themes were performed on the filtered data set using the DATABASE FOR ANNOTATION, VISUALIZATION AND INTEGRATED DISCOVERY (DAVID) (Huang *et al.* 2009) (http://david.abcc.ncifcrf.gov). The UNIPROT accession identifiers of the top protein hits were used as identifiers. Similarly, using BLASTX ($e \leq 10^{-3}$) against Swiss-PROT, functional annotation data were obtained for the whole transcriptome data set; these annotations serve as the background for the enrichment analysis. DAVID uses Fisher's exact test to ascertain statistically significant pathway enrichment amongst DEGs relative to the background transcriptome. For the purpose of the enrichment analysis, GO categories with a Benjamini-corrected *P*-value of ≤0.05 were considered significant (Huang *et al.* 2009). The RNA-Seq reads used in this study have been submitted to the NCBI Gene Expression Omnibus (GEO) database under Accession no. GSE76976

Results

To better understand the molecular response of coral larvae to a competent *Symbiodinium* strain, host gene expression changes were determined when planula larvae of *Acropora digitifera* were exposed to a clade B *Symbiodinium* strain. The progress of the infection was monitored by fluorescence microscopy over the course of the experiment. At 4, 12 and 48 h post-infection, gene expression profiles were compared between *Symbiodinium*-infected and uninfected coral larvae. Sequencing yielded an average of 20.8 million individual paired-end reads per library and an average of 36% of the raw reads were mapped onto the *A. digitifera* transcriptome (see Tables S1 and S2, Supporting information). Hierarchical clustering analysis of transcript expression values revealed a high level of agreement between the biological replicates, as can be seen in the heat map of the pairwise Spearman correlations between samples in Fig. S1 (Supporting information). Moreover, multidimensional scaling (based on the 500 genes that best differentiate the samples) separates the *Symbiodinium*-infected and control

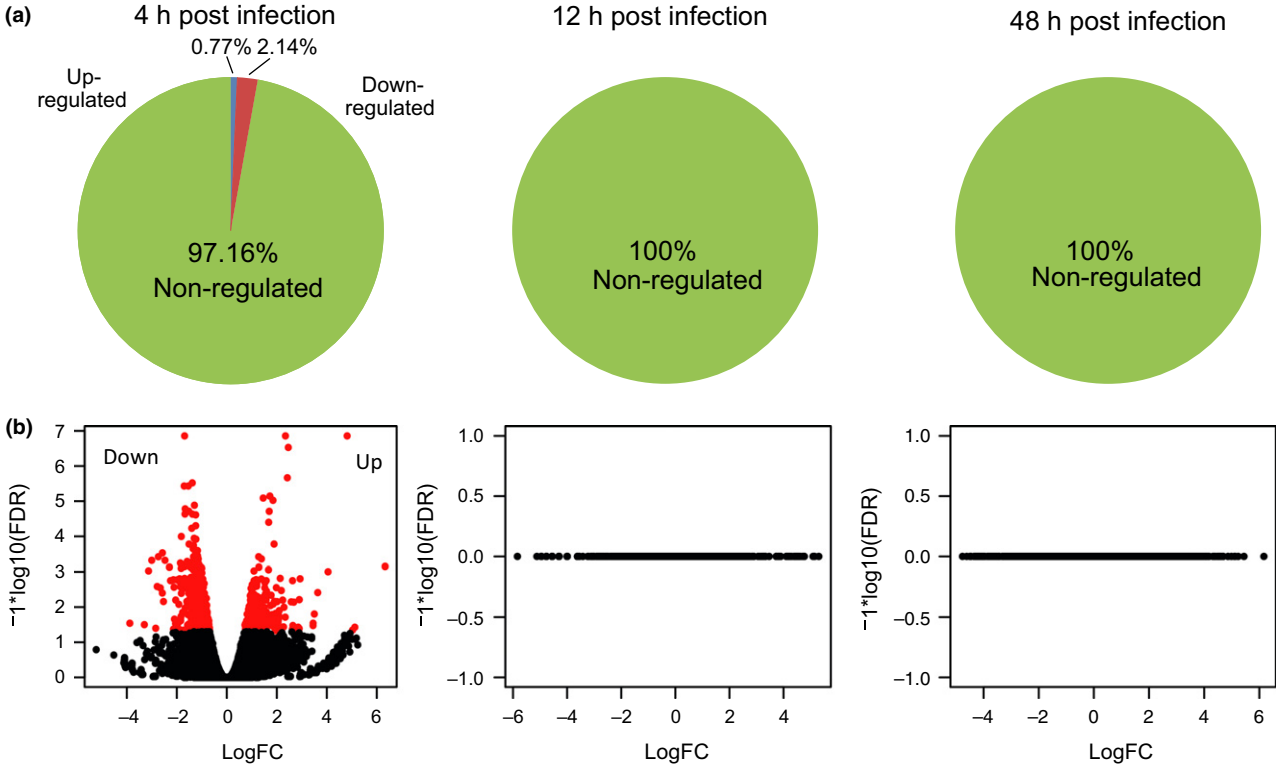
samples along the BCV distance 1 (Fig. S2, Supporting information).

Overall transcriptomic response

Results, summarized as Fig. 1, indicate a transient and relatively small transcriptomic response by the coral larvae early in the infection process. Significant differences in host gene expression were detected only at 4 h post-infection. At this time, 1073 (2.91%) of the *A. digitifera* contigs were differentially expressed, of which 788 (2.14%) were down- and 285 (0.77%) up-regulated (adjusted *P*-value ≤0.05). The range of $\log_2$ fold changes and false discovery rate data are summarized in Fig. 1. Using the criteria above, no significant differences between the infected and uninfected controls were detected at the subsequent time points (12 and 48 h post-infection; Fig. 1). Hierarchical clustering of the significant DEGs at the 4-h time point revealed distinctive expression profiles for the *Symbiodinium*-infected and uninfected larvae (Fig. S3, Supporting information).

