MOLECULAR ECOLOGY

Molecular Ecology (2012) **21**, 2440–2454 doi: 10.1111/j.1365-294X.2012.05554.x

Whole Transcriptome Analysis of the Coral *Acropora millepora* Reveals Complex Responses to CO_2-driven Acidification during the Initiation of Calcification

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Abstract

The impact of ocean acidification (OA) on coral calcification, a subject of intense current interest, is poorly understood in part because of the presence of symbionts in adult corals. Early life history stages of *Acropora* spp. provide an opportunity to study the effects of elevated CO_2 on coral calcification without the complication of symbiont metabolism. Therefore, we used the Illumina RNAseq approach to study the effects of acute exposure to elevated CO_2 on gene expression in primary polyps of *Acropora millepora*, using as reference a novel comprehensive transcriptome assembly developed for this study. Gene ontology analysis of this whole transcriptome data set indicated that CO_2-driven acidification strongly suppressed metabolism but enhanced extracellular organic matrix synthesis, whereas targeted analyses revealed complex effects on genes implicated in calcification. Unexpectedly, expression of most ion transport proteins was unaffected, while many membrane-associated or secreted carbonic anhydrases were expressed at lower levels. The most dramatic effect of CO_2-driven acidification, however, was on genes encoding candidate and known components of the skeletal organic matrix that controls $CaCO_3$ deposition. The skeletal organic matrix effects included elevated expression of adult-type galaxins and some secreted acidic proteins, but down-regulation of other galaxins, secreted acidic proteins, SCRiPs and other coral-specific genes, suggesting specialized roles for the members of these protein families and complex impacts of OA on mineral deposition. This study is the first exhaustive exploration of the transcriptomic response of a scleractinian coral to acidification and provides an unbiased perspective on its effects during the early stages of calcification.

Keywords: *Acropora*, calcification, coral, ocean acidification, transcriptome

Received 28 November 2011; revision received 26 January 2012; accepted 6 February 2012

Introduction

Reef-building scleractinian corals are among the most prominent marine calcifying organisms, creating ecosystems known for their extraordinary productivity, biodiversity and economic value (Wilkinson 2008). During the last few decades, declines in coral calcification have been observed on a global scale, correlated with the rapid increase in atmospheric CO_2 that has occurred as a consequence of anthropogenic emissions (De'ath *et al.* 2009; Pandolfi *et al.* 2011). As the world's oceans are

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sinks for CO_2 released into the atmosphere, elevated emissions are leading to decreases in sea water pH in a process known as ocean acidification (OA; Caldeira & Wickett 2003). Recent estimates imply that the ocean surface pH has dropped by an average of 0.13 units since preindustrial times and is expected to decrease by a further 0.3–0.5 units in the 21st century (Orr 2011).

Although several studies have demonstrated the negative effects of OA on calcification rates in corals (Langdon & Atkinson 2005; Orr *et al.* 2005; Kleypas & Yates 2009; Pandolfi *et al.* 2011), the mechanisms remain unknown, in part owing to the presence of symbionts in adult corals complicating the understanding of skeleton deposition. Early life history stages of *Acropora* spp. provide an opportunity to study the effects of elevated CO_2 on coral calcification without the complication of symbiont metabolism. Moreover, calcification begins in newly settled coral polyps (Figure 1A–C), immediately after the motile larvae initiate metamorphosis (Harrison & Wallace 1990; Harrison 2011), a critical transition that may be more susceptible to environmental perturbations than adult corals (Gilmour *et al.* 2009). Exposure to reduced pH has been shown not only to suppress metamorphosis of larvae of the coral *Acropora digitifera* (Nakamura *et al.* 2011), but also to affect primary polyp growth (Albright *et al.* 2008, 2010; Cohen *et al.* 2009; Suwa *et al.* 2010) and alter the size, shape, orientation and composition of aragonite crystals in the skeleton (Kurihara 2008; Cohen *et al.* 2009). Early initiation of calcification and subsequent rapid growth of the early skeleton protect the young coral polyp and are thus critical determinants of larval recruitment (Babcock & Mundy 1996; Zilberberg & Edmunds 2001). Negative impacts on early life history biology are particularly significant for the maintenance of genetic diversity and for the re-establishment of corals after disturbances such as mass bleaching events. Predicting how OA will impair coral calcification is dependent upon a better understanding of biomineralization mechanisms at both physiological and molecular levels (Allemand *et al.* 2011; de Putron *et al.* 2011).

Models of coral calcification suggest that the overall process may be controlled at two levels (Figure 1D): (i) regulation at the physicochemical level of ion transport and control of carbonate chemistry, and (ii) regulation of the secretion of a skeletal organic matrix into a hydrogel-like environment between the calcifying cells and the skeleton (the Extracellular Calcifying Medium; Allemand *et al.* 2011). Recently, significant progress has been made towards understanding the molecular bases of metamorphosis and the initiation of calcification in corals, much of the work focusing on *Acropora millepora*. Microarray analysis followed by transcript localization via *in situ* hybridization has provided candidate genes

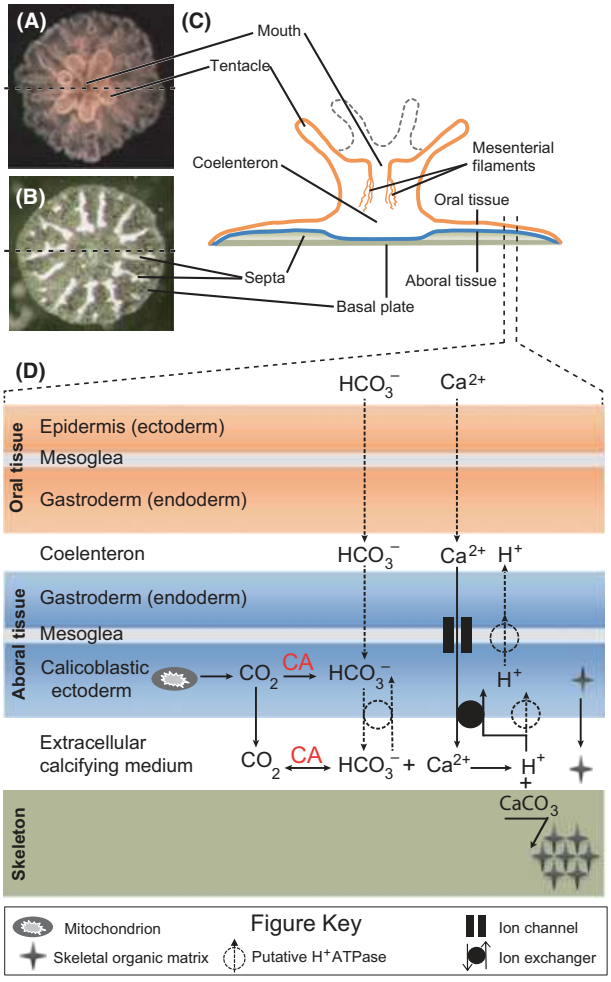

Fig. 1 Calcification in primary coral polyps. (A) Oral view of a primary polyp of *Acropora millepora* and (B) the corresponding calcified skeleton after tissue removal. (C) Schematic representation of a vertical section through a primary polyp (D). Hypothetical model for the organization of the proteins involved in the process of calcification: ion transport, control of carbonate chemistry and secretion of a skeletal organic matrix into the extracellular calcifying medium (after Allemand *et al.* 2011). Putative or unknown transport pathways are indicated with dotted arrows. CA: carbonic anhydrase. The cell layers and extracellular spaces are not shown to scale.

