Symbiosis-dependent gene expression in coral–dinoflagellate association: cloning and characterization of a P-type H^+ -ATPase gene[†]

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We report the molecular cloning of a H^+ -ATPase in the symbiotic dinoflagellate, *Symbiodinium* sp. previously suggested by pharmacological studies to be involved in carbon-concentrating mechanism used by zooxanthellae when they are in symbiosis with corals. This gene encodes a protein of 975 amino acids with a calculated mass of about 105 kDa. The structure of the protein shows a typical P-type H^+ -ATPase structure (type IIIa plasma membrane H^+ -ATPases) and phylogenetic analyses show that this new proton pump groups with diatoms in the Chromoalveolates group. This *Symbiodinium* H^+ -ATPase is specifically expressed when zooxanthellae are engaged in a symbiotic relationship with the coral partner but not in free-living dinoflagellates. This proton pump, therefore, could be involved in the acidification of the perisymbiotic space leading to bicarbonate dehydration by carbonic anhydrase activity in order to supply inorganic carbon for photosynthesis as suggested by earlier studies. To our knowledge, this work provides the first example of a symbiosis-dependent gene in zooxanthellae and confirms the importance of H^+ -ATPase in coral–dinoflagellate symbiosis.

Keywords: symbiodinium; coral; proton pump; carbon-concentrating mechanism

1. INTRODUCTION

Most transport proteins in photosynthetic organisms are energized by electrochemical gradients of protons across the plasma membrane. The formation of these gradients is due to the action of P-type ATPases that are a large, physiologically important family of membrane proteins that couple ATP hydrolysis to the active transport of cations across cell membranes (reviewed by Møller *et al.* 1996). A sequence analysis of conserved core sequences of all P-type ATPases has grouped them in five subfamilies designated Types I–V (Axelsen & Palmgren 1998). Plasma membrane H^+ -ATPases (PMA) belong to the Type IIIA of P-type ATPases. These pumps have been found in the plasma membrane of plants, fungi, protists and Archaea (Morsomme *et al.* 2000; Palmgren 2001; Luo *et al.* 2002). Structurally, the plasma-membrane H^+ -ATPase consists of a 100 kDa polypeptide subunit anchored in the lipid bilayer by four transmembrane segments at the N-terminal end of the molecule and six at the C-terminal end (Serrano *et al.* 1986; Kuhlbrandt *et al.* 2002). Plant H^+ -ATPases belong to multigene families (Harper *et al.* 1990, 1994), with individual members expressed in particular cell types. In some cases, up to three H^+ -ATPase genes may be expressed in the same cell type at the same developmental stage, suggesting that isoforms with distinct catalytic or regulatory properties may coexist (Palmgren 2001). In unicellular organisms,

the presence of several genes encoding H^+ -ATPases is also frequent (Morsomme *et al.* 2000).

Dinoflagellates belong to the Alveolates group, which also includes parasitic protists (*Plasmodium*, *Toxoplasma* and *Eimeria*). They play key roles in a number of ecosystems, but one of their most ecologically significant roles is in the symbiotic association of *Symbiodinium* sp. (colloquially termed zooxanthellae) with Cnidarians, particularly scleractinian corals. This symbiosis is the biological driving force that underpins establishment and maintenance of tropical coral reef ecosystems (Cesar 2000).

Recent changes to the environment surrounding coral reefs (e.g. global warming) have demonstrated that the endosymbiotic relationship between corals and *Symbiodinium* is particularly sensitive to environmental perturbation. The phenomenon known as ‘coral bleaching’ (for reviews, see Douglas 2003; Hoegh-Guldberg 2004) is characterized by the loss of symbiotic algae, their pigments or both and is a major contributor to the global decline of coral reefs. Despite the ecological importance of dinoflagellates, our knowledge of their genome is very limited.

The low level of CO_2 in the ocean (about 10 μM) has led marine phototrophs to develop CO_2 -concentrating mechanisms (CCM; see review by Giordano *et al.* 2005). However, in contrast to most marine phototrophs, symbiotic *Symbiodinium* sp. are not in direct contact with sea water since they live in the endodermal cells of corals or sea anemones (Muscatine & Hand 1958). As such, these dinoflagellates possess pathways to take advantage of host resources (Allemand *et al.* 1998;

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[†] Nucleotide sequence of the dinoflagellate proton pump gene cloned in this study is available in the GenBank under the accession number FJ807389 and the genome fragment is FJ807390.