Gene Ontology (GO) enrichment analysis reveals suppression of ribosomal and mitochondrial functions

As a preliminary approach to data analysis, the DEGs (adjusted *P*-value ≤0.05) were annotated using the GO database. Over-representation of GO terms amongst



(c)

Coral transcripts	DEGs	Up-regulated	Down-regulated	DEGs logFC>1	Swiss-Prot-annotated DEGs
36 780	1073 2.91%	285 0.77%	788 2.14%	625 58.3% DEGs	335

Fig. 1 Changes in the *Acropora digitifera* transcriptome during the onset of symbiosis with *Symbiodinium* sp clade B1 (*S. minutum*; culture ID= CCMP3345). (a) Percentages of transcripts up- (blue) and down-regulated (red) in response to *Symbiodinium* infection. A relatively small number of genes were differentially expressed at 4 h (adjusted $P \leq 0.05$), while no significant differences were observed in the host transcriptome at 12 and 48 h post-infection. (b) Volcano plots for the gene expression profiles in part (a) of this figure, showing false discovery rate ($-\log_{10}$FDR) as a function of $\log_2$ (fold change). The red dots represent the differentially expressed transcripts at adjusted $P \leq 0.05$. (c) Summary of the differential gene expression profile at the 4-h time point. An adjusted *P*-value ≤0.05 and an *e*-value of $\leq 10^{-15}$ were used to filter differentially expressed genes (DEGs) and in BLASTX searches against the Swiss-PROT database, respectively.

DEGs was used to infer molecular function (GO-MF), cellular component (GO-CC) and biological process (GO-BP) categories that were most affected during *Symbiodinium* infection. Of the DEGs, 625 (58.3%) had an absolute $\log_2$ fold change >1, of which 335 had reliable Swiss-PROT annotations (BLASTX $e \leq 10^{-15}$) (Fig. 1) that could be used to characterize the transcriptomic response of *A. digitifera* during onset and establishment of symbiosis with a competent strain of *Symbiodinium*.

Using a corrected *P*-value ≤0.05, no significant GO enrichment related to biological process, molecular function or cellular component could be detected amongst up-regulated genes. The GO-MF terms 'voltage gated channel activity' (GO:0022832) and 'voltage gated ion-channel activity' (GO:0005244) were over-represented amongst up-regulated genes, but with a slightly larger corrected *P*-value (0.058) than the threshold applied (Table S3, Supporting information). Amongst down-regulated genes, significant enrichment was detected in the GO terms 'cellular component' and 'molecular function', but not in 'biological process'. Amongst down-regulated genes, five of the seven over-represented GO-MF terms relate in some way to membrane transport, although the range of transporter activity types involved was very broad (Tables 2 and S3, Supporting information).

In the case of the cellular component (GO-CC) classification, enrichment was observed in relation to 17 terms (Table 2), many of which relate to either mitochondrial (8) or ribosomal (6) functions (Tables S4 and S5, Supporting information). Overall, these data imply that protein synthesis and oxidative metabolism are likely to be temporarily suppressed during the initial interaction with competent *Symbiodinium*. Although there are no direct precedents for this, metabolic suppression is a common feature of the responses of corals to stress (see, for example, Moya *et al.* 2012).

Despite being comprised of only three genes, amongst down-regulated genes, the most highly over-represented (44.91-fold) term was that associated with

Table 2 Gene Ontology (GO) categories (GO-MF, CC) which were enriched (with corrected *P*-value ≤0.05 in the down-regulated DEGs)

	Annotation Term	Gene Ontology (GO) ID	No. of genes	Fold enrichment
Down-regulated cellular components (GO_CC)	Ribonucleoprotein complex	GO:0030529	28	4
	Ribosome	GO:0005840	24	7.11
	Organelle membrane	GO:0031090	21	2.09
	Organelle envelope	GO:0031967	16	2.40
	Envelope	GO:0031975	16	2.37
	Organelle inner membrane	GO:0019866	13	3.76
	Mitochondrial membrane	GO:0031966	13	3.10
	Mitochondrial envelope	GO:0005740	13	2.86
	Mitochondrial inner membrane	GO:0005743	12	3.55
	Ribosomal subunit	GO:0033279	10	7.04
	Cytosolic part	GO:0044445	9	6.65
	Respiratory chain	GO:0070469	8	11.97
	Cytosolic ribosome	GO:0022626	8	9.03
	Small ribosomal subunit	GO:0015935	7	10.22
	Cytosolic small ribosomal subunit	GO:0022627	6	14.37
	Mitochondrial membrane part	GO:0044455	5	8.31
	Rough endoplasmic reticulum membrane	GO:0030867	3	44.91
Down-regulated molecular functions (GO_MF)	Structural constituent of ribosome	GO:0003735	24	9.41
	Structural molecule activity	GO:0005198	24	5.24
	Hydrogen ion transmembrane transporter activity	GO:0015078	6	8.63
	Monovalent inorganic cation transmembrane transporter activity	GO:0015077	6	7.39
	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	GO:0015450	4	30.69
	Protein transmembrane transporter activity	GO:0008320	4	30.69
	Macromolecule transmembrane transporter activity	GO:0022884	4	30.69

the GO-CC category 'rough endoplasmic reticulum membrane'. In addition to the three genes associated with the GO-CC category, five other genes potentially involved in ER functions were identified by manual inspection of the data, all of which were down-regulated (Table S6, Supporting information).

