View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Developmental Biology 353 (2011) 411–419

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Genomes & Developmental Control

The biology of coral metamorphosis: Molecular responses of larvae to inducers of settlement and metamorphosis

L.C. Grasso [a,1], A.P. Negri [b], S. Fôret [a,1], R. Saint [a,2], D.C. Hayward [a,d], D.J. Miller [c,*], E.E. Ball [a,d,**]

[a] Centre for the Molecular Genetics of Development, Molecular Genetics and Evolution Group, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra, ACT, 2612, Australia
[b] Australian Institute of Marine Science, PMB 3, Townsville MC, Townsville 4810, Australia, Queensland, Australia
[c] Centre for Molecular Genetics of Development and ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Qld 4811, Australia
[d] Evolution, Ecology and Genetics, Research School of Biology, Bldg 46, Australian National University, Canberra, ACT 0200, Australia

ARTICLE INFO

Article history:
Received for publication 9 August 2010
Revised 22 December 2010
Accepted 12 February 2011
Available online 19 February 2011

Keywords:
Coral
Acropora
Settlement
Metamorphosis
Microarray
LWamide

ABSTRACT

Like many other cnidarians, corals undergo metamorphosis from a motile planula larva to a sedentary polyp. In some sea anemones such as *Nematostella* this process is a smooth transition requiring no extrinsic stimuli, but in many corals it is more complex and is cue-driven. To better understand the molecular events underlying coral metamorphosis, competent larvae were treated with either a natural inducer of settlement (crustose coralline algae chips/extract) or LWamide, which bypasses the settlement phase and drives larvae directly into metamorphosis. Microarrays featuring >8000 *Acropora* unigenes were used to follow gene expression changes during the 12 h period after these treatments, and the expression patterns of specific genes, selected on the basis of the array experiments, were investigated by in situ hybridization. Three patterns of expression were common—an aboral pattern restricted to the searching/settlement phase, a second phase of aboral expression corresponding to the beginning of the development of the calicoblastic ectoderm and continuing after metamorphosis, and a later orally-restricted pattern.

© 2011 Elsevier Inc. All rights reserved.

Introduction

Sexual reproduction in reef-building corals gives rise to a motile planula larva, which must undergo a complex metamorphosis after location of an appropriate substrate, founding a juvenile coral colony (Fig. 1A–G). During the transition from motile planula to sessile polyp, two phases are distinguished—a searching/settlement phase, during which the planula is thought to use chemical cues to locate appropriate sites, and metamorphosis proper, which involves extensive tissue remodelling, particularly of the aboral ectoderm (Vandermeulen, 1975; Le Tissier, 1988; Clode and Marshall, 2004; Hirose et al., 2008).

The complex process of settlement and metamorphosis typical of corals is most likely a derived trait, the evolution of which was presumably driven by the need for a high level of specificity in the selection of appropriate settlement sites (Morse et al., 1988). Several species of crustose coralline algae (CCA) are capable of inducing settlement of larvae from three different coral families, implying the existence of a common mechanism (Morse et al., 1996), although brooding corals may not require settlement cues (Baird and Morse, 2004; Erwin and Szmant, 2010). In *Acropora millepora*, seven different CCA species are capable of inducing metamorphosis, as are some branching species of coralline algae and non-coralline crustose algae (Heyward and Negri, 1999). Methanolic CCA extracts and decalcified CCA are also effective inducers of metamorphosis in *A. millepora* (Harrington et al., 2004). In contrast with the complexity of these processes in corals, settlement and metamorphosis are non-specific and relatively simple in some sea anemones. For example, in *Nematostella vectensis* no specific settlement cues are required, and the transition from planula to polyp is continuous rather than discrete (Hand and Uhlinger, 1992; Müller and Leitz, 2002).

It is likely that the internal mechanism by which the metamorphosis signal is mediated is common across the Cnidaria. In both *Anthopleura elegantissima* (Anthozoa) and *Hydractinia echinata* (Hydrozoa), neuropeptides of the LWamide family are released following receipt of settlement cues by aboral sensory cells and act to initiate metamorphosis (reviewed by Müller and Leitz, 2002). In the case of *A. millepora*, in situ hybridization reveals LWamide-containing neurons (unpublished results), and metamorphosis can be induced by LWamide II from *Hydra* (Iwao et al., 2002). Treatment of

* Correspondence to: Comparative Genomics Centre and ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Qld. 4811, Australia. Fax: +61 7 47816078.
** Correspondence to: Evolution, Ecology and Genetics, Research School of Biology, Bldg 46, Australian National University, Canberra, ACT 0200, Australia. Fax: +61 2 61255095.
E-mail addresses: lauretta.grasso@jcu.edu.au (L.C. Grasso), a.negri@aims.gov.au (A.P. Negri), rsaint@unimelb.edu.au (R. Saint), david.hayward@anu.edu.au (D.C. Hayward), david.miller@jcu.edu.au (D.J. Miller), eldon.ball@anu.edu.au (E.E. Ball).
[1] Current address: ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Qld 4811, Australia.
[2] Current address: Faculty of Science, The University of Melbourne, Melbourne, Victoria 3010, Australia.

0012-1606/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2011.02.010

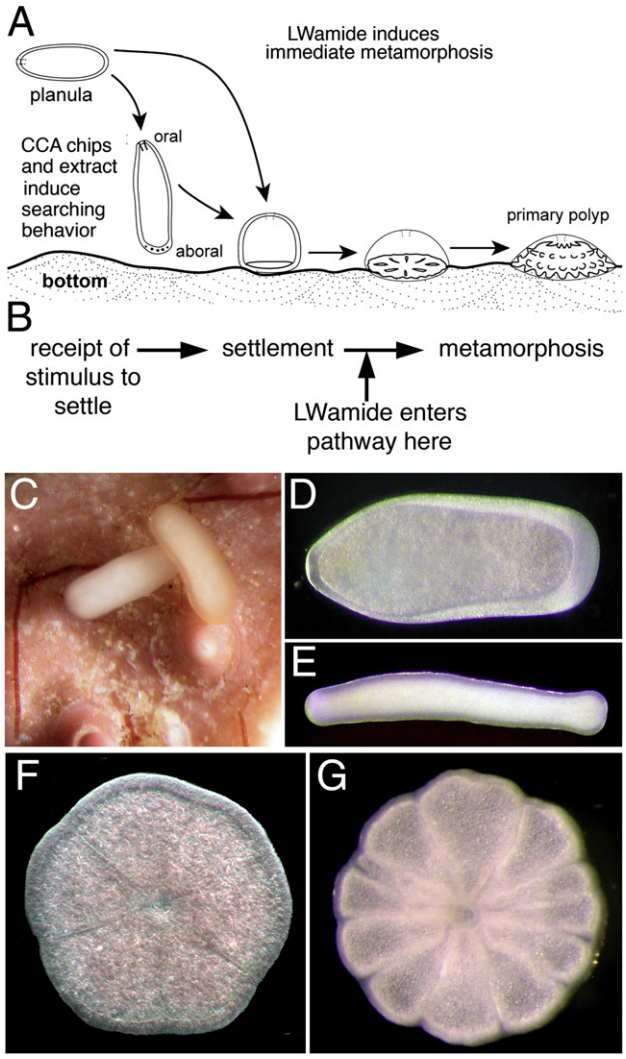

Fig. 1. (A) Schematic diagram of larval responses to crustose coralline algae (CCA) and LWamide. The responses of *Acropora* larvae differ markedly depending on the stimulus applied. CCA extract or chips initiate larval searching behavior prior to metamorphosis while LWamide initiates immediate metamorphosis. (B) LWamide is believed to act downstream of the settlement receptors to transmit the message to undergo metamorphosis. (C) Planulae performing searching behavior on a chip of CCA. (D) The swimming, pre-induction planula developmental stage. (E) An elongated planula following induction with CCA or CCA extract. (F and G) Juveniles after metamorphosis and settlement.

larvae with LWamide II appears to bypass the normal searching/settlement phase (Iwao et al., 2002), providing evidence for the discrete nature of settlement and metamorphosis (Fig. 1A and B).