Leggat *et al.* 1999). One possibility is that some of these pathways are identical in parasitic dinoflagellates, such as Blastodinales and Syndinales (Snyder *et al.* 2003). However, in addition to their role as obligatory endosymbionts for symbiotic anthozoans, zooxanthellae are also capable of existing as free-living organisms and so should also possess genes for most of the pathways present in free-living dinoflagellates. Thus, *Symbiodinium* serves as an intermediate form between the purely free-living photosynthetic dinoflagellates and the heterotrophic parasitic dinoflagellates. Using a pharmacological and physiological approach, Al-Moghrabi *et al.* (1996) investigated the mechanism for dissolved inorganic carbon transport by *in hospite* and cultured *Symbiodinium*. They have shown that *Symbiodinium* sp. displays considerable plasticity to change its mechanism of carbon supply depending on the environment. In symbiosis, the symbiont was found to express a proton pump on its membrane to mediate HCO_3^- dehydration for carbon supply in photosynthesis, while in a free-living state in culture, the symbiont expresses a $\text{Na}^+/\text{HCO}_3^-$ antiport (see Allemand *et al.* 1998 for a review).

In the present study, we report the cloning and sequencing of a *Symbiodinium* sp. gene that encodes a protein that is homologous to P-type H^+ -ATPases and its differential expression between the symbiotic and free-living stage.

2. MATERIAL AND METHODS

(a) Biological material

Experiments were conducted in the laboratory using the branching zooxanthellate scleractinian coral *Stylophora pistillata* (Esper, 1797). Colonies were cultivated as indicated in Tambutté *et al.* (1996). Cultured zooxanthellae (CZ) started from one single cell (Shick *et al.* 2005) and were obtained from the same coral species that was used for the experiments. The clonal cultures of *Symbiodinium* sp. were maintained in 500 ml screw-top polycarbonate Erlenmeyer flasks (Corning) in modified ASP-8A medium (Blank 1987) at pH 8.2. The zooxanthellae were grown as indicated in Goiran *et al.* (1996). *Symbiodinium* sp. isolated from *S. pistillata* belongs to tropical clade A1 as determined by polymerase chain reaction (PCR) fragment sequencing (Rowan & Powers 1991; Santos *et al.* 2002).

(b) Cloning of proton pump

Coral total RNA and poly(A)⁺ RNA were prepared as described previously (Zoccola *et al.* 1999). Oligonucleotide primers were designed to recognize two highly conserved amino acid motifs of cation ATPase genes, the ATP phosphorylation site, DKTGTLT, and the ATP binding site, TGDGVND, i.e. 5'-GAYAAGACKGGYACKCTKAC-3' and 5'-TCRTT MACRCCGTCRCCMGT-3' as the forward and reverse primers, respectively (Meade *et al.* 2000). PCR was performed at 94°C for 1 min, 55–62°C for 2 min and 72°C for 2 min per cycle (30 cycles) using *Taq* polymerase. PCR products were cloned into the pGEM-T vector according to the manufacturer's instructions. After determining the sequence of this fragment, specific primers were designed for 5' RACE (Rv1: 5'-CGTTTCGATGGCGATATTCAGG-3'; Rv2: 5'-CCGCTGTGTGCAATCGAGGGGG-3'; Rv3: 5'-TCGGCAGCAAGCCCAA GAAGTG-3') and 3' RACE (Fd1: 5'-CACTTCTTGGGCTTGCTGCCGA-3'; Fd2: 5'-CCTGAATATCGCCATCGAAACG-3') using the Roche

5'/3' RACE Kit. DNA sequencing of three independent clones was carried out on both strands with SP6 and T7 primer sequences by Macrogen Inc. Contig sequences were assembled using the Lasergene software (SEQMAN LASERGENE 7.1, DNASTar Inc).