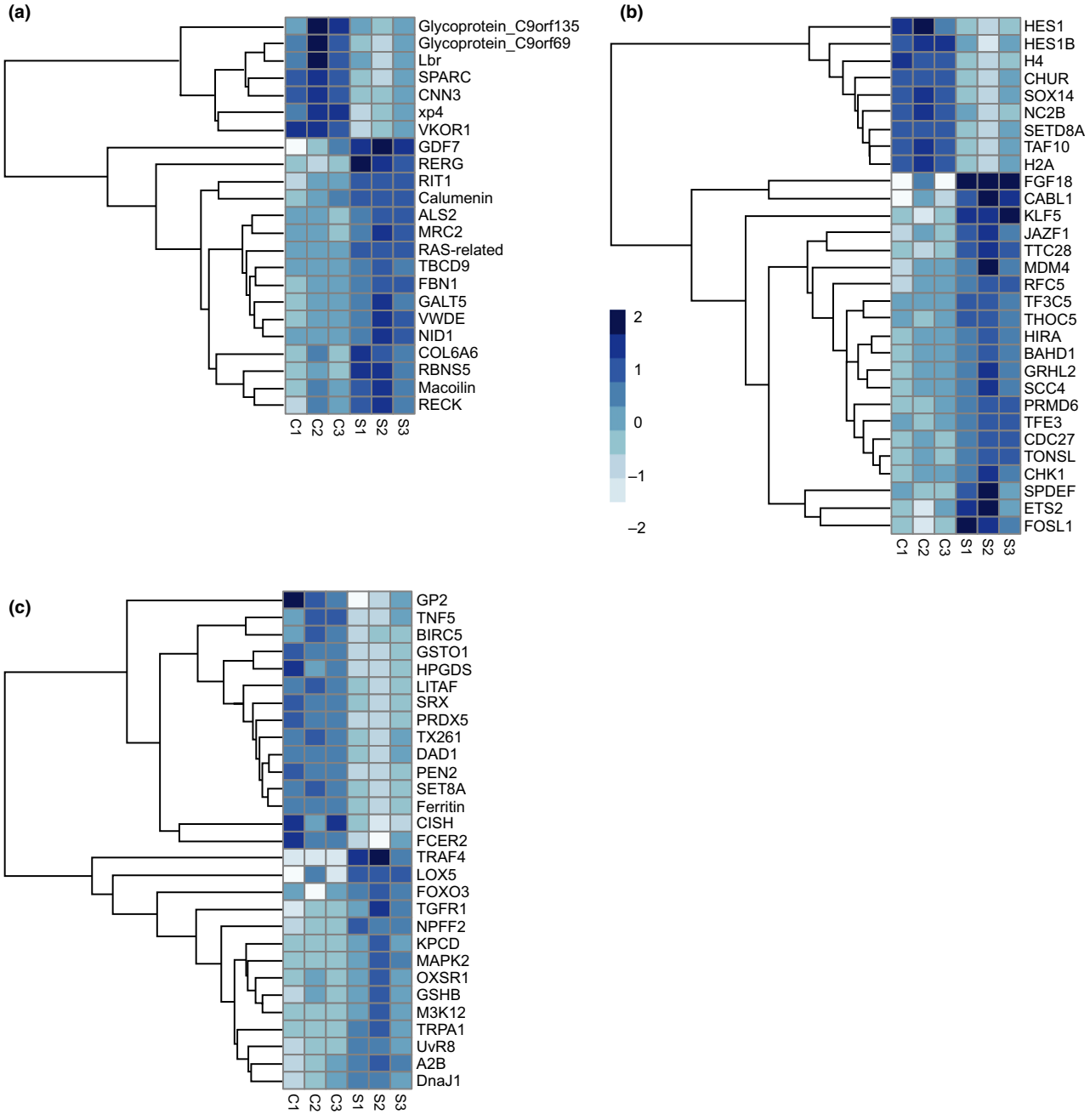


Fig. 2 Heat maps of specific categories of genes that were differentially expressed in *Symbiodinium*-infected (S1, S2, S3) and control larvae (C1, C2, C3) at the 4-h time point. (a) Genes likely to be involved in pattern recognition, cell adhesion and vesicle trafficking. (b) Genes likely to be involved in transcriptional control and cell cycle regulation. (c) Genes likely to be involved in immunity, apoptosis and stress responses. The blue scale represents the relative expression values as $\log_2$ (fold change). The hierarchical clustering shown was obtained by comparing the expression values (fragments per kilobase of transcript per million; FPKM) for *Symbiodinium*-infected samples against the control at 4 h post-infection. Expression values are $\log_2$-transformed and then median-centred by transcript. The heat maps are shown directly as generated using the R package pheatmap (https://cran.r-project.org/web/packages/pheatmap/index.html).