Although the morphological changes that occur during metamorphosis are reasonably well understood, until very recently almost nothing was known about the molecular events underlying either settlement or metamorphosis in corals. A recent microarray study (Grasso et al., 2008) enabled the identification of a number of genes that are differentially expressed during the planula to polyp transition, as are three galaxin-related genes (Reyes-Bermudez et al., 2009a). Building on this work, here we report the transcriptional responses of competent *A. millepora* larvae to inducers of settlement and metamorphosis using arrays corresponding to 8606 unique cDNAs (previous analyses were based on 5081 unique cDNAs). CCA chips or extract were used to stimulate settlement/metamorphosis, and LWamide was used to drive larvae directly into metamorphosis. Based on predicted function and differential expression in the array analyses, genes were chosen for in situ hybridization analysis across the period during which settlement and metamorphosis occur. This led to the identification of three groups of genes characterized by their spatial expression patterns; "early aboral" genes, in which expression is predominantly at the aboral end of planulae and is temporally restricted to settlement/early metamorphosis, a "later aboral" group, expressed aborally in planulae and polyps both before and after metamorphosis, and a "later oral" group whose expression typically begins at the oral end once metamorphosis has commenced.

Materials and Methods

Microarray description

The array used for this experiment contained 18,432 spots including 96 cDNA clones derived from unfertilized eggs (all printed in duplicate), 3456 cDNA clones from the pregastrula, 4836 cDNA clones from the presettlement planula larva, 4128 cDNA clones from the postsettlement primary polyp, and 4800 cDNA clones derived from an adult colony bleached with diuron for the removal of *Symbiodinium* (Negri et al., 2005) from within the tissue (of which 672 were printed in duplicate). 348 spots were positive or negative controls. All of the material used for making the libraries came from Nelly Bay, Magnetic Island, Queensland, Australia (19°08′S 146°50′E). All cDNAs spotted onto the slides were derived from cDNA libraries of the appropriate developmental stage that had been constructed in Lambda ZAP (Stratagene). They were isolated by TempliPhi (GE Life Sciences) on excised clones except for 2000 postsettlement polyp clones which were PCR amplified directly from individual phage suspensions and 3012 planula larva cDNAs which were isolated as previously described (Kortschak et al., 2003).

Generation and spotting of cDNAs

PCR [1× HotMaster Taq Reaction buffer, 0.25 mM each dNTP, 25 pmol of each of M13 Forward and M13 Reverse primer and 1.25 U of HotMaster Taq Polymerase (Eppendorf) spiked with Pfu (Promega) in a 25 μl reaction] was used to generate DNA for spotting onto microarray slides. Phage suspension was used as template by adding 4 μl to the PCR mix. TempliPhi was used as a template by dipping a pin into the TempliPhi reaction and then into the PCR mix. PCR was carried out in 96-well plates (ABGene) under the following conditions: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min, for 30–35 cycles. PCR products were purified using 96 well Multiscreen plates (Millipore).

Microarrays were generated by spotting the amplified cDNA onto GAPSII slides using a Biorad Chipwriter Pro, and then fixed by UV light exposure (150 mJ) followed by baking at 80 °C for 3 h. All cDNA clones represented on the arrays were sequenced from the 5′ direction using standard Sanger (ABI Big Dye) sequencing technology. Sequences were run on an ABI 3730 sequencer at the Biomolecular Resource Facility, JCSMR, ANU.

EST analyses

After data filtering, ESTs were clustered using CAP3 (Huang and Madan, 1999), giving rise to 8606 unique genes (unigenes). The unigenes were then used to search the GenBank database using BlastX (Altschul et al., 1990) with a threshold of $e = 1 \times 10^{-5}$.

Experimental design

Larvae used in this experiment were the offspring of two colonies collected from Nelly Bay, Magnetic Island (19°08′S 146°50′E). Two samples of ~100 larvae, 8 days old, were collected and snap frozen in liquid nitrogen. The remaining larvae were placed in 150 ml filtered sea water (FSW) in 16×150 mm diameter polystyrene Petri dishes at a density of 1 larva/ml. At time point zero, the following different stimuli were added: LWamide, an oligopeptide of sequence EPLPIGLWa (Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University, Canberra) to a final concentration of 1 μM in filtered sea water (FSW); 150 μl ethanolic extract of the CCA

Neogoniolithon fosliei (Heydrich) prepared as per Harrington et al. (2004); 10×1 cm^2 chips of the *N. fosliei* and 150 µl ethanol as a negative control (0.1% v/v). There were two replicates of each stimulus. All experiments were run at 28 °C. Samples of ~50 larvae were taken at 0.5 h post induction (hpi), 4 hpi and 12 hpi and snap frozen in liquid nitrogen. cDNA for probing arrays was produced from unamplified total RNA which was extracted using TRI Reagent (Ambion) according to the manufacturer's instructions. The quality was assessed by running 1 µg on the Agilent 2100 Bioanalyzer. Total RNA was amplified with the Superscript Indirect RNA Amplification System (Invitrogen) and labeled according to the manufacturer's instructions.

Each sample was labeled with Cy3 and co-hybridized with the same quantity of a Cy5-labeled pooled common reference sample consisting of equal microgram amounts of amplified RNA from all samples. Each replicate consisted of 13 samples: planula larvae, LWamide 0.5 hpi, LWamide 4 hpi, LWamide 12 hpi, CCA extract 0.5 hpi, CCA extract 4 hpi, CCA extract 12 hpi, CCA chips 0.5 hpi, CCA chips 4 hpi, and CCA chips 12 hpi, control 0.5 hpi, control 4 hpi and control 12 hpi. A biological replicate was carried out; thus a total of 26 slides were used. Hybridization and washing were done according to standard protocols (http://www.microarray.adelaide.edu.au/) and the slides were scanned with a Genepix 4200A Scanner.

Data analysis and verification

Data were extracted from the slide images using Spot (http://www.cmis.csiro.au/iap/Spot/spotmanual.htm). All analyses were done using the limma package (Smyth, 2005) for the R system (http://www.r-project.org/). 'Normexp' background correction (Ritchie et al., 2007) and print-tip loess normalization (Smyth and Speed, 2003) were performed on each slide. The methodology used for statistical analysis is described in Smyth (2004). The prior probability of differential expression, for each pair of comparisons between stages, was taken as 0.1. The method of Benjamini and Hochberg (1991) was used to adjust the sequence-wise p-values, so that a choice of sequences for which the adjusted p-value is at most 0.05 identifies a set of differentially expressed genes in which 5% may be falsely identified as differentially expressed. For each stimulus, this method was applied to compare samples from each time point to samples from the preceeding timepoint.