(c) Phylogenetic constructions

BLASTX analyses were conducted on the NCBI server (Altschul *et al.* 1997) on GenBank database. Phylogenetic trees were constructed with both the maximum-likelihood (ML) and Bayesian methods in order to check for concordance of the results. The alignments of all amino acid sequences were performed with the Multalin server (Corpet 1988). Based upon the amino acid alignment, ML estimates of the topology and branch length were obtained using PHYML v. 3.0 (Guindon & Gascuel 2003) with the WAG+I+G model as recommended by alignment analysis with PROTTEST v. 2.0 (Abascal *et al.* 2005). Support for individual branches was inferred by bootstrap analyses (1000 replicates). Further phylogenetic relationships were investigated using Bayesian techniques as implemented in the computer program MrBAYES v. 3.1.2 (Huelsenbeck & Ronquist 2001) starting from a random tree, using the WAG model of amino acid substitution (WAG+I+G) generating trees for 800 000 generations with sampling every 100 generations, and with four chains in order to obtain the final (consensus) tree and to determine the posterior probabilities at the different nodes.

(d) PCR amplification from genomic DNA and cDNA

To determine whether the H^+ -ATPase was animal or algal in origin, PCR was performed on genomic DNA obtained from coral tissue and from CZ. Genomic DNA from the host and dinoflagellate was obtained by a method from Sambrook & Russell (2006). For PCR, the H^+ -ATPase primers consisted of the forward primer 5'-GACGCCCGCTGATGTGGAGTGG-3' and the reverse primer 5'-GCACCAGGACAATGATGATGCC-3' giving an amplification product of 320 bp. For the dinoflagellate-specific gene, we chose primers designed on the RuBisCO (ribulose-1,5-bisphosphate carboxylase oxygenase) gene. A forward primer, 5'-ACCGGCGTGGGCAAGCTGTTCTCT-3', and a reverse primer, 5'-TGGGAGTGGTCTGCTTCATG-3', were designed to amplify a product with a size of 433 bp. The host-specific gene calcium ATPase, described in detail in Zoccola *et al.* (2004) was amplified using primers forward 5'-ACCATGGCAGAACCTTCAATTAA-3' and reverse 5'-CCATCGATCCAGCCAGTGTGTCT-3' with an amplified product size of 438 bp. Fifty nanograms of template were used for each reaction and all reactions were cycled 35 times with an annealing temperature of 62°C.

To compare the expression of H^+ -ATPase in symbiotic coral versus CZ, a present/absent PCR assay was performed on cDNA using the primers cited above. The RuBisCO protein gene expression was used as a control for both expressions in symbiotic and CZ.

3. RESULTS

(a) Identification and characterization of a P-type proton pump

Degenerate oligonucleotides corresponding to two conserved domains of P-type ATPases, a phosphorylation site and a site involved in ATP binding (Luo *et al.* 2002), were used to amplify, by PCR, specific sequences

from *S. pistillata* holobiont (host + symbiont) cDNA. The PCR products were cloned and sequenced. GenBank comparisons using BLASTX indicate that our partial sequence corresponds to P-type-like H^+ -pumps (for example, $p = 4e^{-28}$ and 33% identity with the green algae *Dunaliella bioculata*). RACE experiments allowed us to obtain the full-length cDNA. The complete sequence is 3136 bp long and contains, at 55 bp from the 5' end, a methionine in a Kozak's (1984) context followed by an open reading frame (ORF) of 2925 bp. This ORF codes for a protein of 975 amino acids and a calculated molecular mass of approximately 105.5 kDa. By BLAST analysis, the best alignment of the deduced amino acid sequence is obtained with the *Phaeodactylum tricornutum* PMA protein (Bowler et al. 2008), 44 per cent in identity and 63 per cent in conservative substitutions.

Figure 1 shows the amino acid sequence of the predicted PMA protein isolated from the holobiont and its 10 transmembrane domains obtained with the Phobius algorithm (Kall et al. 2004) together with an alignment with *Saccharomyces cerevisiae* PMA1 and *Arabidopsis thaliana* AHA1 gene products. Our sequence contains two motifs common to all P-type ATPases, which were the basis of the original PCR primers (dashed lines in figure 1). The first sequence, DKTGTLT, starts with the aspartate that is phosphorylated during the catalytic reaction. The second is TGDGVND (Axelsen & Palmgren 1998), which links the large cytosolic domain to the membrane-associated C-terminal domain of P-type ATPases (Møller et al. 1996). Our PMA sequence also contains most of the amino acid residues and short peptides that are common to type IIIA P-type ATPases but are not conserved in other subgroups of P-type ATPases (boxed in figure 1; Axelsen & Palmgren 1998). However, there are three discrepancies: a methionine instead of a tryptophane (aa 506), a lysine instead of an arginine (aa 554) and a lysine instead of arginine (aa 636).