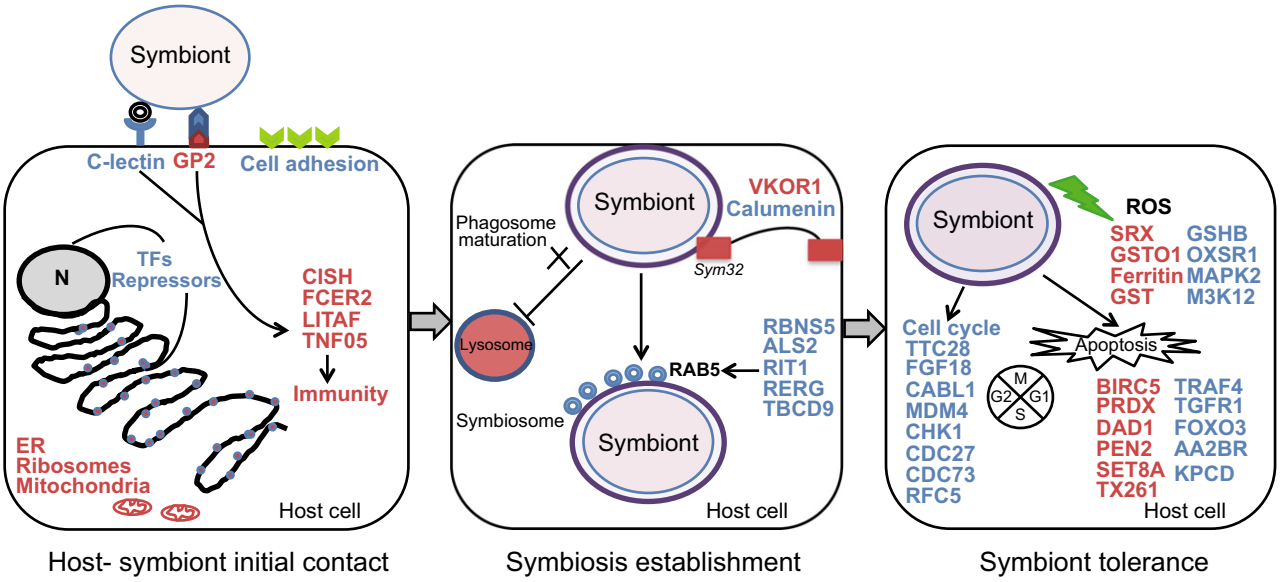


Fig. 3 An integrative model summarizing the roles of genes discussed in the text during the establishment and onset of symbiosis in larvae of *Acropora digitifera*. Up-regulated genes and functions are in blue text while genes that were down-regulated are in red. The initial uptake process (left panel) involves differential expression of a number of cell adhesion genes, including the up-regulation of a C-type lectin and down-regulation of the GP2 glycoprotein, as well as suppression of immunity and translation and mitochondrial processes. The establishment phase (central panel) involves blocking the maturation of the early phagosome containing the symbiont. Rabenosyn 5 and the Rab5-specific GEF ALS2 are likely to prevent the displacement of RAB5 by RAB7 and hence interfere with phagosome maturation. Differential expression of vitamin K epoxide reductase (VKOR) and calumenin is significant because both are thought to modify Sym32, a symbiosis-specific fasciclin (Ganot *et al.* 2011). The symbiont tolerance phase (right panel) involves complex changes required to handle the production of reactive oxygen species (ROS) by the symbionts, as well as transient modifications in cell cycle regulation and the apoptotic network.

Symbiosis-related genes

The GO database is strongly biased towards well-described processes in model organisms, limiting its usefulness in interpreting the identities of genes involved in coral–*Symbiodinium* interactions. Consequently, another phase of data analysis was undertaken. Those annotated transcripts differentially expressed at 4 h post-infection were grouped into the following categories based on literature searches highlighting functions likely involved in the coral- *Symbiodinium* symbiosis: recognition receptors, cell adhesion, symbiosome formation, regulation of transcription, cell cycle, innate immunity, apoptosis and stress responses (Figs 2 and 3).

Pattern recognition, cell adhesion and vesicle trafficking

The initial interaction with *Symbiodinium* must involve pattern recognition and cell adhesion molecules (Weis *et al.* 2008; Davy *et al.* 2012), hence genes involved in these processes are of particular interest in the context of symbiosis. Pattern recognition molecules or pattern recognition receptors (PRRs) recognize specific molecules (known as microbe-associated molecular patterns; MAMPs), such as peptidoglycans, glycans and lipopolysaccharides, which are present on the surfaces of microbes. MAMP–PRR interactions initiate signalling pathways that ultimately either attempt to eliminate pathogens or lead to tolerance of mutualistic organisms. In the case of cnidarian–*Symbiodinium* interactions, lectins are known to be involved (Lin *et al.* 2000; Wood-Charlson *et al.* 2006) although, except in the case of the Kvennefors *et al.* (2008) study, the identities of the interacting molecules are unclear. In this context, the observed up-regulation of the mannose receptor 2 (MRC2) gene (a C-type lectin) at the 4-h time point (Fig. 2a, Table 3) may be particularly significant.

Six proteins potentially involved in cell adhesion were differentially expressed at the 4-h time point (Fig. 2a, Table 3); four of these (fibrillin-1 [FBN1], nidogen-1 [NID1], collagen alpha-6 chain [CO6A6] and calumenin [calumenin]) were up-regulated, while vitamin K epoxide reductase complex subunit 1 (VKOR) and calponin-3 (CNN3) were down-regulated. Calumenin and VKOR participate in regulating the gamma-carboxylation of a range of specific targets which include fasciclin I proteins (Coutu *et al.* 2008). The differential expression of calumenin and VKOR is significant in the context of

Table 3 Differential expression of *Acropora digitifera* DEGs (n = 17) (FDR ≤0.05) likely involved in cell adhesion and pattern recognition. Note that data for these genes are also shown in Fig. 2a

Transcript ID	Best BLAST hit	UniProt ID	E-Value	logFC
adi_EST_assem_3458	FBN1_fibrillin-1 (*Bos taurus*)	P98133	7.36E-23	2.60
adi_EST_assem_9121	GDF7 (*Homo sapiens*)	Q7Z4P5	9.03E-22	1.94
adi_EST_assem_9676	C-type mannose receptor 2_ MRC2 (*Mus musculus*)	Q64449	5.62E-18	1.17
adi_EST_assem_5646	von Willebrand factor d and EGF domain-containing protein (*H. sapiens*)	Q8N2E2	4.40E-41	1.08
adi_EST_assem_3713	Macoilin-transmembrane protein 57 (*M. musculus)*	Q7TQE6	9.51E-161	1.08
adi_EST_assem_5440	Reversion-inducing cysteine-rich protein with kazal motifs_ RECK (*H. sapiens*)	O95980	0	1.20
adi_EST_assem_9498	Polypeptide n-acetylgalactosaminyltransferase GALT5 (*Drosophila melanogaster*)	Q6WV17	2.36E-171	1.17
adi_EST_assem_8472	Nidogen-1_ NID1 (*H. sapiens*)	P14543	3.77E-88	1.77
adi_EST_assem_5774	Collagen alpha-6 chain_ CO6A6 (*H. sapiens*)	A6NMZ7	2.87E-67	1.12
adi_EST_assem_9424	Calumenin_Calu (*H. sapiens*)	O43852	2.68E-51	1.05
adi_EST_assem_2644	Uncharacterized protein C9orf135 (*H. sapiens*) glycoprotein	Q5VTT2	1.71E-46	−1.20
adi_EST_assem_8996	upf0565 protein c2orf69 homolog (*Danio rerio*) glycoprotein	A0JMH2	3.15E-67	−1.20
adi_EST_assem_7512	Osteonectin-related_SPARC (*Caenorhabditis elegans*) |	P34714	1.37E-39	−1.20
adi_EST_assem_10377	Lamin-b receptor_ LBR (*M. musculus*)	Q3U9G9	2.05E-04	−1.10
adi_EST_assem_1377	Gastrointestinal growth factor XP4 (*Xenopus laevis*)	Q00223	1.06E-15	−1.70
adi_EST_assem_17327	Vitamin k epoxide reductase complex subunit 1 VKOR1 (*B. taurus*)	Q6B4J2	1.21E-23	−1.20
adi_EST_assem_5414	Calponin-3_CNN3 (*Rattus norvegicus*)	P37397	2.18E-19	−1.10