for roles in these processes (Grasso *et al.* 2008, 2011), as has subtractive hybridization (Hayward *et al.* 2011). Genes implicated in calcification are of two distinct types – those involved in ion transport and regulation of carbonate chemistry, and taxonomically restricted genes (TRGs). The chemistry of calcium carbonate deposition dictates that some proteins (the 'usual suspects') are required in all calcifying organisms (for example, carbonic anhydrases (Jackson *et al.* 2007)), whereas the independent evolution of calcification in diverse animals has led to the recruitment of

taxonomically restricted genes to control the specific architecture of $CaCO_3$ deposition in each case. To date, galaxins are the only skeletal organic matrix proteins to have been unambiguously identified in coral (Fukuda *et al.* 2003). Three galaxin-related *A. millepora* genes have patterns of expression during metamorphosis consistent with roles in skeleton deposition (Reyes-Bermudez *et al.* 2009). Originally, galaxins were thought to be coral-restricted genes, but similar proteins have now been identified in noncalcifying organisms, including the sea anemone *Anemonia* (Ganot *et al.* 2011) and the vestimentiferan tubeworm *Riftia* (Sanchez *et al.* 2007), consistent with the idea that the galaxins are derived from an ancestral gene with a role in the extracellular matrix (Sanchez *et al.* 2007; Forêt *et al.* 2011). Other coral-specific gene families implicated in early calcification on the basis of bioinformatic analyses, include the small cysteine-rich proteins (SCRiPs) (Sunagawa *et al.* 2009), several secreted acidic repetitive proteins (Shinzato *et al.* 2011) and a number of orphan taxonomically restricted genes, which gave *in situ* hybridization patterns consistent with their involvement (Grasso *et al.* 2008, 2011).

Given the significance of early life history stages, we analysed the impact of OA on gene expression in *A. millepora* in the early postsettlement phase during which calcification is initiated. Primary polyps were exposed to elevated concentrations of CO_2 for 3 days, reflecting short-term exposure to the medium and high future carbon predictions by the Intergovernmental Panel on Climate Change (IPCC 2007). mRNA from these polyps was subjected to high-throughput sequencing (using the Illumina RNAseq method), and levels of gene expression were compared by mapping the sequences onto a reference transcriptome that was assembled for this study (Supporting Information). Both gene ontology (GO) and candidate gene analyses were used to interpret the results, the former providing a global view of the overall transcriptomic (and, by inference, metabolic) response to acidification, whereas the latter was specifically directed at better understanding the impact on biomineralization, by targeting individual genes or classes of genes implicated in this process either on the basis of known or implied function or *in situ* hybridization analyses.

Acute exposure to elevated CO_2 levels resulted in significant changes in the expression of many (~19%) coral genes. In addition to the general metabolic suppression implied by the GO analysis, elevated CO_2 levels resulted in major changes in the expression of genes implicated in skeletal organic matrix synthesis and the control of internal carbonate chemistry. The expression of genes encoding ion transport proteins was relatively unaffected. By demonstrating which processes are most sensitive to OA and significantly increasing the set of candidate calcification genes for further studies, this project provides a major advance in the understanding of early coral calcification in the context of altered sea water chemistry linked to climate change.

Materials and methods

Maintenance of coral material and experimental design

Fifteen adult colonies of *Acropora millepora* were collected off the coast of Orpheus Island, Queensland, Australia (GBRMPA Permit No G10/33232.1), prior to spawning in November 2009 and transported to the aquaculture facilities at James Cook University in Townsville, where they were maintained in outdoor flow-through aquaria filled with 5 μm filtered sea water. Immediately after spawning, eggs and sperm from these 15 colonies were collected and used to make 3 independent crosses (3 × 5 colonies) corresponding to three biological replicates.

Larvae were cultivated and settled under ambient conditions (380 ppm $pCO_{2(air)}$, pH_{NBS} 8.16 ± 0.01, 27.5 °C, total alkalinity (TA) 2028.8 ± 5, salinity 33‰). Five days after fertilization, settlement was induced by the introduction of unglazed terracotta tiles conditioned with crustose coralline algae. Twenty-four hours after induction of settlement, tiles on which *Acropora millepora* primary polyps settled were transferred to experimental tanks, where they were exposed for 3 days to sea water that had been equilibrated with air containing 380 ppm (pH~8.16), 750 ppm (pH~7.96) or 1000 ppm CO_2 (pH~7.86), reflecting the control condition, medium and high CO_2 scenarios for the 21st century predicted by the IPCC (2007) (Figure 2). The desired CO_2 concentration was produced using a CO_2 mixing system developed by Munday *et al.* (2009). pH was measured on the National Bureau of Standards (NBS) scale with a portable meter (Hach HQ11D) calibrated daily with pH4 and pH7 buffers. Temperature, pH, oxygen concentration and TA were monitored daily at 11 am. Aragonite saturation states as well as CO_2 concentration in sea water were estimated using the program CO2SYS (Lewis & Wallace 1998). Average $pCO_{2(sea\ water)}$ was estimated to be 414, 669 and 917 μatm. All sea water carbonate parameters are shown in Table S1 (Supporting information).

Sampling and RNA extraction

Primary polyps (~50 per condition) were removed from the tiles with a sterile scalpel, immediately snap-frozen in liquid nitrogen and stored at −80 °C until further treatment. Total RNA was extracted using Trizol

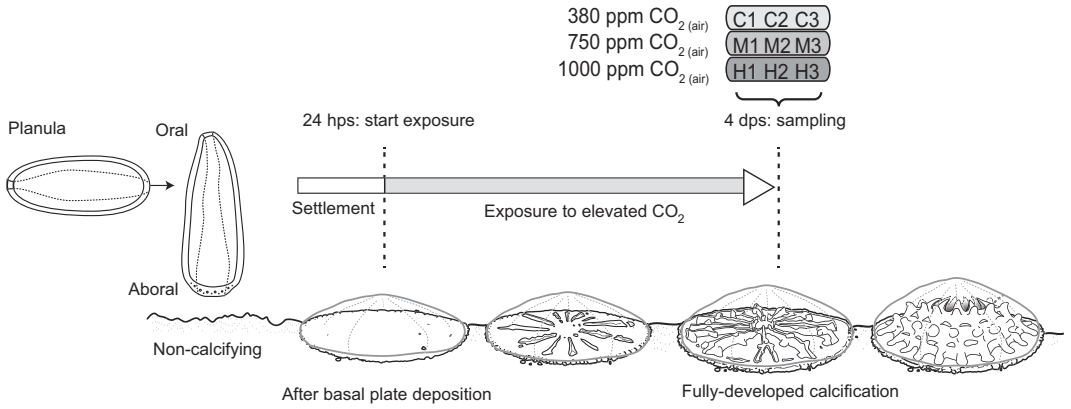

Fig. 2 Summary of the experimental design used. Three independent pools of *Acropora millepora* larvae, each generated from crosses between eggs and sperm from 5 colonies (3 × 5 colonies), were maintained in aquaria under ambient conditions (380 ppm $pCO_{2(air)}$, pH_{NBS} 8.16 ± 0.01, 27.5 °C, TA 2028.8 ± 5, salinity 33‰) for 5 days, by which time they were competent to settle. Settlement was induced by the introduction of (autoclaved) unglazed terracotta tiles conditioned with crustose coralline algae. Twenty-four hours after settlement, the primary polyps were exposed to sea water equilibrated with air containing 380 ppm CO_2 (C, pH∼8.16), 750 ppm CO_2 (M, pH∼7.96), or 1000 ppm CO_2 (H, pH∼7.86), reflecting the control condition, and the medium and high CO_2 scenarios for the 21st century predicted by the Intergovernmental Panel on Climate Change (IPCC 2007). After 3 days of treatment, primary polyps (∼50 per condition, three biological replicates per condition) were removed from the tiles using a sterile scalpel and snap-frozen in liquid nitrogen prior to RNA extraction for RNAseq analysis. hps = hours postsettlement and dps = days postsettlement.