Analysis of phylogenetic construction, after a MULTALIN alignment (Corpet 1988) of complete amino acid sequences of 84 previously cloned proteins, shows 11 subfamilies (figure 2): (i) the first subfamily corresponds to Archaeobacterium soluble P-type ATPase and might represent an ancestral P-type ATPase subfamily (for a review, see De Hertogh et al. 2004), (ii) two subfamilies represented in the Fungi, Ascomycota and Basidiomycota, (iii) five distinct subfamilies identified in plant Streptophyta species (Palmgren 2001), (iv) a single subfamily represented in plants Chlorophyta, (v) another single subfamily for the Euglenozoa and (vi) finally the Chromoalveolata subfamily where the cloned holobiont H^+ -ATPase groups, suggesting that the PMA gene that we cloned belongs to the symbiotic dinoflagellate rather than to the host.

(b) Expression of *Symbiodinium* sp. proton pump

To confirm that the proton pump belongs to the symbiont, PCRs performed with specific primers show that the gene is present in CZ genome (figure 3a). RuBisCO gene amplification was used as a positive control of dinoflagellate gene amplification. Considering the evidence from the Blast comparison, phylogenetic tree and genomic DNA PCR, this new cloned gene appears to belong to *Symbiodinium*

sp. and not the coral host, and codes a P-type H^+ -ATPase. Thus, the cloned gene is referred to as *SspPMA1* for *Symbiodinium* sp. P-type membrane ATPase 1 (accession number FJ807389). The genomic PCR product of the proton pump is longer (700 bp) than the cDNA (320 bp) owing to the presence of two small introns in the PCR fragment (accession number FJ807390).

Finally, we investigated the expression of the *SspPMA1* gene in total RNA extracted from the holobiont or from cultured *Symbiodinium* sp. For this purpose, we performed reverse transcription (RT) PCR (figure 3b). Results show that the proton pump is expressed *in hospite* but not in CZ. We used the Ca^{++} -ATPase gene as a control since it belongs to the animal part of the coral (Zoccola et al. 2004). As expected, the Ca^{++} -ATPase pump is not present in zooxanthellae. RuBisCO, used as a control for the dinoflagellate genes expression, is present both in the holobiont and in the CZ.

4. DISCUSSION

In this work, we have demonstrated that a gene encoding a protein highly related to P-type H^+ -ATPase, referred to as *SspPMA1*, is present in the *Symbiodinium* genome. This is supported by (i) PCR experiments on the holobiont and CZ that demonstrated the presence of the *SspPMA1* gene in the zooxanthellae genome and a specific expression only when they are in symbiosis with coral and (ii) phylogenetic analysis that groups the *Symbiodinium* ATPase with other Chromoalveolate sequences.

To date, the Chromoalveolates include six phyla, divided into two subgroups: Alveolata, which include dinoflagellates, apicomplexans and ciliates, and Chromista represented by haptophytes, cryptophytes and stramenopiles (Reeb et al. 2009). The apicomplexan group contains parasitic organisms of significant interest, such as *Plasmodium* that causes malaria. Apicomplexan parasites are characterized by the loss of photosynthetic function with unpigmented chloroplast remnants termed apicoplasts. P-type proton pumps have already been characterized in apicomplexan parasites (Holpert et al. 2001) but *SspPMA1* is the first P-type H^+ -ATPase characterized in a phototrophic alveolate.

If we compare the dinoflagellate proton pump with either yeast (*S. cerevisiae*) or plant (*A. thaliana*), we observe some specific characteristics (figure 1). The *Symbiodinium* pump possesses the C-terminal peptide present in yeast PMA1 that constitutes the non-essential inhibitory domain involved in the regulation of the enzyme by glucose metabolism (Portillo et al. 1989). Indeed, in this peptide, phosphorylation of serine and threonine is clearly related to glucose activation in yeast (Lecchi et al. 2007). These residues are present in *SspPMA1* (Ser-929 and Thr-930). In plants, the R-domain of P-type H^+ -ATPase consists of approximately 100 amino acid residues and is composed of two important regions (I and II) for the auto-inhibitory role of the C-terminus (Axelsen et al. 1999). In the *Arabidopsis* proton pump, AHA1, these domains are located between Lys863 to Leu885 (I) and Ser904 to Leu919 (II). These regions in *Symbiodinium* (I, Lys882 to Val908; II, Ser924 to Asn945) lack homology with the *Arabidopsis* ones (figure 1). Furthermore, the penultimate Thr (Thr947 in *Arabidopsis*), for which phosphorylation is needed for a 14-3-3 protein binding (Maudoux et al.