Table 4 Up-regulation of *Acropora digitifera* DEGs (n = 6) (FDR ≤0.05) likely involved in symbiosome formation. Note that data for these genes are also shown in Fig. 2a; see also the central panel of Fig. 3 ('symbiosis establishment')

Transcript ID	Best BLAST hit	UniProt ID	E-Value	logFC
adi_EST_assem_26789	Ras-related and oestrogen-regulated growth inhibitor_ RERG (*Bos taurus*)	Q0VCJ7	1.41E-15	1.8
adi_EST_assem_22740	RBNS5 (*Mus musculus*)	Q80Y56	8.21E-85	1.63
adi_EST_assem_27206	GTP-binding protein RIT1 | Ras-like without CAAX (*M. musculus*)	P70426	8.52E-39	1.37
adi_EST_assem_12817	ALS2 (*Rattus norvegicus*)	P0C5Y8	0.00E00	1.07
adi_EST_assem_5272	Ras-related and estrogen-regulated growth inhibitor RERG (*M. musculus*)	Q8R367	1.99E-33	1.04
adi_EST_assem_559	TBC1 domain family member 9_ TBCD9 (*H. sapiens*)	Q6ZT07	0.00E00	1

the establishment of symbiosis because it has been suggested (Ganot *et al*. 2011) that both are involved in modifying the adhesion protein Sym32, a cnidarian fasciclin thought to play a key role in host–*Symbiodinium* interactions.

In most cases, *Symbiodinium* cells are initially acquired through phagocytosis, but competent strains evade the normal process by which late phagosomes fuse with lysosomes, instead forming the stable symbiosome structure. Consistent with the idea that the symbiosome is derived from an early phagosome (Davy *et al*. 2012), the expression of six genes encoding proteins with known or potential roles in vesicle trafficking was up-regulated (Fig. 2a, Table 4, symbiosome formation) in response to *Symbiodinium* infection. This category of genes comprises two distinct members of the Ras superfamily of GTPases, the four others (amyotrophic lateral sclerosis2 [ALS2], GTP-binding protein RIT1, TBC1 domain family member 9 [TBCD9] and rabenosyn-5 [RBNS5]) potentially having effector activities on Rab proteins.

Transcriptional control and cell cycle regulation

Complex changes occur amongst the transcription regulatory machinery during the initial interaction with competent symbionts (Fig. 2b, Table S7, Supporting information). Some of the changes observed are consistent with a general suppression of replication (down-regulation of the core histones H2A and H4 and of the lysine methyltransferase SET8A/SETD8, whose activity is required for cell cycle progression). Other data support suppression of transcription, that is down-regulation of the TFIID subunit TAF10 and the

up-regulation of the transcriptional repressor PRDM6. However, other data are inconsistent with this; for example, the observed down-regulation of NC2B, a negative regulator of transcription and up-regulation of HIRA, which is a highly conserved chaperone that places H3.3 in nucleosomes (H3.3 is typically associated with active chromatin).

Similarly complex changes occur amongst genes regulating the cell cycle. In addition to SET8A/SETD8 down-regulation (see above), ten genes involved in the control of cell proliferation and at cell cycle checkpoints were up-regulated during the interaction with symbionts (Fig. 2b, Table S8, Supporting information).

Immunity, apoptosis and stress responses

Previous studies suggest that the host immune response may be suppressed during establishment of coral–algal symbiosis (Weis *et al.* 2008; Schnitzler & Weis 2010). Consistent with this idea, five genes that are likely to have immune-related functions were significantly down-regulated (Fig. 2c and Table 5, immune response) in *Symbiodinium*-infected larvae, including homologs of mammalian glycoprotein2 (GP2; zymogen granule membrane2), the low affinity immunoglobulin epsilon Fc receptor (FCER2) and two tumour necrosis factor (TNF) ligands; TNF superfamily member 5 (TNF5; CD40 ligand) and lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF)-alpha factor (LITAF).

Apoptosis of host cells is thought to have an important role in the specificity of cnidarian–dinoflagellate associations (Voolstra *et al.* 2009; Meyer & Weis 2012; Lehnert *et al.* 2014). Eleven genes with apoptosis-related functions were differentially expressed between *Symbiodinium*-infected and uninfected coral larvae, including the TRAF4 gene, which was up-regulated by 6.3-fold (Fig. 2c, Table 6). The down-regulation of the anti-apoptotic proteins baculoviral IAP repeat-containing protein 5 (BIRC5), and defender against cell death-1 (DAD1) observed in *Symbiodinium*-infected larvae suggests that cells may be more sensitive to apoptotic stimuli during the infection process.