Following statistical analysis, the data were separated into groups of interest, namely up- and down-regulated genes from each comparison (Supplementary Table 1). All array data have been deposited in the Gene Expression Omnibus (GEO) database, accession number GSE11709.

In situ hybridization

Templates for riboprobe production were generated by PCR or restriction digest. Riboprobe synthesis and in situ hybridization were performed as previously reported (Hayward et al., 2001). cDNA sequences for genes characterized by in situ hybridization have been deposited in GenBank. In the following list the cDNA sequence name is first, followed by the GenBank accesssion (C_A009-A3, HO088735; C_A036-C3, HO088736; S_A039-E10, HO088737; C_D009-G7, HO088738; C_D052-C12, HO088739; S_A045-H12, GQ228825; C_D034-A4 (D013-H3), GQ228826; C_D020-D3, GQ228827; S_D023-C7, GQ228828; C_A027-E11 (GS01TE02), GQ228829; C_A009-C1, (A040-H7), GQ228830 ; C_A049-A7 (A047-G9), GQ228831; C_A043-H1 (B041-G7), GQ228832 and C_A005-G11 (D021-G4), GQ228833). Clearing and photography were as described in de Jong et al (2006). The specimens used for in situ hybridization were undergoing normal settlement, with a searching/settling phase prior to metamorphosis proper.

5′RACE and 454/Illumina data

Where a cDNA clone was 5′ truncated despite having sequenced the entire plasmid insert, 5′RACE was used to isolate the missing 5′ end. This was done using the Clontech SMART RACE cDNA Amplification Kit according to the manufacturer's protocol. Clones in which 5′ RACE failed to extend were completed using 454/Illumina data (http://www.bio.utexas.edu/research/matz_lab/matzlab/454.html, and unpublished).

Results

Changes in morphology and gene expression occurring post-induction

In contrast to control larvae, which showed no changes in behavior, at 0.5 hpi with CCA chips or CCA extract, 100% of larvae had settled to the bottom of the dish (and some onto CCA chips if present) and were either showing 'searching' behavior by testing the substratum with the aboral end (Fig. 1C), or were thinner and more elongate than previously and were moving laterally along the substratum (cf. Fig. 1D and E). Although larvae appeared to respond more rapidly to CCA extract than to CCA chips (data not shown), at 4 hpi a higher proportion of those induced by CCA chips had undergone metamorphosis (70%) than in the case of the CCA extract (50%; Fig. 2). By 12 hpi, 90% of the larvae induced using CCA chips or extract had metamorphosed. As predicted, LWamide treatment appeared to bypass the seaching/settlement phase; at 0.5 hpi, all of the larvae exposed to LWamide had contracted along their long axis (forming a sphere) and by 4 hpi all had undergone metamorphosis. The morphological responses to LWamide and CCA based stimuli are consistent with the broad effects seen by Meyer et al. (2009) with *A. millepora* and by Erwin and Szmant (2010) with *Acropora palmata*, although these authors did not analyze transcript levels, precluding direct comparison with the work presented here.

To better understand transcriptional changes associated with specific stimuli, comparisons were made between consecutive time points within treatments. These comparisons implied a consistent relationship between larval morphology and gene expression profiles that was independent of treatment. LWamide produced the most dramatic effect, both in terms of morphological change and in terms of changes in gene expression. By 4 hpi the expression of 397 genes had changed significantly in LWamide treated embryos (306 up plus 91 down), while no significant changes occurred over this time period in response to CCA chips and only 167 genes changed upon stimulation with CCA extract (Fig. 2). These results are consistent with the idea

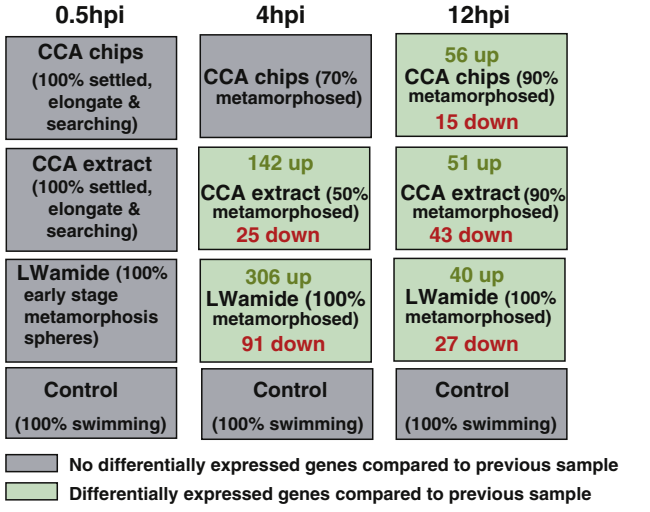

Fig. 2. Numbers of differentially expressed genes. Each sample was compared to the previous time point with that treatment, and 0.5 hpi samples were compared to a common sample of competent planula larvae. The number of unigenes showing upregulation is given as a green number, while the number down-regulated is given by a red number. The lists of genes in each category can be found in Supplementary Table 1.

that LWamide feeds into a stimulus–response cascade of gene activation downstream of the point at which CCA acts (Fig. 1B).

There are some anomalies in the results of differential gene expression analysis. For example, although 70% of larvae had metamorphosed at 4 hpi after exposure to CCA chips, the corresponding changes in gene expression profiles were below the significance cut-off used here, which was ≤0.05. This was particularly surprising given the extent of morphological change during metamorphosis. By contrast, at the corresponding time point 167 genes were either up- or down-regulated in response to CCA extract despite the fact that somewhat fewer (50%) of the larvae had metamorphosed (Fig. 2).

Identification and characterization of candidate metamorphosis genes

Classes of genes that could be identified on the basis of similarity to known proteins and were differentially regulated, were selected for further characterization by in situ hybridization. Of particular interest were genes likely to be involved in signalling and its regulation, immunity and cell adhesion, and apoptosis, as genes in these functional categories are involved in metamorphosis in other animals (summarized in Heyland and Moroz, 2006). In addition, transcription factors, RNA binding and processing factors and a highly regulated gene involved in metabolism (NADH dehydrogenase 1 alpha subcomplex 4) that were found to be strongly differentially expressed were investigated. Coral calcification, which is of particular current interest because of ocean acidification, begins at settlement, so a number of differentially expressed genes potentially involved in this process are also listed in Table 1. As well as genes matching the above criteria, genes without database matches that were highly up- or down-regulated in reponse to one or more stimuli were subjected to in situ analysis on the basis that these might be taxonomically restricted genes (TRGs) required for coral metamorphosis or the beginnings of calcification (Table 1). The genes listed in Table 1 are a subset of the complete dataset presented in Supplementary Table 1.

Three patterns of gene expression can be recognized at the time of settlement and metamorphosis

Among the 14 genes for which in situ hybridization revealed a spatially restricted expression, three types of pattern could be recognized. We refer to these three patterns here as the “early aboral” (Fig. 3), “later aboral” (Fig. 4) and “later oral” patterns (Fig. 5) based on the timing of regulation relative to the stimulus and the assumption that the LWamide stimulus invokes the later stages of a stimulus–response pathway.

