Identification of differentially expressed genes during early growth of *Acropora tenuis*

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Abstract. During early growth of *Acropora*, the planula larvae metamorphose into polyps, skeletogenesis occurs, and a symbiotic relationship is established with *Symbiodinium*. Therefore, investigation of gene expression pattern during early growth stages of corals may help elucidate bone morphogenesis and symbiosis. To identify the coral genes that play important roles in symbiosis and skeletogenesis, mRNA expression profiles were compared among juvenile corals at varying stages of growth, specifically the nonsymbiotic planular larvae, aposymbiotic polyps (day 4 and 18), and symbiotic polyps (day 18). The genes coding for BMP-1, Vam6/Vps39-like protein, and cryptochrome were differentially expressed during the early growth of *Acropora tenuis*. The expression level of each transcript was higher in 18-day-old polyps than in the planula larvae and 4-day-old polyps. The expression level of the BMP-1 transcript was low in planula larvae but markedly elevated in 18-day-old polyps, suggesting that the BMP-1 may be involved in biomineralization.

Key words: Symbiosis, Bone morphogenesis, Coral, *Symbiodinium*, Gene expression.

Introduction

Reef-building corals live in obligatory mutualistic symbiosis with the symbiotic dinoflagellates *Symbiodinium* spp. (generally known as zooxanthellae). These corals receive photosynthetic products from the endosymbionts as nutrients (Muscatine, 1990). Photosynthesis by *Symbiodinium* cells results in enhancement of the calcification rate in the host skeleton, although the mechanisms linking the photosymbiosis to skeletogenesis remain largely unknown. In *Acropora tenuis*, a gene coding for a sulfate transporter was identified as a symbiosis-related gene, and the resultant protein was found to be localized between the coelenteron and skeleton of the adult colony (Yuyama et al., 2009). This observation suggests that the transporter is involved in the uptake of SO_4^{2-} for the synthesis of sulfated macromolecules contained in the organic matrix of the calcified skeleton. Orthologs of the bone morphogenetic proteins (BMPs) 2 /4 were observed in corals (Hayward et al., 2002; Zoccola et al., 2009), and their synthesis localised in the calcifying epithelium. In addition, carbonic anhydrase and calcium ATPase were shown to be related to skeletogenesis; these enzymes were also localized in the coral calcifying cell layer (Zoccola et al., 2004; Moya et al., 2008).

In hermatypic corals, naturally aposymbiotic larvae and the juveniles of certain coral species are useful for molecular analysis (Yuyama et al., 2005; deDore et al., 2007). One can collect symbiont-free planula larvae of *A. tenuis* and develop them into aposymbiotic polyps (Iwao et al., 2002). Symbiosis can be established experimentally between these polyps and some culturable *Symbiodinium* strains. Therefore, determining the origin (host, symbiont or others) of mRNAs isolated from the holobionts becomes easy by using juvenile polyps with monoclonal *Symbiodinium.* The polyps begin skeletogenesis after metamorphosis, so these juvenile polyps are useful for the study of coral skeletogenesis. To identify the coral genes that play important roles in the symbiosis and skeletogenesis of *A. tenuis*, mRNA expression profiles were compared among juveniles at varying stages of growth.

Material and Methods

Symbiodinium and coral samples.

Symbiodinium strain PL-TS-1 (MBIC10802) was obtained from Marine Biotechnology Institute Cultures Collection (Kamaishi, Japan, http://seasquirt.mbio.co.jp/mbic/). The collection of *A. tenuis* larvae and the induction of metamorphosis were performed as previously described (Iwao et al., 2002). *Symbiodinium* (approximately 1000 cells per

Clone No.	DDBJ/EMBL/ Gene Bank Accession No.	Length (bp)	Blastx search	Organism	E-value
AtSym35	BB999977	377	Bone morphogenetic protein 1	*Xenopus leavis*	1.00E-09
AtSym36	BB999978	420	Vam6/Vps39-like protein	*Heterocephalus glaber*	1.00E-09
AtSym37	BB999979	463	Cryptochrom CRY1	*Acropora millepora*	9.00E-45

Table 1: Homology/Similarity matches of genes with altered expression levels during early growth in *A. tenuis.*

polyp) were added to a half of polyps 120–144 hours after settlement. The juvenile polyps were grown in glass petri dishes (55 mm in diameter) at 24°C under a 12 h light (20 μE m^{-2} s^{-1})/12 h dark cycle. Each dish contained approximately 50 polyps in 40 ml of filtered (pore size 0.22 μm) seawater, which was changed daily.

RNA extraction

Coral larvae and juvenile polyps were homogenized using a T8.01 homogenizer (IKA-Werke GmbH & Co.) prior to the glass beads treatment. Total RNA was prepared from the samples using an absolutely RNA RT-PCR Miniprep Kit (Stratagene).