Photosynthesis by *Symbiodinium* imposes oxidative stress on the host cells, and the symbiotic states of cnidarians are characterized by elevated expression of antioxidant activities (Richier *et al.* 2005). Consistent with this, six genes involved in oxidative stress

Table 5 Down-regulation of *Acropora digitifera* DEGs ($n = 5$) (FDR ≤0.05) likely involved in immune response. Note that data for these genes are also shown in Fig. 2c. See also Fig. 3 left panel

Transcript ID	Best BLAST hit	UniProt ID	E-Value	logFC
adi_EST_assem_1403	Glycoprotein 2 (zymogen granule membrane) GP2 (*Homo sapiens*)	P55259	2.00E-22	−2.1
adi_EST_assem_22228	Cytokine-inducible SH2-containing protein_ CISH (*H. sapiens*)	Q9NSE2	1.85E-15	−1.83
adi_EST_assem_5336	Low affinity immunoglobulin epsilon Fc receptor_ FCER2 (*Mus musculus*)	P20693	1.09E-15	−1.4
adi_EST_assem_16400	CD40 ligand | TNF05_Tumor necrosis factor ligand superfamily member 5 (*Gallus gallus*)	Q9I8D8	3.00E-95	−1
adi_EST_assem_4310	Lipopolysaccharide (LPS)-induced tumour necrosis factor (TNF)-alpha factor homolog_ LITAF (*G. gallus*)	Q8QGW7	1.44E-23	−1.04

Table 6 Differential expression of *Acropora digitifera* DEGs ($n = 11$) (FDR ≤0.05) likely involved in regulation of apoptosis. Note that data for these genes are also shown in Fig. 2c. See also Fig. 3, right panel ('symbiont tolerance')

Transcript ID	Best BLAST hit	UniProt ID	E-Value	logFC
adi_EST_assem_30449	TRAF4 (*H. sapiens)*	Q9BUZ4	9.67E-47	6.3
adi_EST_assem_26435	Adenosine receptor a2b_ AA2BR (*Gallus gallus*)	O13076	1.10E-17	1.2
adi_EST_assem_8165	FOXO3 (*H. sapiens*)	O43524	2.80E-30	1.58
adi_EST_assem_19964	TGFR1 (*Sus scrofa*)	Q5CD18	1.94E-173	1.9
adi_EST_assem_4877	protein kinase c delta type_ KPCD (*Rattus norvegicus*)	P09215	00E00	1.3
adi_EST_assem_15760	BIRC5 (*Felis catus*)-Apoptosis Inhibitor	Q6I6F4	5.05E-36	−1.03
adi_EST_assem_7793	Peroxiredoxin-mitochondrial_ PRDX5 (*H. sapiens*)	P30044	6.09E-59	−1.2
adi_EST_assem_9908	DAD1 (*S. scrofa*)	Q29036	2.39E-51	−1.03
adi_EST_assem_17635	Gamma-secretase subunit PEN2 (*Danio rerio*)	Q8JHF0	6.96E-45	−1.12
adi_EST_assem_7152	Histone-Lysine N-methyltransferase SET8A (*Xenopus laevis*)	Q08AY6	6.74E-56	−1.04
adi_EST_assem_18666	protein testis expressed tex261_TX261 (*R. norvegicus*)	Q5BJW3	3.17E-53	−1

responses were differentially expressed at 4 h post-infection (Table S9, Supporting information). Glutathione metabolism appears to be of particular importance during infection, as a glutathione synthetase gene (GSHB) was up-regulated whereas a glutathione S-transferase (GST01) was down-regulated (Fig. 2c, Table S9, Supporting information). Three protein kinases likely involved in the response to stress were also amongst the DEGs.

Discussion

Whereas previous studies detected differential expression of very few genes during infection of a coral with a competent *Symbiodinium* strain (Voolstra *et al.* 2009; Schnitzler & Weis 2010), in the work described here 1073 genes were differentially expressed when larvae of *Acropora digitifera* were exposed to clade B *Symbiodinium* cells. A major factor in this difference is the use in the present case of high-throughput sequencing, rather than microarray technology, but timing is also clearly important. Differential gene expression was clearly observed only at 4 h post-infection, a time window not previously investigated. The lack of differential expression at the 12-h and 48-h time points observed in the present case is consistent with previous work on cnidarian–algal symbioses (Rodriguez-Lanetty *et al.* 2006; Ganot *et al.* 2011; Lehnert *et al.* 2014) as well as on other mutualistic associations (Dale & Moran 2006; Chun *et al.* 2008; Heller *et al.* 2008). For example, a similar number of host genes (781) was differentially expressed during the establishment of the symbiosis between the squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri* (Chun *et al.* 2008).

Metabolic suppression during the initial interaction between coral and Symbiodinium

Most DEGs (73.4%) were down-regulated and GO analysis revealed that many of these encoded proteins associated with ribosomes, the rough endoplasmic reticulum and the respiratory chain/mitochondria. For example, 22 ribosomal proteins and a probable translation initiation factor were down-regulated. While these results imply that translation is suppressed, the down-regulation of RNA polymerase subunits and up-regulation of at least five transcriptional repressors imply that there may also be an overall down-regulation of transcription during the initial coral–*Symbiodinium* interaction. Meyer *et al.* (2011) reported that the long-term exposure of *Acropora millepora* larvae to elevated temperatures resulted in down-regulation of ribosomal proteins. In the case of the Caribbean coral *Orbicella* (*Montastraea) faveolata*, down-regulation of protein synthesis has also been observed during thermal stress and bleaching (DeSalvo *et al.* 2008).

Mitochondrial metabolism appears to be specifically down-regulated during the interaction, as GO terms related to mitochondria, mitochondrial membranes and respiratory chains were enriched. The expression of many genes coding for proteins involved in mitochondrial metabolism was suppressed, as was expression of Tom7, a key component of the complex that transports proteins into mitochondria. It is unclear why such suppression of mitochondrial metabolism should occur. Although there are no direct precedents for this, Moya *et al.* (2012) reported a similar suppression when *A. millepora* juveniles were exposed to elevated levels of CO_2. In the case of the Moya *et al.* study, it was suggested that acute suppression of oxidative metabolism might enable the redistribution of energy to stress and immune responses, but in the present study suppression occurs in the absence of an immune response.