Genes in the “early aboral” category are expressed in the aboral region of the planula (Fig. 3 A1 and B1) or metamorphosing larva (Fig. 3, A2, B2 and C2). Expression of these genes declined rapidly following metamorphosis (Fig. 3, A3, B3 and C3) and in each case no expression was evident in the primary polyp (Fig. 3, A4, B4 and C4). By contrast, while the “later aboral” genes are also expressed on the aboral side of the planula (Fig. 4, B1–E1) and metamorphosing larva (Fig. 4, A2–E2), expression of these genes is maintained much longer (Fig. 4, A3–E3 and A4–E4). The expression of the “later oral” genes was typically restricted to the oral half of the metamorphosing larva and early primary polyp (Fig. 5, A–F 2–4), in some cases declining dramatically in older primary polyps (Fig. 5 A5–D5).

Discussion

Acropora planulae appear to be primed for settlement

The coral planula appears to anticipate metamorphosis as, by contrast with the dramatic changes in morphology occurring during the initial stages of the planula/polyp transition, the extent of transcriptional change is surprisingly small; 30 min after treatment with CCA chips or extract,

Table 1
Selected differentially expressed genes showing the fold change in response to LWamide and CCA extract or chips. In cases where unigenes are represented on the array by more than two different cDNA spots, the median value of regulation is shown (underlined). Values of less than 1 indicate a down-regulation of expression. Genes showing early aboral expression are indicated in red, those in the later aboral group in blue, and those in the oral expression group in green. The expression of the CEL-III gene marked with an asterisk is shown in Grasso et al. (2008).Genes showing early aboral expression (Fig. 3) are indicated in red, those in the later aboral group (Fig. 4) in blue, and those in the oral expression group (Fig. 5) in green.

Unigene	Best Blast	e-value	Description	Fold change/Treatment				
				LW 30min-4hr	LW 4hr-12hr	CCA ext 30min-4hr	CCA ext 4hr-12hr	CCA chips 4hr-12hr
Signalling and its Regulation								
C_A043-H1	Q8WPM5	6E-38	Transmembrane receptor		40.3		33.2	53.6
C_B027-F10	Q5SYE6	1E-85	Nemo-like kinase	11.9		17.7		
C_D009-G7	Q5TCZ1	9E-48	SH3 and PX domain containing	12.5		10.8		
C_D020-D3	Q69ZL6	6E-75	Patched-like	2.5				
S_A023-C10	Q8SPU3	2E-84	BMP-receptor			5.7		
Immunity and Cell Adhesion								
C_A009-A3	Q95X07	6E-72	Peroxinectin	32		23.1		
C_A009-D7	Q868M7	1E-137	CEL-III lectin	2.9	11		8.8	13.6
C_A049-E7*	Q868M7	8E-62	CEL-III lectin		8.9		6.8	13
C_D034-A4	Q4SXP1	2E-44	Plexin domain containing			0.3		
S_A035-E3	Q28EH0	6E-16	Tetraspanin CD9		6.7		5.7	
S_A045-H12	O76470	7E-22	Echinonectin	3.7				
Apoptosis								
C_A036-C3	Q5XGJ4	2E-24	Bcl2-like	5.5		8.3		
Regulators of Transcription								
C_A031-G1	Q6DIG5	8E-43	Pax 3/7 related			7.5		
C_B032-D10	Q9PSX1	1E-151	Groucho/TLE	3.8				
C_D034-D7	Q703F5	3E-16	SAM/pointed domain containing	4.5		10.2		
C_D052-C12	Q7YZD1	1E-47	Homeodomain protein NK-4	5.8		10.1		
S_A037-A6	Q703F5	3E-13	SAM/pointed domain containing	6.2		6.6		
S_A039-E10	Q2MJB4	0.000004	Doublesex/mab-3 related	14.9				
S_A047-F7	Q4SLA8	3E-43	OVO Zinc finger transcription factor	13.4		7.8		
S_D039-A2	Q6YLV8	0.000001	Kruppel-like		3			
S_D053-G3	Q9U739	1E-27	Homeodomain protein Otx	4.5		4		
RNA Binding and Processing								
C_B030-H1	O95205	4E-42	Zinc-finger protein muscleblind	22.5		18		
C_D028-G6	Q8I9V8	2E-34	Acheron family			5		
Metabolism								
S_D023-C7	Q6PBH5	3E-13	NADH dehydrogenase 1 alpha sub-complex 4	64	0.1	134.5	0.2	
Calcium Handling								
C_A006-D10	Q8I6S1	6E-16	Galaxin-like1		5.4			
C_A022-E1	Q9U6S0	1E-83	Calreticulin	3.6		4.7		2.6
C_C006-F1	Q4RRL0	9E-35	Carbonic anhydrase	0.1		0.2		
C_D013-C10	Q3Y546	9E-62	Carbonic anhydrase	33.8		32.6		
C_GS01MG03	O96949	1E-42	Calmodulin	2.2				
C_GS01VA05	Q4XXN0	8E-34	Calmodulin	0.4				
S_D015-C8	O96949	2E-40	Calmodulin	2.7				
Taxonomically restricted genes (TRGs)								
C_A005-G11	Q7PTG9	0.000007	DOMON domain containing	5.5		4.4	4.3	6.3
C_A009-C1			SCRiP	5.4	13.9	9.2	8.5	14
C_A027-E11			No recognizable domain	0.1	0.2	0.3	0.1	0.2
C_A049-A7		3E-84	Similar to Nematostella EDO40958	14.9	46.9	10.4	21.2	22.8
C_GS01UH10			C type lectin domain containing	5.5		4.6		