Reagent (Invitrogen) according to the Chomczynski method (Chomczynski & Sacchi 1987) and dissolved in RNase-free water. RNA quantity and quality were assessed using a NanoDrop ND-1000 spectrometer and denaturing gel electrophoresis using standard methods (Sambrook & Russell 2001). Before being shipped on dry ice to the Macrogen sequencing facilities in Seoul, South Korea, each RNA sample was precipitated in ethanol and sodium acetate (2× and 0.1× sample volume, respectively), and stored at −80 °C.

High-throughput sequencing and data analysis

The reference transcriptome was generated using around 2 million 454 reads (GSFlx and Titanium; >2 Gbp), >400 million single-end Illumina reads (>15 Gbp) and >125 million paired-end Illumina reads (>6.5 Gbp) based on mRNA from a wide range of developmental stages. The assembly was generated using a modified version of Oases (http://www.ebi.ac.uk/~zerbino/oases/); for full details, see Appendix S1.

For the acidification experiment, mRNA isolation and library construction were performed by Macrogen (South Korea). The libraries were sequenced using the Illumina GAIIx platform, producing an average of 28 million short sequence reads (38 bp) per sample. Sequencing reads were mapped onto an *A. millepora* transcriptome assembly (Supporting Information) using the Bowtie mapping software v0.12.7 (Langmead *et al.* 2009). Differential gene expression was inferred based on these mapping counts using the edgeR package (Robinson *et al.* 2010). Gene Ontology enrichment analysis was performed using the GOseq package (Young *et al.* 2010) that accounts for selection bias for long and highly overexpressed sequences. The RNA-seq reads used in this study have been submitted to the NCBI Gene Expression Omnibus (GEO) database under accession number GSE33016.

Real-time quantitative PCR

Specific primers amplifying around 100 bp and spanning intron–exon boundaries were designed using the software Primer3 (Rozen & Skaletsky 2000). Primer sequences used in this study are listed in Table S9. Expected length of the amplicons was checked by agarose gel electrophoresis after regular PCR amplification. Primer efficiencies were determined using standard curve analysis with a 10-fold dilution series of pooled cDNA from both control and treated samples, and ranged from 0.95 to 1.02.

cDNAs were prepared using SuperScriptII reverse transcriptase (Invitrogen) and a mixture of oligodT and random primers, according to the manufacturer's instructions. Transcript level quantification was performed using the GoTaq qPCR mastermix from Promega and a Rotor-gene 6000 (Corbett). PCR conditions were as follows: 1× GoTaq qPCR mastermix (Promega), 100 nM primers and 2.5 ng of cDNA in a total volume of 15 µl. Each sample was run in triplicate using the following PCR parameters: 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C, 60 s at 60°C and a dissocia-

tion curve step (60–95 °C) to confirm the absence of nonspecific products. The dissociation curves showed a single amplification product and no primer dimers.

Several control genes were chosen based on the Illumina results as a whole (most stably expressed genes, in all tested conditions), and the expression stability of ten putative control genes was evaluated using the GeNorm software (Vandesompele *et al.* 2002). The control genes finally selected are Cluster007383 and Cluster017385. We used the relative expression software tool [REST©] for group wise comparison and statistical analysis of relative expression results (Pfaffl *et al.* 2002).

Results

Transcriptome assembly

To enable comprehensive gene expression profiling, it was necessary to generate as complete a reference transcriptome as possible for *Acropora millepora*. A transcriptome assembly (generated from 600 000 reads of 454 GS-Flx data; 44 400 contigs) is available for *A. millepora* (Meyer *et al.* 2011), but was prepared exclusively from coral larvae and hence is sub-optimal for expression profiling of postsettlement corals. To generate a more comprehensive reference transcriptome, RNA from a wide range of developmental stages was used as starting material for both 454 and Illumina sequencing. In total, the assembly used >125 million paired-end Illumina reads and >400 million single-end Illumina reads, combined with a substantial body of 454 data (both GS-Flx and Titanium chemistry; around 2 million reads in total); for full details, see Supporting information. The reference transcriptome consisted of 56 260 contigs with mean size of 1367 bases and N50 of 2023 bases, representing a significant improvement over the transcriptome assembly of Meyer *et al.* (Meyer *et al.* 2011) (mean = 440, N50 = 693). The transcriptome assembly has been submitted to the NCBI Transcriptome Shotgun Assembly Database under accession numbers JR970414–JR999999 and JT000001–JT023377.

General results

Acropora millepora primary polyps (Figure 1A–C) were exposed for 3 days to sea water equilibrated with air containing 380 ppm, 750 ppm or 1000 ppm CO_2, reflecting the current (control) condition, medium and high CO_2 scenarios for the 21st century predicted by the IPCC (2007) (see Table S1, Supporting information for the experimental sea water parameters). Figure 2 summarizes the experimental design. The application of Illumina RNAseq technology revealed a very close correlation between expression levels of transcripts in the three biological replicates (Figure 3). Significant changes in the transcriptome were measured under elevated CO_2; using a cut-off of adjusted $P < 0.01$, 12 and 19% of *A. millepora* transcripts were differentially expressed under medium and high CO_2 conditions, respectively (Figure 4A,B). While 42% of the differentially expressed genes were common between the two experimental conditions (Figure 4C,D), responses to the two treatments were sufficiently different to imply operation of a number of thresholds as well as linear responses. Most changes in levels of expression were relatively small; approximately three quarters of the transcripts in both up- and down-regulated categories changed <6-fold under elevated CO_2, but 5% of those down-regulated and 4% of those up-regulated changed >36-fold in level (Figure S1, Supporting information).

To validate the RNAseq results, levels of expression of genes representing both differentially expressed

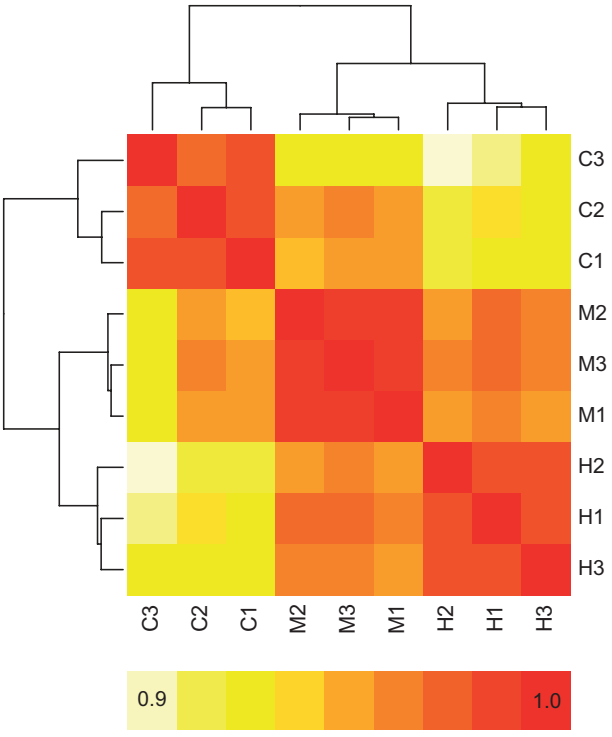

Fig. 3 Level of agreement between the biological replicates. The diagram shows the Pearson correlation coefficient of the transcript expression levels from all samples compared against each other. The level of correlation is represented by a coloured field and ranges from light yellow (correlation coefficient 0.9) to red (correlation coefficient 1). Samples C1, C2, C3: $pCO_{2\ (air)}$ 380 ppm (controls), M1, M2, M3: $pCO_{2\ (air)}$ 750 ppm, H1, H2, H3: $pCO_{2\ (air)}$ 1000 ppm. Clustering of samples based on their correlation coefficient clearly indicates the consistency between biological replicates; samples having undergone the same treatment are more correlated than samples from different treatment groups.