Pattern recognition, cell adhesion and symbiont recognition

In the case of symbiotic cnidarians, in addition to roles in pathogen recognition, it is likely that specific PRRs interact with potential symbionts (Weis *et al.* 2008). Other symbiotic associations provide precedents for this – for example, associations of symbiotic squid with bacteria (Nyholm & McFall-Ngai 2004) and plants with nitrogen-fixing bacteria (Cullimore & Denarie 2003) have been found to be initiated through MAMP–PRR interactions between host and microbes. The first coral PRR to be identified is a distinct C-type lectin protein known as 'Millectin' (Kvennefors *et al.* 2008). This protein family includes PRRs that recognize mannose and fucose residues on bacterial or eukaryotic glycoproteins (Stahl & Ezekowitz 1998). This protein has been shown to bind to both the pathogenic bacterium *Vibrio coralliilyticus* and the dinoflagellate *Symbiodinium*, indicating a dual function in recognizing both pathogenic and mutualistic microorganisms (Kvennefors *et al.* 2008, 2010). Although in the present case Millectin was not differentially expressed, another C-type lectin, a homolog of the mammalian mannose receptor 2 (MRC2), was highly up-regulated during the infection process. Consistent with a role in recognition in corals, MRC2 was one of four macrophage receptors that were up-regulated in *Acropora cervicornis* infected with white band disease (Libro *et al.* 2013). There are precedents for the observed up-regulation of C-type lectin genes during onset of cnidarian–dinoflagellate symbiosis (Grasso *et al.* 2008; Schwarz *et al.* 2008; Sunagawa *et al.* 2009).

Previous work directly investigated the role of glycan–lectin interactions during establishment of the

cnidarian–dinoflagellate symbiosis. Lin *et al.* (2000) and Wood-Charlson *et al.* (2006) altered *Symbiodinium* cell surfaces by enzymatic removal of glycans and by glycan masking before algae were inoculated into aposymbiotic hosts (using the anemone *Aiptasia pulchella* and the mushroom coral *Fungia scutaria*, respectively). In both studies, glycan removal significantly decreased infection success, measured in *A. pulchella* by quantifying algal cells per tentacle and in *F. scutaria* larvae by quantifying both the percentage of larvae infected and the density of *Symbiodinium* in larvae.

Two other DEGs of particular interest in the context of symbiont recognition are calumenin and VKOR, as the corresponding proteins are thought to interact with fasciclins.

A fasciclin I protein known as Sym32 was first described in the sea anemone *Anthopleura elegantissima* (Reynolds *et al.* 2000), and Sym32 homologs are highly expressed in the symbiotic state relative to the aposymbiotic state in *A. elegantissima* (Weis & Levine 1996; Reynolds *et al.* 2000). Sym32 was localized to the symbiosome surrounding resident *Symbiodinium* in *A. elegantissima* and it was hypothesized that this protein functions in signalling between host and alga (Schwarz & Weis 2003). In *Anemonia viridis*, calumenin was highly expressed in the tissues harbouring *Symbiodinium* (Ganot *et al.* 2011). In mammals, calumenin and VKOR participate in regulating the gamma-carboxylation of a range of specific targets which includes fasciclin I proteins (Coutu *et al.* 2008). Assuming that the adhesion protein Sym32 is important in host–symbiont interactions, the differential expression of calumenin and VKOR is significant in the context of the establishment of symbiosis because it has been suggested that both are involved in modifying the adhesion protein Sym32 (Ganot *et al.* 2011). In *Anemonia viridis* both calumenin and Sym32 are highly expressed in symbiotic anemones relative to aposymbiotic individuals (Ganot *et al.* 2011). Note that while the potential modifiers VKOR and calumenin were differentially expressed during our *A. digitifera* infection experiment, Sym32 was not, although Sym32 has been reported in a range of corals, including *A. digitifera* (Meyer & Weis 2012).

The symbiosome as an arrested phagosome

Symbiosomes, where competent *Symbiodinium* cells reside, are thought to be derived from the plasma membrane of the host cell via phagocytosis, but the resulting structures evade the process of phagosome maturation that would normally result in fusion with lysosomes (Desjardins *et al.* 1994). Phagosomal maturation is a conserved process that involves the sequential acidification of the vesicle and the recruitment of particular proteins, including specific Rab GTPases, onto the phagosomal membrane at precise times (Kinchen & Ravichandran 2008). The normal process of endocytosis involves internalization and fusion with endosomes, resulting in the formation of an early phagosome that is marked by the presence of Rab5, a protein critical to endocytic trafficking (Gruenberg & van der Goot 2006). Maturation to a late phagosome involves loss of Rab5 and recruitment of Rab7, a process brought about by Mon1/SAND-1 (Poteryaev *et al.* 2010). Rab7 alone is unable to execute the early/late phagosome transition; other components required include the LAMP-1 and -2 proteins. The content of the late phagosome is in the range pH 4.5 – 5, and its fusion with lysosomes brings about the digestion of the contents (Luzio *et al.* 2007).