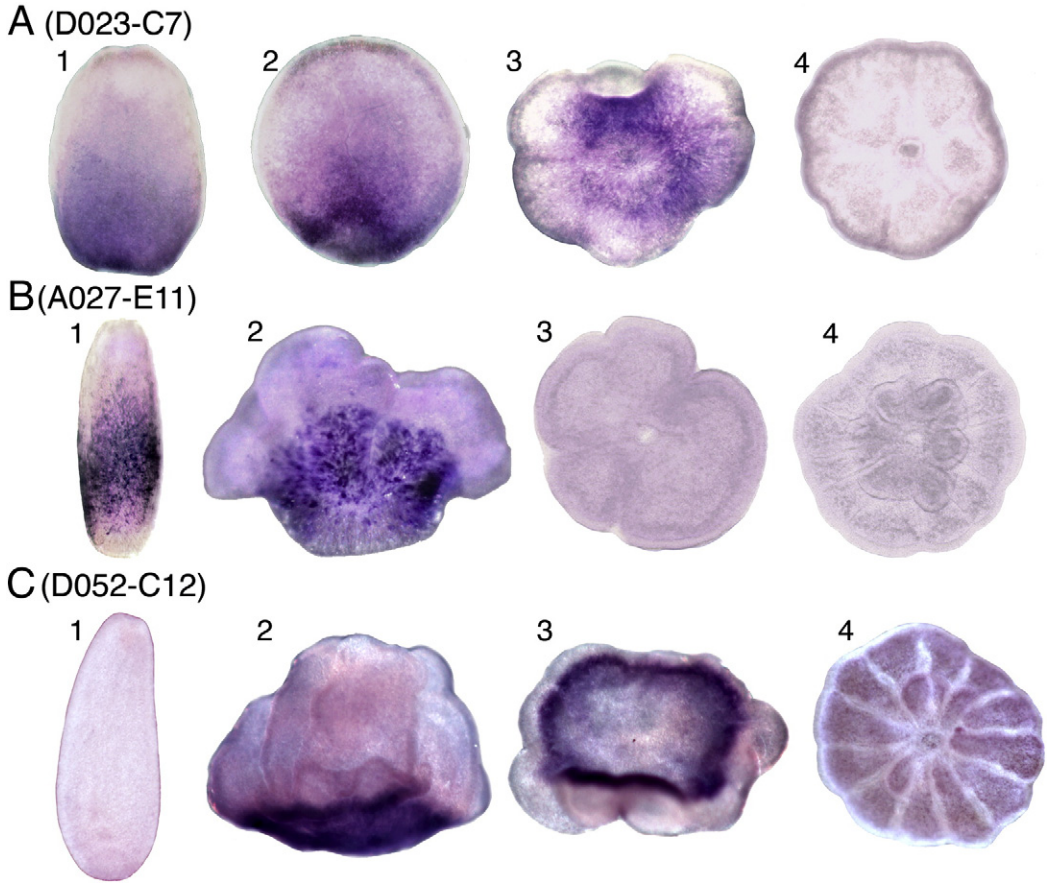

Fig. 3. Whole mount in situ hybridization of the 'early aboral' genes. The 'early aboral' genes are unified by similarities in their expression patterns—they are all predominantly expressed at the aboral end of the planula and/or metamorphosing larva, with expression decreasing after metamorphosis. Localization of transcripts (dark purple) is shown in (1) planula larva (oral upward), (2) metamorphosing larva (oral upward), (3) primary polyp (looking onto aboral end) and (4) an older postsettlement polyp (looking onto oral end).

larvae had settled, and after treatment with LWamide had commenced metamophosis, yet no changes in gene expression were detected. However, there are precedents for the pre-established competence implied by our array experiments. Hadfield (2000) and Hadfield et al. (2001) have pointed out that metamorphoses of marine invertebrates are rapid compared to those of terrestrial animals. For example, newly settled hydroids can capture prey with their nematocysts 5–6 h after metamorphosis and sea anemones with carnivorous planulae can capture prey almost immediately after settlement (Hadfield, 2000). The speed with which these transitions are accomplished is based on a pre-established competence; the larvae are "primed" for settlement, and Hadfield et al. have shown that transcription is not required once competence is achieved in several marine invertebrates. With the caveats that our arrays are based on approximately half the transcriptome, and some changes are presumably below the threshold for detection, the speed with which metamorphosis occurs in *Acropora* is likely to be enabled by pre-established competence. *Acropora* larvae are effectively "primed" for settlement.

Differentially regulated genes at metamorphosis

Many types of genes are differentially regulated at metamorphosis but the number with roles which can be predicted from sequence alone is considerably more limited. Some of the genes that fall into this category, and which are of particular relevance to metamorphosis, are listed in Table 1. The taxonomically restricted genes, or TRGs (Khalturin et al., 2009) which by definition have no strong hits in the sequence databases outside of the Cnidaria, are potentially of great interest, in part because some of them may be specific to the calcification process in corals. A number of such genes were therefore selected for in situ hybridization. C_A027-E11 (Fig. 3B), C_A009-C1 (Fig. 5B), C_A049-A7 (Fig. 5C) and C_A005-G11 (Fig. 5E), all fell into this category and in each case the spatial expression patterns were consistent with roles in settlement/metamorphosis. C_A005-G11 (Fig. 5E) encodes a DOMON protein; members of the DOMON superfamily may be haem or sugar recognition proteins (Iyer et al, 2007). C_A049-A7 (Fig. 5C) encodes a trans-membrane protein that has a clear *Nematostella* homolog but matches nothing else significantly. C_A009-C1 (Fig. 5B) is a member of the small cysteine rich protein (SCRiP) family, a group of coral genes of unknown function (Sunagawa et al., 2009).

Comparison with metamorphosis in other animals

Although there has been little work directly comparable to that presented here, genes involved in calcium handling and the processes of apoptosis, immunity and stress responses have been implicated in metamorphosis in a phylogenetically diverse range of animals (Heyland and Moroz, 2006; Williams et al., 2009; Reyes-Bermudez et al., 2009b).

Calcium handling and organic matrix components

This category of genes is of particular significance in the case of corals, as one of the most obvious characteristics of the transition from coral larva to polyp is the initiation of calcification by the calicoblastic ectoderm that arises from the presettlement aboral ectoderm. Several genes with roles in calcium metabolism or the formation of the skeletal organic matrix were among those differentially expressed during metamorphosis (Table 1), including two carbonic anhydrases identified in our previous array experiments (Grasso et al., 2008). The