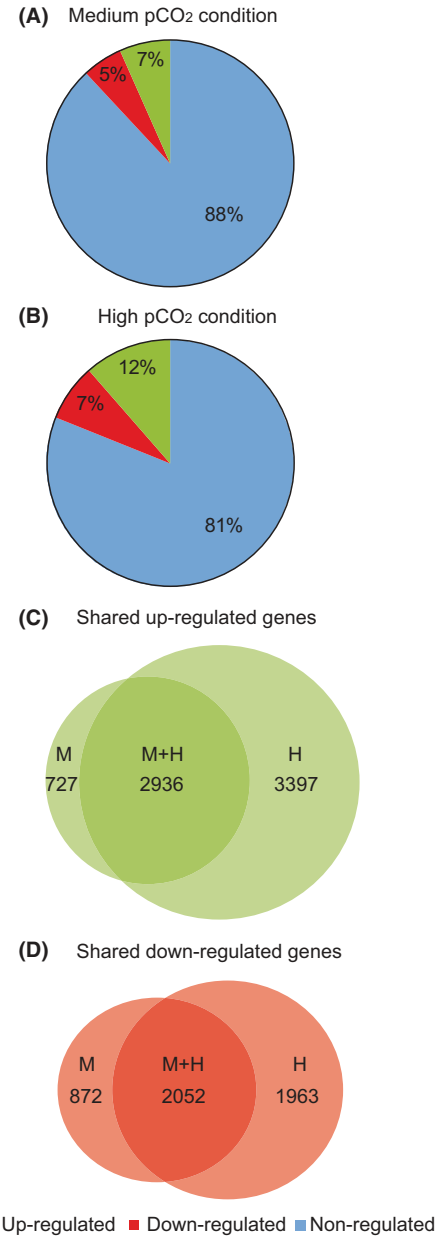

Fig. 4 Effects of the elevated CO_2 treatments on the *Acropora millepora* transcriptome. Parts (A) and (B) of the figure show the percentages of transcripts up- (green) and down-regulated (red) in response to the medium (A) and the high (B) pCO_2 treatments (adjusted $P < 0.01$). The Venn diagrams shown as (C) and (D) indicate the numbers of up-regulated (C) and down-regulated (D) transcripts common between the medium (M) and high (H) pCO_2 treatments (adjusted $P < 0.01$). In (C) and (D), each of the classes M, M+H and H is mutually exclusive. Subsets of transcripts are differentially regulated only under either treatment, implying the operation of distinct thresholds as well as linear responses.

($n = 6$) and stable ($n = 2$) profiles on the basis of the RNAseq data were analysed by real-time quantitative PCR (RT-qPCR) (Figure S2, Supporting information). A remarkably close correlation was observed between fold differences in expression in this experiment (regression $P < 0.001$; Pearson's correlation coefficient $R^2 = 0.93$; Figure S3, Supporting information), validating the RNAseq methodology described here for quantitative analysis of the coral transcriptome.

GO analysis implies that acidification enhances extracellular organic matrix synthesis and suppresses metabolism

As a preliminary approach to the analysis of the data, the differentially expressed genes were annotated using the GO database (Ashburner *et al.* 2000). Over-representation of GO terms in the set of differentially expressed genes was evaluated to infer which molecular functions, cellular components and biological processes were most affected by the experimental conditions. The two GO categories most highly over-represented were cellular components 'extracellular region GO:0005576' and 'mitochondrion GO:0005739', these categories being significantly up- and down-regulated, respectively (adjusted $P < 0.05$, Goseq analysis, see Materials and methods).

The set of transcripts in *A. millepora* belonging to the category 'extracellular region' comprises 71 genes encoding glycoproteins, collagens, lipoproteins, lectins and cysteine-rich proteins, as well as exopeptidases and metalloproteases (see Table S2, Supporting information for a detailed list), 68 and 92% of genes in this category were differentially expressed under the medium and high pCO_2 conditions, respectively. In corals, two functionally distinct extracellular organic matrices can be distinguished (Helman *et al.* 2008): (i) an extracellular organic matrix, which facilitates cell–cell and cell–substrate adhesion and (ii) a Skeletal Organic Matrix (SOM) that facilitates controlled deposition of the calcium carbonate skeleton (see section Acidification alters the expression of many known and candidate Skeletal Organic Matrix proteins). Most of the differentially expressed genes in the category 'extracellular region' could be identified as either skeletal organic matrix or extracellular organic matrix coding genes, but often it is not clear to which of these matrices individual genes belong because spatial localization data are not yet available. The 'extracellular region' proteins could also be related to mucus production. Changed environmental conditions often trigger mucus production in corals (Brown & Bythell 2005; Jatkar *et al.* 2010), which could potentially act as a mechanism to insulate coral tissue from the increased acidity. Elevated expression of exopeptidases and matrix metalloproteases was also observed under acidification suggesting the degradation of proteins in response to stress. This is consistent with the results in a recent study of thermal stress impact on

Montastrea faveolata, which led the authors to propose that an increased degradation of skeletal organic matrix proteins is responsible for a thermal stress-induced reduction of calcification (DeSalvo *et al.* 2008).

'Mitochondrion' is the second category highlighted by the GO analysis. Many components of the electron transport chain (including proteins from the four mitochondrial inner membrane complexes as well as several ATP synthase subunits, see Table S3, Supporting information) were affected, suggesting that oxidative metabolism is suppressed under elevated CO_2. In many cases, metabolic suppression is an adaptive strategy for survival of short-term energy limitation in aquatic organisms (Seibel & Walsh 2003) and is accomplished, at least in part, by shutting down processes such as protein synthesis (Guppy & Withers 1999) and mitochondrial protein synthesis in particular (Kwast & Hand 1996). In the present study, 37 metabolic genes were down-regulated (Table S3, Supporting information), but most (79%) of these responded only at high pCO_2 conditions, implying that strong metabolic suppression occurs only in response to the highest pCO_2 stress applied.

Focus on biomineralization

Although global GO analysis provided an initial insight into the metabolic impacts of OA, the GO database is strongly biased towards highly conserved, well-described processes in model organisms. This bias is particularly significant for *A. millepora* because 73% of the transcripts lack GO annotation. Therefore, GO analysis allowed only a general overview of the stress response. Because many genes involved in calcification are likely to be unique to corals or otherwise taxonomically restricted (specifically, skeletal organic matrix genes, see above), these will be ignored under GO analysis. For this reason, a second phase of data analysis was undertaken, focusing specifically on genes implicated in the two sub-processes of biomineralization: (i) physicochemical regulation of ion transport and control of carbonate chemistry and (ii) secretion of a skeletal organic matrix into the extracellular calcifying medium. Ion transport and control of carbonate chemistry are not exclusive to calcification, as the classes of enzymes involved play a variety of other cellular roles. On the other hand, macromolecules secreted into the extracellular calcifying medium of calcifying organisms are specifically involved in biomineralization and are often phylum- or species-restricted.