Virtual Northern analysis

A SMART cDNA Synthesis and Library Construction kit (BD Biosciences) was used for reverse transcription-PCR of coral total RNA. cDNA samples (1 μg per lane) were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. The fluorescence of the separated bands was measured using a computerized densitometer-scanner (FLA2000; FujiFilm). Subsequently, cDNA samples were electrically blotted onto NYTRAN membranes (Schleicher and Schuell). We selected genes of interest from a subtracted cDNA library constructed previously (Yuyama et al., 2005). As probes, we used each of the cDNA fragments with the pGEM T-Easy vector (Promega). The probes were generated by PCR using T7 and SP6 primers. Radiolabeling of the probes and hybridization were performed as previously described (Watanabe et al., 2000). The membranes were washed in three changes of 0.5× SSC and 0.1% SDS at 50°C, and the hybridization signals were measured using the FLA2000. To calculate the expression levels of coral mRNAs, the hybridization signals were normalized against the fluorescence levels of loading cDNA in the each lane of ethidium bromide - stained agarose gels. As a control experiment, equivalent blots were hybridized with a probe derived from presumptive housekeeping gene of *A. tenuis* (a fragment of the ribosomal protein L5 (AtRibo-L5) cDNA, (Fig.1).

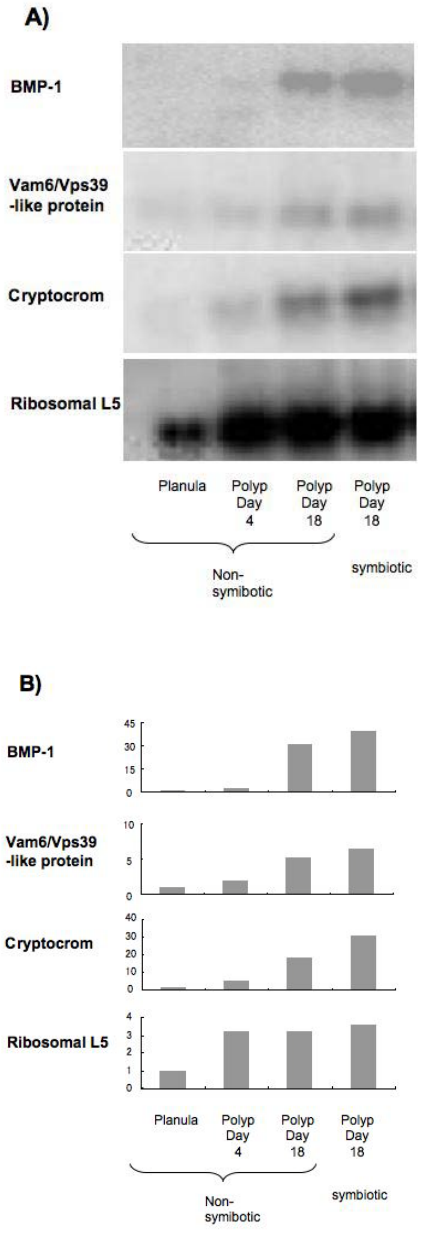

Figure 1: Virtual Northern Hybridization of gene coding BMP-1, Vam6/Vps39 –like protein, cryptochrom, and ribosomal protein L5(A). Each band shows ^{32}P-labeled cDNA probe hybridized to planula and polyps cDNA. The ratio of signal intensity (polyps/planula) by virtual Northern Hybridization is shown in (B).

Results

We prepared juvenile polyps at varying stages of growth to investigate the genes related to skeletogenesis and symbiosis. Metamorphosis of *A. tenuis* planula larvae into polyps was induced by Hym-248. One day after metamorphosis, *Symbiodinium* strain PL-TS-1 was introduced to a half of polyps. After settlement, the polyps began to undergo skeletogenesis. During the collection year (2005), only the PL-TS-1 strains naturally inhabited the host at high density. Symbiosis with the PL-TS-1 cells promoted the growth of the host (Yuyama et al., 2005). For expression analysis, we prepared nonsymbiotic planular larvae, aposymbiotic polyps (days 4 and 18), and symbiotic polyps (day 18).