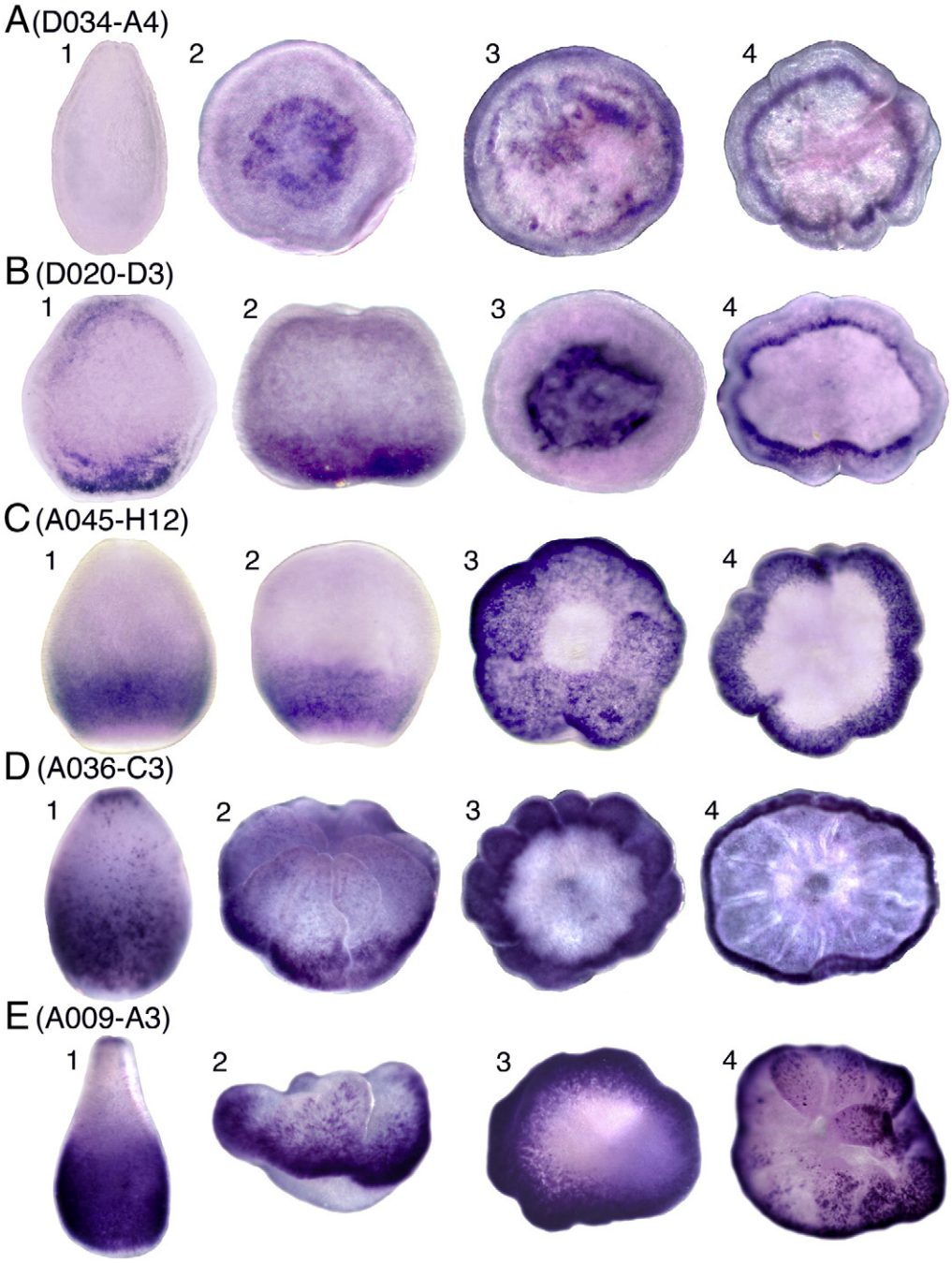

Fig. 4. Whole mount in situ hybridization of the 'later aboral' genes. The 'later aboral' group genes are unified by similarities in their expression patterns—they are predominantly expressed at the aboral end of the planula and/or metamorphosing larva, and continue to be expressed well after metamorphosis. Localization of transcripts (dark purple) is shown in (1) planula larva (oral upward), (2) metamorphosing larva (oral upward, except A2, which is viewed from aboral), (3) primary polyp (looking onto aboral end) and (4) an older postsettlement polyp (looking onto aboral end, except E4, which is viewed from oral.

calcium-sequestering protein calreticulin, responsible for storing calcium within ER vesicles, is consistently up-regulated early regardless of the stimulus. Other regulated genes potentially involved in calcium handling include calmodulins and one of the *Acropora* galaxin genes *AmGalaxin-like1* (Reyes-Bermudez et al., 2009a), a putative organic matrix component.

Apoptosis

Metamorphosis of the hydrozoan cnidarian *Hydractinia* (Seipp et al., 2001, 2006) involves extensive apoptosis, suggesting that programmed cell death might also play a significant role during coral metamorphosis. However, the only apoptosis-related gene consistently up-regulated during *Acropora* metamorphosis was a Bcl2-like gene which is strongly expressed in a restricted pattern in the ectoderm during metamorphosis (Fig. 4D). The *Acropora* protein has the same domain structure as vertebrate Bcl-2 which consists of BH4, BH3, BH1 and BH2 domains followed by a predicted C-terminal transmembrane domain, indicating that this Bcl2-like molecule is likely to be anti-apoptotic. Note that the failure to detect up-regulation of pro-apoptotic genes does not rule out a role for apoptosis in coral metamorphosis as caspase activation occurs primarily at the protein level in both vertebrates and insects (Logue and Martin, 2008).

Stress responses and immunity

Several chaperone and heat shock proteins were differentially expressed in at least one comparison. However, the interpretation of

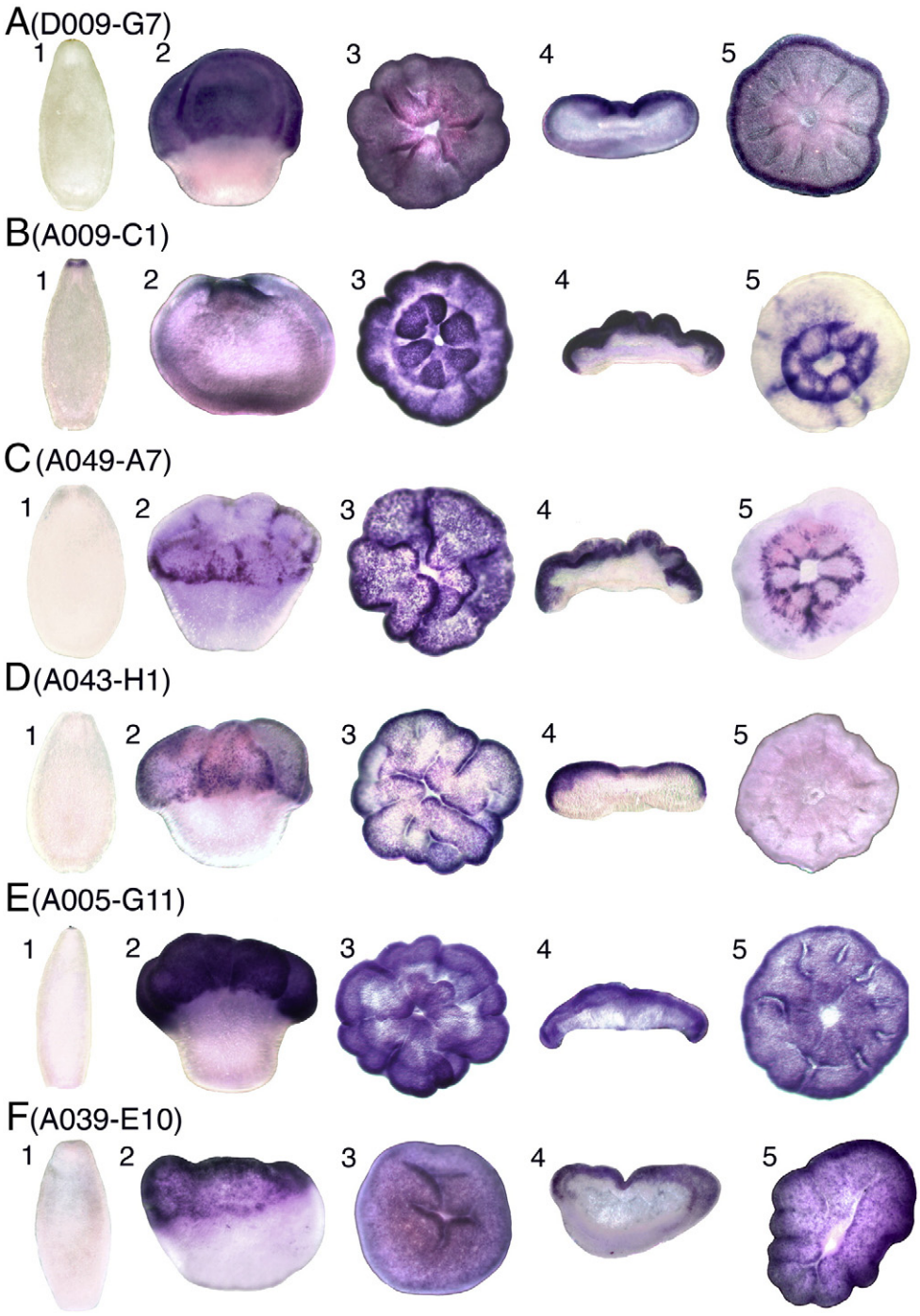

Fig. 5. Whole mount in situ hybridization of the 'later oral' genes. The 'later oral' group genes are unified by similarities in their expression patterns—they are generally not expressed in the planula but start to be expressed at the oral end of metamorphosing larvae, with expression continuing directly after metamorphosis, and starting to decrease well after metamorphosis. Localization of transcripts (dark purple) is shown in (1) planula larva (oral upward), (2) metamorphosing larva (oral upward), (3) primary polyp (looking onto oral end) (4) primary polyp (sectioned vertically) and (5) an older postsettlement polyp (looking onto oral end).