The expression of most ion transporter proteins is unaffected by acidification. Expression patterns of 67 transcripts implicated in calcium, bicarbonate or proton transport were analysed, but few significant changes were observed (16% of genes responded, Figure 5 and Tables S4–S6, Supporting information), and overall neither this category of genes nor its individual components (Ca^{2+}-, H^+- and HCO_3^- transporters) was significantly enriched in differentially expressed genes in comparison with the entire data set (Fisher exact test $P > 0.05$). Within the category of ion transporters, calcium transporters had the largest fraction of genes responding to elevated CO_2. However, only a small subset (8 of 38) responded and none of the responding genes has obvious counterparts among the calcification-related calcium transporters known from *Stylophora pistillata* (Figure 5A, Table S4, Supporting information). According to a current view on calcification mechanisms (Figure 1D), aragonite precipitation involves a plasma membrane Ca^{2+}-ATPase (PMCA) acting as a $Ca^{2+}/2H^+$ exchanger (Allemand *et al.* 2011) possibly operating in conjunction with other H^+ transporters. In this study, none of the Ca^{2+}-ATPases was differentially regulated in response to OA. However, two of the five T-type calcium channels were up-regulated at 1000 ppm of $CO_{2(air)}$. In vertebrates, T-type Ca^{2+}-channels are associated with bone development (Shao *et al.* 2005; Bergh *et al.* 2006), making the corresponding coral genes interesting candidates for further study. Among the down-regulated genes are two putative transient receptor potential cation channels (TRPC), transporters that mediate the release of calcium from intracellular stores (Cook & Minke 1999; Hardie 2011). Two-pore calcium channels, of which 2 were up-regulated, have similar roles in the release of stored calcium. Although these results do not suggest a direct role in transport of calcium to the extracellular calcifying medium, the acidification-induced alteration of intracellular calcium stores might have an indirect effect on calcification by affecting the central calcium signalling pathways.

Acidification leads to decreased expression of many Carbonic Anhydrases. Carbonic anhydrases (CAs) are the second major conserved component of the calcification repertoire, and a considerably higher percentage (53%) of this class of genes responded to elevated CO_2. CAs are ubiquitous enzymes that catalyse the interconversion of HCO_3^- and CO_2 and are involved in a range of physiological processes such as pH regulation in addition to biomineralization (see for review Pastorekova *et al.* 2004; Supuran 2008). In calcifying corals, carbonic anhydrases are involved in dissolved inorganic carbon dynamics at the site of calcification and in carbon exchange between the zooxanthellae and the coral (Furla *et al.* 2000). In *A. millepora*, two carbonic anhydrases have previously been implicated in biomineralization on the basis of microarray analysis and spatial localization by *in situ* hybridization (Grasso *et al.* 2008). In the pres-

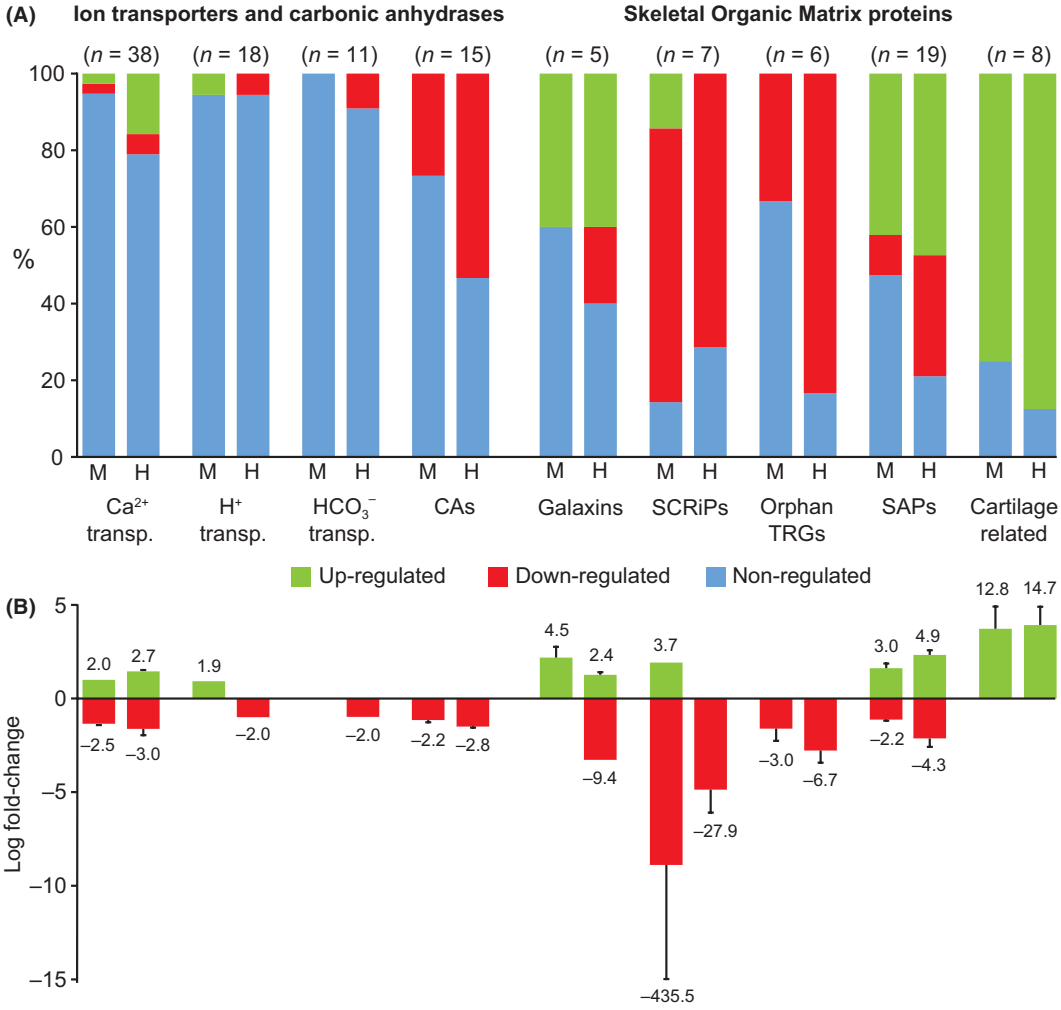

Fig. 5 Changes in levels of expression of candidate and known components of the biomineralization machinery. The bar graphs shown in (A) summarize the impact of medium (M) and high (H) pCO_2 treatments on the total complement of *Acropora millepora* transcripts in various categories (ion transporters, carbonic anhydrases and skeletal organic matrix (SOM) proteins). Transcripts whose levels changed significantly (adjusted $P < 0.01$) are indicated as follows: up-regulated in green, down-regulated in red and unchanged in blue. Total numbers of transcripts in each category are indicated above the corresponding bars. (B) Summary of the corresponding mean log fold-change values for each of the classes of genes responding in part (A). Actual fold-change values are indicated above or below each bar. Transp. = transporter, CA = carbonic anhydrase, SCRiP = small cysteine-rich protein, TRG = taxonomically restricted genes and SAP = secreted acidic proteins.

ent study, 4 and 8 of the total of 15 CAs identified in the *A. millepora* transcriptome were differentially expressed under medium and high pCO_2 exposure, respectively (27 and 53%, Figure 5A and Table S7, Supporting information). All of the differentially expressed CAs were down-regulated (on average 2.5-fold; Figure 5B). Maximum-likelihood phylogenetic analysis (Figure 6) suggests that 7 of 8 carbonic anhydrases that are differentially expressed under high pCO_2 conditions are likely to be secreted or membrane-associated forms, whereas only one of the differentially expressed CAs is likely to be a cytosolic type. As can be seen in Table S7 (Supporting information), the two carbonic anhydrases that were previously suggested to be associated with calcification by Grasso *et al.* (2008) (Cluster023744 and Cluster013858) differed with respect to their sensitivity thresholds.