Some functionally annotated genes were chosen from a subtracted cDNA library that had been previously constructed (Yuyama et al., 2005) and analyzed by virtual Northern blotting (Table 1, Fig. 1). As a result, BMP-1, Vam6/Vps39-like protein, and cryptochrome were identified as differentially expressed genes during the different growth stages of *A. tenuis*. As shown in Fig. 1, the expression level of each transcript was higher in 18-day-old polyps than in the planula or 4-day-old polyps. The transcript level of the BMP-1 ortholog was very low in the planula larvae. The BMP-1 ortholog exhibited a 30-fold greater expression level in the 18-day-old aposymbiotic polyps as compared with the planula larvae. Additionally, the expression level in the symbiotic polyps was 1.3-fold higher than in the aposymbiotic polyps (Fig. 1b). The expression levels of the Vam6/Vps39-like protein and cryptochrome orthologs were 5-fold and18-fold higher, respectively, in the 18-day-old aposymbiotic polyps than in the planula larvae. The transcript levels of these proteins tended to be higher in symbiotic than in aposymbiotic polyps (Fig. 1).All three transcripts were detected in aposymbiotic polyps and planula larvae, indicating their origin to be in the host corals.

Discussion

During early growth of *Acropora*, the planula larvae metamorphose into polyps, skeletogenesis occurs, and a symbiotic relationship is established with *Symbiodinium*. Therefore, investigation of the gene expression pattern during the early growth stages of corals may help to elucidate bone morphogenesis and symbiosis. In this study, three genes that were upregulated during early growth and symbiosis were analysed. Here, we discuss how these genes could be involved in growth and symbiosis.

The *Symbiodinium* strain PL-TS-1 densely inhabited *A. tenuis* polyps, and its population in the host increased. During preliminary studies, the sizes of symbiotic and aposymbiotic polyps were compared by height (Watanabe et al., 2007). The symbiotic polyps were found to be significantly taller than the aposymbiotic polyps, indicating that symbiosis enabled faster growth. Thus, the symbiotic polyps very likely utilize the photosynthetic products from the symbionts for skeletogenesis. Coral skeletogenesis begins after metamorphosis and is promoted during symbiosis. Therefore, genes with increased expression levels during metamorphosis and symbiosis may be related to skeletogenesis.

Most BMPs are multifunctional growth factors that belong to the transforming growth factor (TGF)-β superfamily. BMPs play important roles in postnatal bone formation. Animal studies have shown that BMP signaling plays critical roles in heart, neural, and cartilage development (Chen et al., 2004). Unlike other BMPs, BMP-1 does not belong to the TGF-β superfamily, but rather is the prototype of a family of putative proteases implicated in pattern formation during development in diverse organisms. BMP-1 is a protease involved in the processing of a series of extracellular structural proteins, including collagen and laminin (Amano et al., 2000). No previous report of BMP-1 in corals has been published. On the other hand, the Dpp/BMP-2/4 ortholog, central to the specification of the dorsoventral axis, was identified in *Acropora millepora* (Heyward et al., 2002). BMP-2/4-Am expression was localized in the ectodermal region adjacent to the blastopora in coral embryos. In *Stylophora pistillata*, BMP-2/4 antibody specifically labels the calicoblastic ectoderm (Zoccola et al., 2008). Therefore, BMP-2/4 might play a role in biomineralization in coral. In our study, the transcript level of the BMP-1 was elevated in 18-day-old polyps, but the expression level was very low in planula larvae (Fig. 1), suggesting that the BMP-1 may also be involved in growth of polyps or biomineralization.

The Vam6/Vps39-like protein and cryptochrome orthologs were expressed in planula larvae, and these expression levels increased in the polyps (Fig. 1). These proteins might not be directly concerned with skeletogenesis or symbiosis because these transcripts were expressed in planula larvae. The VAM has been shown to be involved in vesicle trafficking (Nakamura et al., 1997). Additionally the Vam6/Vps 39 has recently been shown to be involved in the TOR (target of rapamycin) activation pathway, a central regulation pathway for eukaryotic cell growth (Binda et al., 2009). Messler et al., (2010) reported Vps39 inactivation in the mouse germ lines. The data demonstrate essential roles of vps39 genes in early murine development as no embryos with gene-deficiency on both alleles survive beyond gastrulation. The gene coding for Vam6/Vps 39 were differentially expressed during the early growth of *A. tenuis* (Fig.1). This protein could have also essential roles in early

development of corals. Cryptochromes are blue light-sensing photoreceptors mediating various light responses in plant and animals (Cashmore et al., 1999). Cryptochrome expression in *A. millepora* increased on nights with a full moon versus a new moon (Levy et al., 2007). The cryptochromes may mediate the spectacular mass-spawning event in corals. In this study, the transcript level of cryptochrome was elevated in polyps compared with planula larvae, suggesting that the blue light-sensing photoreceptor may become activated after settlement.

The three genes coding for BMP-1, Vam6/Vps39 like-protein, and cryptochrome were genes that changed in expression during the early growth of corals. Further studies will be needed to investigate the localization of these proteins to help identify their functions in coral tissue.

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