these data is complicated both by the complexity of the coral chaperone repertoire and the fact that the expression of many chaperones cycles in a circadian pattern in *Acropora* (Levy et al., 2011). Differential expression of several genes with presumed roles in immunity at metamorphosis in *A. millepora* has previously been described (Miller et al., 2007; Grasso et al., 2008). Lectins, including CEL-III (Grasso et al., 2008), a previously undescribed taxonomically restricted lectin (C_GS01UH10), and a clear counterpart of the key arthropod immune effector peroxinectin (Fig. 4E), were all upregulated. In addition to immune functions, many of these lectins are likely to have roles in cell adhesion, so understanding their significance will require characterization of the corresponding proteins in vivo and in vitro.

In situ hybridization reveals three patterns of gene expression during settlement and metamorphosis

During the transition from planula to polyp, several successive but overlapping phases of gene expression can be recognized: an aboral early phase, an aboral wave often beginning somewhat later in development, and a later oral phase. The early aboral pattern correlates with the searching/settlement phase of behavior, whereas

the latter two phases correspond to metamorphosis proper. Although showing broadly similar patterns of expression, the early aboral genes are diverse in function; one of the genes up-regulated in the "early aboral" pattern (Fig. 3) encodes a transcription factor, another is a subunit of NADH dehydrogenase and the third is a taxonomically restricted gene of unknown function. From previous studies the atypical lectin A043-D8 (C_GS01UH10) (Grasso et al., 2008) and one of the *Acropora* galaxin-related genes, *AmGalaxin-like1* (Reyes-Bermudez et al., 2009a) were expressed in a similar pattern.

The group of genes shown here to be upregulated in the "later aboral" pattern (Fig. 4), consists of genes likely to function in cell adhesion/immunity, cell signalling, and the regulation of apoptosis. On the basis of previous work *AmGalaxin-like2* (Reyes-Bermudez et al., 2009a) can be added to this group. In the present study, C_C006-F1 (carbonic anhydrase) expression was initially down-regulated (0–4 h) after exposure to either LWamide or CCA extract. The expression patterns of these genes are consistent with roles in transduction and implementation of the metamorphosis program, including the development of the calicoblastic ectoderm.

The "later oral" genes are characterized by expression on the oral side of the metamorphosing and juvenile polyp (Fig. 5), upregulation occuring only when metamorphosis is well under way. To the six genes shown here to have this pattern can be added the CEL-III lectins C_A009-D7 (represented by A036-E7 in Grasso et al., 2008) and C_A049-E7, and the soluble carbonic anhydrase C_D013-C10 (represented by A030-E11 in Grasso et al., 2008).

Metamorphosis in Acropora—more pieces of the puzzle

The data presented here represent the most comprehensive analysis to date of the motile larva/sedentary polyp transition in any cnidarian, and provide some more pieces in the puzzle of coral metamorphosis. The data are broadly consistent with earlier studies of coral metamorphosis (Grasso et al., 2008; Reyes-Bermudez et al., 2009b) and identify several of the same groups of genes. The discussion above is concerned mainly with up-regulated genes simply because few of the down-regulated genes have been annotated.

The two major findings presented are, first, that at a transcriptional level coral larvae appear to anticipate metamorphosis, since dramatic morphological changes occur in the absence of comparable changes in transcription. Second, three kinds of expression patterns are commonly seen for genes up-regulated during metamorphosis—early aboral-restriction, a second aborally-restricted type, and a later oral pattern. Genes expressed somewhat later in the aboral region include a membrane-associated carbonic anhydrase and galaxin-like1, both of which presumably function in the initiation of calcification. The orally-restricted phase includes a number of genes with putative roles in cell signalling, genes taxononically restricted to corals, the CELIII lectins, and other lectins previously discussed (Grasso et al., 2008). These three patterns correlate with an initial substrate recognition/signalling phase, the beginning of the metamorphic transformation of the aboral ectoderm into the calicoblastic ectoderm (the tissue responsible for secretion of the coral skeleton) and the subsequent stabilization/protection of the oral ectoderm, respectively.

Several of the genes that are highly expressed during the metamorphosis of *Acropora* do not have orthologs in the presently available *Nematostella* data (Grasso et al., 2008). While this is consistent with the idea that the complex version of metamorphosis seen in many corals is a derived trait within Anthozoa (Fôret et al., 2010), counterintuitively, several of these (apextrin, CELIII lectins, dispatched) have orthologs in other animals, so their apparent absence from *Nematostella* would appear to reflect gene loss. Some other genes implicated in metamorphosis in *Acropora* (e.g. the SCRiP) are unique to corals. The jigsaw puzzle of coral metamorphosis appears to have been assembled from whatever was to hand, as well as requiring the manufacture of some new pieces. When we see the big picture more clearly, will it resemble a da Vinci masterpiece or a Dada-ist readymade?

Acknowledgments

We thank the Australian Research Council for support through the Centre for Molecular Genetics of Development and the Centre of Excellence for Coral Reef Studies, Dr. Madeleine van Oppen for supplying specimens of bleached adult material, the University of Adelaide Microarray Facility for printing the slides, Sharyn Wragg for assistance with the figures and Rosalind Attenborough for comments on the paper.

Appendix A. Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.02.010.