Figure 1. ClustalW alignment of putative H⁺-ATPase amino acid sequences from *Symbiodinium* sp., *SspPMA1* (GenBank accession number FJ807389), *S. cerevisiae* PMA1 (accession number P05030), and *A. thaliana* AHA1 (accession number P20649). Identical amino acids (AA) among all sequences are shaded in dark grey, AA that *SspPMA1* shares with one of the two other sequences are in light grey, AA that are absent from other sequences are denoted by dashes. The AA sequences corresponding to the conserved catalytic autophosphorylation and ATP-binding regions, and used for the design of cloning primers, are indicated by dashed lines. Transmembrane domains I–X are underlined. Boxes indicate specific motifs to type IIIA P-type ATPases. Arrowheads show conserved residues that have been studied by site-directed mutagenesis.

Figure 2. Phylogenetic analysis of *SspPMA1* (indicated in bold as *Symbiodinium* sp.) and 84 previously cloned H⁺-ATPases sequences inferred from both maximum-likelihood (ML) and Bayesian analyses. The results from ML bootstrap analysis are shown above the branches, only values greater 50% are indicated. The full and dashed branches represent greater than 95% and 50–95% Bayesian posterior probabilities, respectively. The accession numbers are from the protein sequence database Entrez from National Center of Biotechnology Information. The different subfamilies are coming from [Palmgren \(2001\)](#). The branch lengths are proportional to the number of substitutions per site (see scale bar in the figure).

Figure 3. (a) PCR of genomic DNA from coral holobiont and CZ, using RuBisCO, Ca^{2+} -ATPase and H^{+} -ATPase gene primers. (b) PCR of cDNA from coral holobiont and CZ using the same primers. RuBisCO (accession number AY996050) primers are used as specific primers for the dinoflagellate component of the holobiont DNA and Ca^{2+} -ATPase (accession number AY360080) primers as specific primer for animal component.

2000), is missing in *Symbiodinium* sequence, suggesting that there is no regulation of ATPase activity by 14-3-3 protein. Lastly, the serine residue (Ser931 in AHA1), phosphorylated by PKS5 (Fuglsang *et al.* 2007) is not present in *Symbiodinium*. However, the C-terminal region in *Symbiodinium* is longer than that found in yeast. In addition, similarities with *Arabidopsis* are great in this region (from transmembrane segment IX to the C terminus). Moreover, a number of amino acids known, from site-directed mutagenesis studies, to have a role in other H^{+} -ATPases are also conserved in *SspPMA1*. For example, mutation of *S. cerevisiae* Gly158 (Gly95 in the cloned PMA; figure 1, first arrowhead) confers a hygromycin resistance phenotype (Perlin *et al.* 1989). Mutation of Asp730 in *S. cerevisiae* (Asp707 in the cloned PMA; figure 1, second arrowhead) abolishes ATPase activity and proton transport (Perlin *et al.* 1989). In conclusion, the *Symbiodinium* H^{+} -ATPase presents characteristics from both plants and fungi and is related to Chromoalveolates group in which data are still scarce.

Many marine cnidarians, such as stony corals or sea anemones, are engaged in intracellular symbiosis with dinoflagellates, forming the trophic and structural foundation of the coral reef ecosystem. Very little is known about the cellular and molecular mechanisms that are at work in these associations. The coral houses the