Acidification alters the expression of many known and candidate Skeletal Organic Matrix proteins. The skeletal organic matrix forms the structural backbone of the biomineral; interconnected proteins, collagens and ion binder proteins provide sites for nucleation and govern the shape of the inorganic crystals that are precipitated. It is likely that many components of the skeletal organic matrix, including the galaxins, SCRiPs and secreted

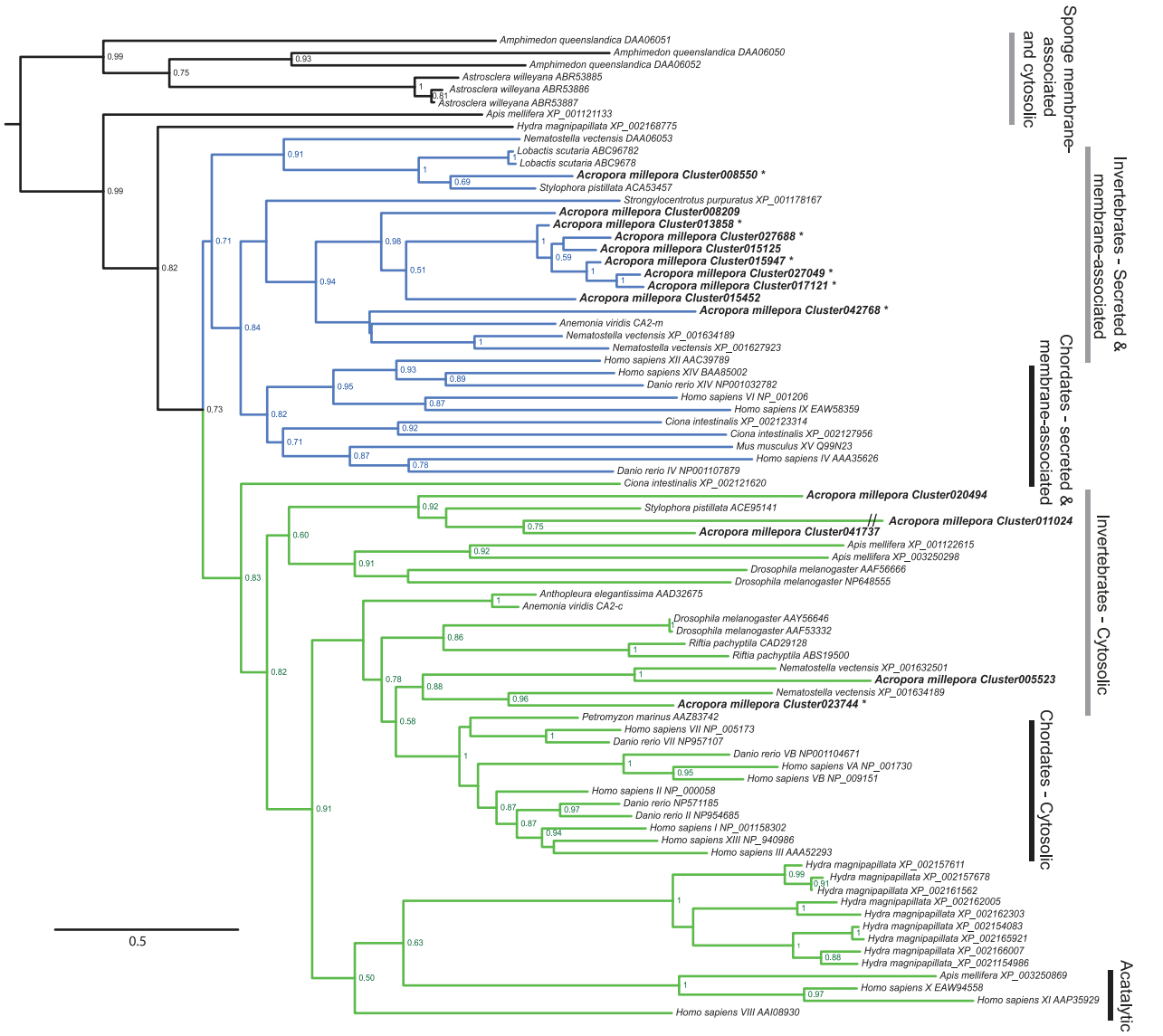

Fig. 6 Phylogenetic analysis of carbonic anhydrase sequences identified in the *Acropora millepora* transcriptome. The tree shown was inferred from maximum-likelihood (ML) analysis. aLRT (approximate likelihood-ratio test) branch support, based on a Shimodaira-Hasegawa-like procedure, was estimated with an LG model using PhyML (Guindon *et al.* 2010). Only values >0.50 are indicated beside nodes. The accession numbers indicated are from the Entrez (NCBI) protein sequence database. Branch lengths are proportional to the number of substitutions per site (see scale bar in the figure). *Acropora millepora* sequences are printed bold. An asterisk (*) indicates the differentially expressed sequences in our experiment. Branches corresponding to secreted and membrane-associated isoforms are in blue and branches corresponding to cytosolic isoforms in green.

acidic proteins, are taxonomically restricted genes or have coral-specific functions in calcification (see Introduction). In addition to the three galaxin-related genes that have been described previously in *A. millepora* (Reyes-Bermudez *et al.* 2009), 2 novel related sequences were identified in the transcriptome (Cluster007440 and Cluster015317, Table S8, Supporting information). The response of the individual galaxins was variable and inconsistent; under the medium CO_2 scenario, two 'adult-type' galaxins – Amgalaxin (Reyes-Bermudez *et al.* 2009) and a novel family member – were up-regulated, whereas under the high CO_2 scenario, an additional member of this group, galaxin-like 1, was down-regulated 9.4-fold (Figure 5B, Table S8, Supporting information). The SCRiPs (Small cysteine-rich proteins) are a family of putatively coral-specific genes for which roles in calcification have been suggested based on molecular features (presence of a signal peptide, high acidic amino acid residues content and cysteine-rich) (Sunagawa *et al.* 2009). For a subset of the

Acropora SCRiPs, *in situ* expression patterns are consistent with roles in calcification (Grasso *et al.* 2011; Hayward *et al.* 2011). In addition to the three SCRiPs previously known from *A. millepora* (Sunagawa *et al.* 2009), four novel SCRiP-related transcripts were identified in the present study and five of the total *A. millepora* repertoire of seven SCRiPs were down-regulated under elevated CO_2, one being completely switched off (Figure 5, Table S8, Supporting information). Although one of the novel SCRiPs was up-regulated and SCRiP1 showed no significant response, the general trend observed for the SCRiPs was down-regulation. In a microarray analysis of the response of *M. faveolata* to thermal stress, some SCRiP family members were among the most down-regulated genes (Sunagawa *et al.* 2009). Hence, the suppression of SCRiP expression seen here in response to CO_2 stress resembles the response of *M. faveolata* to heat stress – another condition known to affect calcification rates in corals (Marshall & Clode 2004).