To persist within macrophages, various intracellular protozoan parasites, including apicomplexans (Sibley 2011), interrupt host membrane trafficking, the net result being that host lysosomes fail to fuse with the vacuoles in which the parasites are housed. Data for the symbiotic sea anemone *Aiptasia pulchella* suggest that *Symbiodinium* cells also evade digestion by interrupting phagosome maturation; orthologs of several Rab proteins localize in specific patterns (Chen *et al.* 2003, 2004, 2005; Hong *et al.* 2009a) that are consistent with the idea that the symbiosome is an arrested phagosome. The *Aiptasia* homolog of Rab5 (ApRab5) is present on vesicles containing either recently ingested or resident *Symbiodinium* (Chen *et al.* 2004), as is the Rab4 homolog ApRab4 (Hong *et al.* 2009b) but, after killing the symbionts with heat or blocking photosynthesis, staining for both Rab4 and Rab5 is lost (Hong *et al.* 2009b). The sea anemone Rab7 homolog (ApRab7) essentially has a reciprocal relationship with ApRab4/ApRab5 in that it was detected on phagosomes harbouring heat-killed or photosynthetically impaired *Symbiodinium*, but not on phagosomes harbouring live *Symbiodinium.* These results imply that in the sea anemone, *Symbiodinium* cells interrupt phagosomal maturation at the early stage by stably recruiting Rab4/5 and excluding Rab7. On the other hand, it has been shown that the mechanism that arrests phagosome maturation, permitting the establishment of symbiosis, was deactivated during thermally induced bleaching of *Pocillopora damicornis* (Downs *et al.* 2009). In this case, the symbiosome is transformed into a digestive organelle via fusion with host lysosomes, a process that involves recruitment of the late phagosome marker Rab7 and has been referred to as 'symbiophagy' (Downs *et al.* 2009).

In this context, the up-regulation of ALS2 and rabenosyn-5 expression observed here at an early stage in the infection of coral larvae with *Symbiodinium* is particularly significant. ALS2 acts as a specific GEF (guanosine nucleotide exchange factor) for Rab5 (Hadano *et al.*

2007), maintaining it in the active state and thus contributing to the stabilization of early endosomes. Rabenosyn-5 (RBNS5) acts early in mammalian endocytosis and plays a critical role in trafficking of endosomes for recycling (Navaroli *et al.* 2012), modulating the Rab5 GTPase activity by binding specifically to the active form of Rab5 and thus regulating the processes of docking and fusion of endosomal membranes, motility of endosomes and intracellular signal transduction. Four other genes that may be involved in endosome trafficking were significantly up-regulated in the larvae exposed to *Symbiodinium* (Table 6), however the literature on the mammalian orthologs of these is limited, thus their connection with symbiosome formation is more tenuous. The TBC1D9/MDR1 protein is thought to act as a Rab GTPase activator, RIT1 is a representative of a highly divergent Ras subfamily, and RERG (two different transcripts up-regulated) proteins are small Ras GTPases predicted to have higher affinity for GDP than GTP.

It appears that similar mechanisms operate during the infection of *Acropora* and *Aiptasia* by competent *Symbiodinium* strains, but whether these developed before the deep evolutionary divergence between corals and sea anemones (Shinzato *et al.* 2011) or by convergent evolution is unclear. Many parasites (including prokaryotes) survive in host cells by subverting phagocytosis, so some similarities in how this is achieved are inevitable.

The data presented here provide the first insight into transcriptional changes in the coral host during the initial interaction with a competent strain of *Symbiodinium*. Importantly, significant changes in gene expression were detected only very early in the infection process. The transcriptome data imply that complex changes occur in the coral during the interaction, including temporary suppression of protein synthesis/transport, mitochondrial metabolism and immune functions. The data presented imply that symbiont uptake by the coral host involves similar mechanisms to those used to identify microbial pathogens and that the symbiosome is an arrested phagosome in both corals and sea anemones. How the arrest occurs is unclear, and whether this is achieved by the same mechanism in corals and anemones remains to be established.

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A.R.M. and D.J.M. conceived and designed the experiments. A.R.M., S.H. and V.C. carried out the fieldwork at Sesoko Island Research Station, Okinawa. A.R.M. and C.S. carried out RNA isolation. C.S. and N.S. provided reagents and generated the RNA-Seq data. A.R.M. analysed the RNA-Seq data, with the assistance of C.X.C. and M.A.R. A.R.M., E.E.B., D.G.B. and D.J.M. interpreted the data and wrote the manuscript, with input from all other authors. All authors read the article and approved the final version.

Data accessibility

The RNA-Seq reads and count tables used for the differential expression studies have been submitted to the NCBI Gene Expression Omnibus (GEO) database under Accession no. GSE76976

Supporting information

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

Fig. S1 There is a high level of agreement amongst the biological replicates.

Fig. S2 A multidimensional scaling (MDS) plot produced by edgeR showing the relationship between all replicates of *Symbiodinium* sp. clade B infection (S1, S2, S3) and negative control condition (C1, C2, C3) at the 4 h time point.

Fig. S3 Infected and uninfected *Acropora digitifera* planulae have distinct differential gene expression profiles at 4 h post infection The heat map shows the expression profiles of *Symbiodinium* infected and control samples.

Table S1 Raw Illumina Hi-Seq sequencing reads.

Table S2 Percent of reads successfully mapped onto the *Acropora digitifera* transcriptome assembly.

Table S3 Differential expression of *Acropora digitifera* DEGs ($n = 19$) (FDR ≤ 0.05) likely involved in voltage gated (ion) channel activity and trans-membrane transporters.

Table S4 Down-regulation of *Acropora digitifera* DEGs ($n = 19$) (FDR ≤ 0.05) likely involved in mitochondrial functions.

Table S5 Down-regulation of *Acropora digitifera* DEGs ($n = 25$) (FDR ≤ 0.05) likely involved in protein synthesis and translation.

Table S6 Down-regulation of *Acropora digitifera* DEGs ($n = 8$) (FDR ≤ 0.05) likely involved in endoplasmic reticulum functions.

Table S7 Differential expression of *Acropora digitifera* DEGs ($n = 21$) (FDR ≤ 0.05) likely involved in transcription regulation.

Table S8 Up-regulation of *Acropora digitifera* DEGs ($n = 10$) (FDR ≤ 0.05) likely involved in cell cycle regulation.

Table S9 Differential expression of *Acropora digitifera* DEGs (FDR ≤ 0.05) likely involved in responses to stress.