References

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
Baird, A.H., Morse, A.N.C., 2004. Induction of metamorphosis in larvae of the brooding corals *Acropora palifera* and *Stylophora pistillata*. Mar. Freshwater Res. 55, 469–472.
Benjamini, Y., Hochberg, Y., 1991. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B 57, 289–300.
Clode, P., Marshall, A., 2004. Calcium localisation by X-ray microanalysis and fluorescence microscopy in larvae of zooxanthellate and azooxanthellate corals. Tissue Cell 36, 379–390.
de Jong, D.M., Hislop, N.R., Hayward, D.C., Reece-Hoyes, J.S., Pontynen, P.C., Ball, E.E., Miller, D.J., 2006. Components of both major axial patterning systems of the Bilateria are differentially expressed along the primary axis of a "radiate" animal, the anthozoan cnidarian *Acropora millepora*. Dev. Biol. 298, 632–643.
Erwin, P.M., Szmant, A.M., 2010. Settlement induction of *Acropora palmata* planulae by a GLW-amide neuropeptide. Coral Reefs 29, 929–939.
Fôret, S., Knack, B., Houliston, E., Momose, T., Manuel, M., Queinnec, E., Hayward, D.C., Ball, E.E., Miller, D.J., 2010. New tricks with old genes: the genetic bases of novel cnidarian traits. Trends Genet. 26, 154–158.
Grasso, L.C., Maindonald, J., Rudd, S., Hayward, D.C., Saint, R., Miller, D.J., Ball, E.E., 2008. Microarray analysis identifies candidate genes for key roles in coral development. BMC Genomics 9, 540.
Hadfield, M.G., 2000. Why and how marine invertebrate larvae metamorphose so fast. Semin. Cell Dev. Biol. 11, 437–443.
Hadfield, M.G., Carpizo-Ituarte, E.J., del Carmen, K., Nedved, B.T., 2001. Metamorphic competence, a major adaptive convergence in marine invertebrate larvae. Am. Zool. 41, 1123–1131.
Hand, C., Uhlinger, K.R., 1992. The culture, sexual and asexual reproduction, and growth of the sea-anemone *Nematostella vectensis*. Biol. Bull. 182, 169–176.
Harrington, L., Fabricius, K., De-ath, G., Negri, A., 2004. Recognition and selection of settlement substrata determine post-settlement survival in corals. Ecology 85, 3428–3437.
Hayward, D.C., Catmull, J., Reece-Hoyes, J.S., Berghammer, H., Dodd, H., Hann, S.J., Miller, D.J., Ball, E.E., 2001. Gene structure and larval expression of cnox-2Am from the coral *Acropora millepora*. Dev. Genes Evol. 211, 10–19.
Heyland, A., Moroz, L.L., 2006. Signalling mechanisms underlying metamorphic transitions in animals. Int. Comp. Biol. 46, 743–759.
Heyward, A., Negri, A., 1999. Natural inducers for coral larval metamorphosis. Coral Reefs 18, 273–279.
Hirose, M., Yamamoto, H., Nonaka, M., 2008. Metamorphosis and acquisition of symbiotic algae in planula larvae and primary polyps of *Acropora* spp. Coral Reefs 21, 247–254.
Huang, X., Madan, A., 1999. CAP3: a DNA sequence assembly program. Genome Res. 9, 868–877.
Iwao, K., Fujisawa, T., Hatta, M., 2002. A cnidarian neuropeptide of the GLWamide family induces metamorphosis of reef-building corals in the genus *Acropora*. Coral Reefs 21, 127–129.
Iyer, L.M., Anantharaman, V., Aravind, L., 2007. The DOMON domains are involved in heme and sugar recognition. Bioinformatics 23, 2660–2664.
Khalturin, K., Hemmrich, G., Fraune, S., Augustin, R., Bosch, T.C., 2009. More than just orphans: are taxonomically-restricted genes important in evolution? Trends Genet. 25, 404–413.
Kortschak, R.D., Samuel, G., Saint, R., Miller, D.J., 2003. EST analysis of the cnidarian *Acropora millepora* reveals extensive gene loss and rapid sequence divergence in the model invertebrates. Curr. Biol. 13, 2190–2195.
Le Tissier, M., 1988. Patterns of formation and the ultrastructure of the larval skeleton of *Pocillopora damicornis*. Mar. Biol. 98, 493–501.
Levy, O., Kaniewska, P., Alon, S., Eisenberg, E., Karako-Lampert, S., Bay, L.K., Reef, R., Rodriguez-Lanetty, M., Miller, D.J., Hoegh-Guldberg, O., 2011. Complex diel cycles of gene expression in coral-algal symbiosis. Science 331, 175.

Logue, S.E., Martin, S.J., 2008. Caspase activation cascades in apoptosis. Biochem. Soc. Trans. 36, 1–9.
Meyer, E., Aglyamova, G.V., Wang, S., Buchanan-Carter, J., Abrego, D., Colbourne, J.K., Willis, B.L., Matz, M.V., 2009. Sequencing and de novo analysis of a coral larval transcriptome using 454 GSFlx. BMC Genomics 10, 219.
Miller, D.J., Hemmrich, G., Ball, E.E., Hayward, D.C., Khalturin, K., Funayama, N., Agata, K., Bosch, T.C.G., 2007. The innate immune repertoire in Cnidaria—ancestral complexity and stochastic gene loss. Genome Biol. 8, R59.
Morse, D.E., Hooker, N., Morse, A.N.C., Jensen, R.A., 1988. Control of larval metamorphosis and recruitment in sympatric agariciid corals. J. Exp. Mar. Biol. Ecol. 116, 193–217.
Morse, A.N.C., Iwao, K., Baba, M., Shimoike, K., Hayashibara, T., Omori, M., 1996. An ancient chemosensory mechanism brings new life to coral reefs. Biol. Bull. 191, 149–154.
Müller, W.A., Leitz, T., 2002. Metamorphosis in the Cnidaria. Can. J. Zool. 80, 1755–1771.
Negri, A., Vollhardt, C., Humphrey, C., Heyward, A., Jones, R., Eaglesham, G., Fabricius, K., 2005. Effects of the herbicide diuron on the early life history stages of coral. Mar. Pollut. Bull. 51, 370–383.
Reyes-Bermudez, A., Lin, Z., Hayward, D.C., Miller, D.J., Ball, E.E., 2009a. Differential expression of three galaxin-related genes during settlement and metamorphosis in the scleractinian coral *Acropora millepora*. BMC Evol. Biol. 9, 178.
Reyes-Bermudez, A., DeSalvo, M.K., Voolstra, C.R., Sunagawa, S., Szmant, A.M., Iglesias-Prieto, R., Medina, M., 2009b. Gene expression microarray analysis encompassing metamorphosis and the onset of calcification in the scleractinian coral *Montastraea fevolata*. Mar. Genomics 2, 149–159.
Ritchie, M.E., Silver, J., Oshlack, A., Holmes, M., Diyagama, D., Holloway, A., Smyth, G.K., 2007. A comparison of background correction methods for two-colour microarrays. Bioinformatics 23, 2700–2707.
Seipp, S., Schmich, J., Leitz, T., 2001. Apoptosis—a death-inducing mechanism tightly linked with morphogenesis in *Hydractina echinata* (Cnidaria, Hydrozoa). Development 128, 4891–4898.
Seipp, S., Wittig, K., Stiening, B., Bottger, A., Leitz, T., 2006. Metamorphosis of *Hydractinia echinata* (Cnidaria) is caspase-dependent. Int. J. Dev. Biol. 50, 63–70.
Smyth, G., 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3 Available: http://www.statsci.org/webguide/smyth/pubs/ebayes.pdf.
Smyth, G., 2005. Limma: linear models for microarray data. In: Gentleman, R., Carey, V., Dudoit, S., Irizarry, R., Huber, W. (Eds.), Bioinformatics and Computational Biology Solutions using R and Bioconductor. New York, pp. 397–420.
Smyth, G., Speed, T., 2003. Normalization of cDNA microarray data. Methods 31, 265–273.
Sunagawa, S., DeSalvo, M.K., Voolstra, C.R., Reyes-Bermudez, A., Medina, M., 2009. Identification and gene expression analysis of a taxonomically restricted cysteine-rich protein family in reef-building corals. PLoS ONE 4, e4865.
Vandermeulen, J., 1975. Studies on reef corals. III. Fine structural changes of calicoblast cells in *Pocillopora damicornis* during settling and calcification. Mar. Biol. 31, 69–77.
Williams, E.A., Degnan, B.M., Gunter, H., Jackson, D.J., Woodcroft, B.J., Degnan, S.M., 2009. Widespread transcriptional changes pre-empt the critical pelagic–benthic transition in the vetigastropod *Haliotis asinina*. Mol. Ecol. 18, 1006–1025.