In addition to galaxins and SCRiPs, several other putative taxonomically restricted genes have been implicated in early calcification in *A. millepora* on the basis of previous experiments (Grasso *et al.* 2011; Hayward *et al.* 2011); spatial patterns of gene expression for some of these (A036-B3, B036-D5 and C012-D9) are shown in Figure 7 and are consistent with roles in early calcification. With the sole exception of one sequence (Cluster019888), each of these candidate genes and two novel sequences closely related to A036-B3 were found to be down-regulated (mean of 4.8-fold) under elevated CO_2, with variable sensitivity thresholds (Figure 5, Table S8, Supporting information).

In the related coral *Acropora digitifera*, eight novel candidate skeletal organic matrix genes were identified by performing a genome-wide search for secreted, acidic

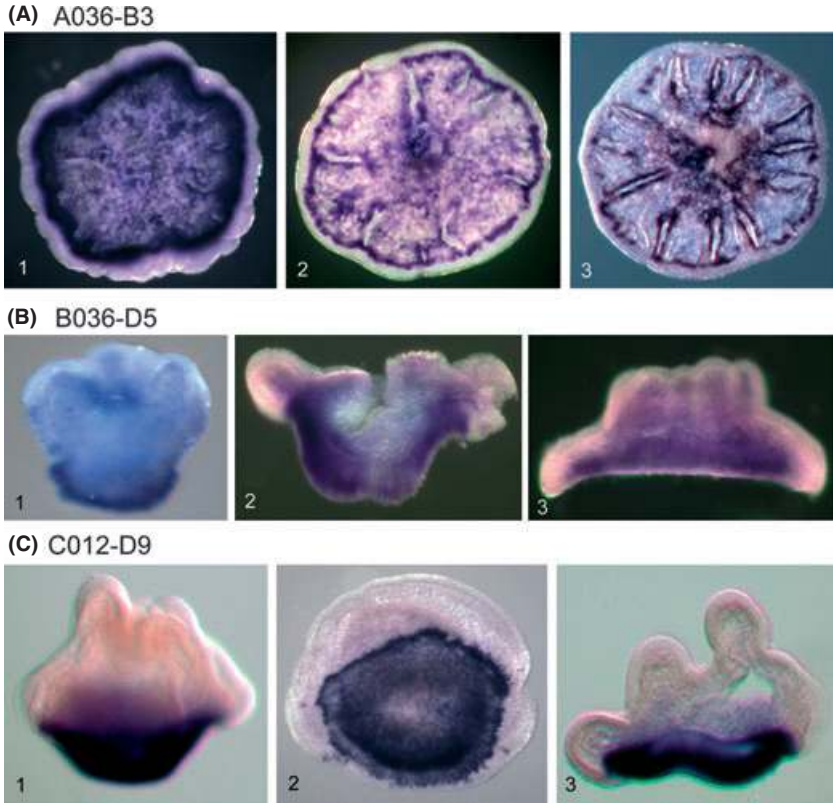

Fig. 7 Expression patterns of three coral-specific genes down-regulated under elevated CO_2 are consistent with roles in early calcification (A) A036-B3 is expressed in the area of the future calicoblastic layer (1) with later expression becoming concentrated at the border of the calicoblastic ectoderm and around the forming septa (2, 3). A036-B3 is down-regulated approximately 5-fold under high pCO_2 (Table S8, Supporting information). (B) B036-D5 is expressed at the aboral end of the metamorphosing polyp (1), after which expression becomes strongest in the developing calicoblastic ectoderm (2, 3). B036-D5 is down-regulated approximately 3-fold under high pCO_2 (Table S8, Supporting information). (C) C012-D9 is expressed at the aboral end of the metamorphosing polyp (1) and slightly later in the developing calicoblastic layer (2). The cut polyp in (3) shows strong expression in aboral ectodermal cells. C012-D9 is down-regulated approximately 5-fold under medium pCO_2 and >40-fold under high pCO_2 (Table S8, Supporting infomation).

sequences containing repetitive motifs (Shinzato *et al.* 2011), the rationale being that the soluble fraction of the skeletal organic matrix is known to be rich in acidic amino acids (Sarashina & Endo 2006). The secreted acidic proteins potentially act as high-affinity, low-sensitivity calcium binders, providing nucleation sites for aragonite precipitation in the skeletal organic matrix (Mitterer 1978; Cuif *et al.* 1999). Nineteen sequences with secreted acidic protein characteristics were identified in the *A. millepora* transcriptome, many of which were differentially expressed under elevated pCO_2 (10 and 15 under the medium and high pCO_2 conditions, respectively; Figure 5A, Table S8, Supporting information).

Whereas all of the putative components of the skeletal organic matrix discussed above are taxonomically restricted genes, some extracellular calcifying medium components may be conserved among metazoan lineages. On this basis, the *A. millepora* transcriptome was queried for counterparts of genes known to be involved in vertebrate bone and cartilage development. This search yielded a total of 8 genes with significant homology to components of the vertebrate cartilage matrix, all but one of which were highly up-regulated (mean of 13.7-fold, Figure 5 and Table S8, Supporting information) in response to elevated pCO_2.

Discussion

In the present study, exposure of *Acropora millepora* polyps to elevated levels of CO_2 led to decreased expression of metabolism-related genes, implying that metabolic suppression occurs. Physiological data for coral larvae (Nakamura *et al.* 2011) and reduced growth rates immediately postsettlement (Albright *et al.* 2010) support this idea, as do gene expression studies in calcifying sea urchin larvae (Todgham & Hofmann 2009; Donnell *et al.* 2010). Decreased expression of metabolic genes suggests that either there is a decreased demand for ATP or that metabolism is actively suppressed as an acute survival response, potentially allowing the reallocation of energy to more immediate stress response needs such as mucus production, the maintenance of intracellular pH homeostasis and/or immune defence. While suppression of metabolism may be an important short-term survival mechanism, chronic metabolic suppression may have negative consequences including compromised immune responses, inability to respond appropriately to additional stressors, and the impairment of gametogenesis (Holcomb *et al.* 2011). Metabolic suppression is likely to indirectly suppress calcification by affecting the energy-requiring transport steps of the process.

Carbonic anhydrases (CAs) play important roles in biomineralization in both invertebrates and vertebrates (Miyamoto *et al.* 2005; Tohse *et al.* 2006). In corals, it has been proposed that CA removes carbonic acid produced by calcification from the calcification site (Goreau 1959), and/or supplies inorganic carbon for calcification (Tambutté *et al.* 1996; Furla *et al.* 2000; Marshall & Clode 2003; Moya *et al.* 2008). The carbonic anhydrase repertoire of *Acropora* is relatively complex, and the expression of a large number of the genes encoding membrane-associated or secreted CAs, including those previously implicated in calcification on the basis of expression patterns (Grasso *et al.* 2008), was decreased significantly under the acidification scenarios employed here. The reasons for this are not clear; presumably, there is a reduced requirement for carbonic anhydrase activity in the extracellular calcifying medium and in the calicoblastic ectoderm under acidification, possibly to maintain appropriate pH at the site of calcification. As well as critical functions in calcification, carbonic anhydrases have other important roles, including in carbon concentrating mechanisms for dissolved inorganic carbon supply to symbiont photosynthesis (Furla *et al.* 2000) and in acid–base regulation by maintaining the availability of H^+ and HCO_3^- for transporters (Henry 1984; Burnett 1997). Organisms living in variably acidic and hypercapnic environments such as deep-sea hydrothermal vents (Goffredi *et al.* 1997) or estuaries (Burnett 1997) typically have a large capacity for acid–base regulation and this may also be the case for corals. It will be informative to examine the expression patterns of various CA isoforms via *in situ* hybridization or immunolocalization.