intracellular symbiont within its gastrodermal tissue layer and receives the nutritional benefits of a resident primary producer (Trench 1979; Muscatine 1980). Following carbon fixation by the zooxanthellae, much of the photosynthates are exported to the host and can contribute up to 100 per cent of the host's energy requirements (Muscatine & Cernichiaro 1969). The supply and fixation of carbon therefore has a major influence on the symbiosis. It has been shown that zooxanthellae use a CCM (Allemand *et al.* 1998; Leggat *et al.* 1999). Such a CCM also exists in diatoms where its functioning is still a matter of debate (for a review, see Roberts *et al.* 2007). As described in §1 based on pharmacological and physiological approaches, Al-Moghrabi *et al.* (1996) and Goiran *et al.* (1996) proposed that *in hospite*, an H^{+} -ATPase located in the plasma membrane of the zooxanthellae may acidify the perisymbiotic space, and then participates, with a putative membrane-bound carbonic anhydrase (CA), in the carbon supply for symbiont photosynthesis. Since P-type proton ATPases are probably involved in CCM and therefore may undergo a specific selective pressure, it could explain why *SspPMA1* does not group with the non-phototrophic dinoflagellates' evolutionary closest relatives (apicomplexans) in our phylogenetic analysis (figure 2) but with another marine phototroph, *P. tricornutum*, that may possess a more closely related proton pump owing to a convergent evolutive phenomenon. The removal of such a selective pressure by the loss of photosynthesis in apicomplexans could have facilitated mutations in conserved domains (i.e. important for protein function) and then explain the weak support values for grouping of *Symbiodinium* sp. and *P. tricornutum* sequences with *Toxoplasma*, either in ML bootstrap (less than 50%) or Bayesian analysis (77%). By contrast, free-living *Symbiodinium* in culture, mediate carbon supply by a Na^{+} -dependent HCO_3^{-} uptake (Al-Moghrabi *et al.* 1996). The data in the present study confirm that zooxanthellae change their mechanism of carbon supply depending on their symbiotic state. Specifically, we have demonstrated a differential expression of the H^{+} -ATPase between *in hospite* and CZ. This protein is only present in the holobiont, and since it does not belong to the host genome (see above), we can conclude that it is specifically expressed by the zooxanthellae. Therefore, this study confirms previous results showing that the physiology of the symbiont, as well as the host, is profoundly modified by symbiosis in terms of CO_2 supply (Furla *et al.* 2005). *In hospite*, the symbionts behave as intracellular organelles adopting a H^{+} -driven uptake mechanism while in free-living stage, they adopt Na^{+} -based mechanisms owing to the high level of Na^{+} in sea water. It has also been shown in giant clams that there is a shift in inorganic carbon uptake (from CO_2 to HCO_3^{-}) when isolated zooxanthellae are cultured in sea water for 2 days (Leggat *et al.* 1999, 2002). Further studies will help us to understand how regulation of the transcriptome occurs during the transition between the symbiotic and the free-living forms.

With regard to cnidarian–algal associations, many studies have recently focused upon animal transcriptomic and/or proteomic changes when studying the onset and establishment of symbiosis in order to uncover symbiosis-specific changes in gene or protein expression

(Barneah *et al.* 2006; Rodriguez-Lanetty *et al.* 2006; deBoer *et al.* 2007). Weis & Reynolds (1999) described the differential expression and synthesis of two animal proteins by comparison between symbiotic and aposymbiotic anemones: a CA and a protein involved in cell–cell interaction (sym32, Reynolds *et al.* 2000). However, these genes are not symbiosis-specific since they are also expressed, at a lower level, in aposymbiotic animals. In the same manner, Richier *et al.* (2005) have shown an increased superoxide dismutase (SOD) activity in symbiotic anemones compared with aposymbiotic. At a genetic level, six expressed sequence tags (ESTs) and 91 host unigenes that are differentially expressed in symbiotic state have been characterized so far (Rodriguez-Lanetty *et al.* 2006; deBoer *et al.* 2007). However, up to now, no protein belonging to zooxanthellae has been shown to be specifically expressed in the symbiotic state. Only Richier *et al.* (2005) have provided an example of a downregulation in SOD activity in zooxanthellae after symbiosis onset. The recent development of EST library from dinoflagellates (Leggat *et al.* 2007) will allow new investigations but, currently, the P-type H^+ -ATPase gene *SspPMA1*, characterized in the present study, is the first symbiosis-specific gene described in zooxanthellate symbionts.

In conclusion, the current study demonstrates that *Symbiodinium* sp. possesses P-type H^+ -ATPases, which are potentially involved in a CCM to supply CO_2 from the host to the symbiont for photosynthesis. Furthermore, this is the first report of an activation of a dinoflagellate gene by the association with coral. Further studies might focus on the mode of regulation of this gene.

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