A current model of coral calcification involves ion transport from the external medium to the extracellular calcifying medium (Figure 1D; see Allemand *et al.* 2011 for a review) and, if this is correct, one might expect changes in the expression of various ion transport proteins under OA. However, this prediction was not fulfilled under the conditions used here; the expression of Ca^{2+}, HCO_3^- and H^+ transporters was essentially unaffected by the acute exposure to acid conditions. As more extreme pH shifts have been documented on reef flats on diurnal timescales (e.g. Ohde & van Woesik 1999), the fact that acute OA exposure has little effect presumably reflects the adaptation of coral metabolism to cope with short-term pH stress without significantly altering the expression of most ion channels.

In contrast to ion transporters, transcription of genes encoding known or putative proteins of the skeletal organic matrix was highly disturbed (78% of genes) by exposure to elevated CO_2. Relationships between CO_2 exposure and direction/fold-change were complex with, for example, similar numbers of skeletal organic matrix genes being up-regulated (40%) and down-regulated (38%) under the high pCO_2 condition. The micro-architecture of the coral skeleton dictates that the

skeletal organic matrix be spatially organized so that calcification is facilitated at some sites and prevented at others, so perhaps such a complex response is to be expected. Effectively, the skeletal organic matrix acts as a network to control, enhance, but also inhibit mineral deposition (Marin *et al.* 2007).

Within the classes of skeletal organic matrix components studied, individual members responded differently to elevated CO_2. For example, within the secreted acidic protein family, some genes were up-regulated and others down-regulated, suggesting specific and unique roles for those proteins in mineral deposition. A similarly complex response was found for the galaxins. The two galaxins whose expression is elevated under elevated CO_2 are Amgalaxin, which begins expression postsettlement but is much more strongly expressed in the adult (Reyes-Bermudez *et al.* 2009) plus a novel member of the galaxin family, whereas the one family member whose expression is strongly down-regulated under the high CO_2 scenario is Galaxin-like 1, which is expressed only during the early pre- and postsettlement period. Although the complexity of the response prevents a reliable reconstruction of the mechanism through which the skeletal organic matrix is affected by acidification, the overall disturbance of the skeletal organic matrix implied by the present study could explain the changes in size, shape, orientation and composition of the aragonite crystals observed in coral juveniles exposed to CO_2 (Cohen *et al.* 2009).

Some components of the skeletal organic matrix may not directly participate in aragonite deposition. It has been suggested that organic molecules may play roles in buffering the calcifying fluid against pH changes owing to fluctuations in internal CO_2 concentrations caused by diurnal cycles in photosynthesis and respiration (Holcomb *et al.* 2009), and some of the putative skeletal organic matrix components identified in the present experiment may function in this context. The observed down-regulation of other putative skeletal organic matrix components may reflect either specific suppression or a more general suppression of metabolism.

While the results presented here superficially appear to contradict the findings of Todgham & Hofmann (2009) with respect to skeletal organic matrix expression, they analysed a limited number of genes, so the results are not necessarily incompatible.

In terms of the impact of OA on calcification in corals, the main a priori concerns have been the likely direct impact of increasing calcium carbonate solubility and decreasing availability of CO_3^{2-}. However, the work presented here implies that decreased calcification under OA may in part reflect the diversion of energy away from $CaCO_3$ deposition to processes such as maintenance of pH homeostasis, and metabolic suppression leading to decreased availability of metabolic CO_2 for calcification. In addition to general metabolic suppression, the major transcriptional consequences of OA are reduced expression of many membrane-associated and secreted carbonic anhydrases, and gross disturbances in the expression of many known and putative components of the skeletal organic matrix. This latter effect presumably underlies the disorganization of aragonite precipitation that is the characteristic of coral juveniles grown under elevated CO_2.

The data presented here provide the first comprehensive overview of gene expression changes in a coral in response to elevated CO_2 but reflect a 'simple' reductionist scenario – acute exposure of the aposymbiotic life stage to a single stressor. They should therefore be viewed with the caveat that some of the changes in gene expression observed in juveniles in response to acidification may simply be irrelevant in the case of the adult coral because some genes are uniquely associated with early postsettlement life. While, as an initial attempt to understand the impact of acidification on coral gene expression, the reductionist approach taken here is appropriate, in the real world, the coral holobiont will face long-term exposure to a variety of stressors. Under global climate change, the combined effects of elevated temperature and OA are likely to be synergistic (Anlauf *et al.* 2011; Pandolfi *et al.* 2011). Sensible next steps for experimentation will be the extension of this approach to adult corals, longer term exposures and analysis of the transcriptomic impact of, and physiological responses to, compound stressors. The availability of whole genome sequences for several coral species (Shinzato *et al.* 2011, http://coralbase.org/) paves the way for this next important phase in the study of coral biology.

Acknowledgements

This research was supported by the Australian Research Council through Discovery Grant DP1095343 to DJM, EEB and SF and via the Centre of Excellence for Coral Reef Studies, and by a Marie Curie International Outgoing Fellowship to AM. LH was supported by the Netherlands Science Foundation (grant No. 645.100.005). This work is a contribution to the 'European Project on Ocean Acidification' (EPOCA), which received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement no 211384. The authors gratefully thank Andrew Baird and Bill Leggat for access to aquaculture facilities, Prof Philip Munday for developing the CO_2 mixing system and Marcelo Kitahara for advice on phylogenetic analyses.

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This project, which was carried out under the direction of Professor David Miller at James Cook University, was a truly international effort including researchers from France, the Netherlands, South Korea and Australia. Three younger scientists: AM, LH and SF performed most of the experimental work and much of the analysis. AM's research focuses on the molecular responses of calcifying cnidarians to environmental changes, LH on the analysis of coral development and calcification from a complex systems perspective and SF on interactions between genomes and their environment.

Data accessibility

Transcriptome sequences: GenBank accessions JR970414-JR999999, JT000001-JT023377.

Gene expression data: NCBI Gene Expression Omnibus (GEO) database accession number GSE33016.

Supporting information

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

Table S1 Summary of seawater parameters in control and acidification treatments.

Table S2 Differentially expressed genes belonging to the GO category ''extracellular region GO:0005576''.

Table S3 List of differentially expressed genes belonging to the GO category ''mitochondrion GO:0005739''.

Table S4 The response of calcium transporters to elevated levels of CO_2.

Table S5 The response of proton transporters to elevated levels of CO_2.

Table S6 The response of bicarbonate transporters to elevated levels of CO_2.

Table S7 The response of carbonic anhydrases to elevated levels of CO_2.

Table S8 The response of skeletal organic matrix proteins to elevated levels of CO_2.

Table S9 Primer sequences for the genes tested by real-time quantitative PCR.

Fig. S1 Fold-change distribution of the differentially expressed genes.

Fig. S2 Validation of the RNA-seq results by quantitative PCR.

Fig. S3 Quantitative comparison of expression profiles obtained from RNA-seq and RT-qPCR analysis.

Appendix S1 Supplementary methods.

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