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Dual recognition activity of a rhamnose-binding lectin to pathogenic bacteria and zooxanthellae in stony coral *Pocillopora damicornis*

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ABSTRACT

Rhamnose-binding lectin (RBL) is a type of Ca^{2+}-independent lectin with tandem repeat carbohydrate-recognition domain, and is crucial for the innate immunity in many invertebrates. In this study, the cDNA sequence encoding RBL in coral *Pocillopora damicornis* (PdRBL-1) was cloned. The PdRBL-1 protein shared highest amino acid sequence similarity (55%) with the polyp of *Hydra vulgaris*, and contained a signal peptide and two tandem carbohydrate-recognition domains in which all cysteine residues were conserved. Surface plasmon resonance method revealed that the recombinant PdRBL-1 protein bound to LPS and Lipid A, but not to LTA, β-glucan, mannose and Poly (I:C). Results also showed that it bonded with zooxanthellae using western blotting method, and that the bound protein was detectable only at concentrations higher than 10^2 zooxanthellae cell mL^{-1}. When recombinant PdRBL-1 protein was pre-incubated with LPS, lower amounts of protein bound to zooxanthellae compared to cells not pre-incubated with LPS. Furthermore, PdRBL-1 mRNA expression increased significantly at 12 h, and declined to the baseline at 24 h after heat stress at 31 °C. These results collectively suggest that PdRBL-1 could recognize not only pathogenic bacteria but also symbiotic zooxanthellae, and that the recognition of zooxanthellae by PdRBL-1 could be repressed by pathogenic bacteria through competitive binding. This information allows us to gain new insights in the mechanisms influencing the establishment and maintenance of coral-zooxanthella symbiosis in coral *P. damicornis*.

1. Introduction

Stony corals form complex mutualistic symbiosis with unicellular photosynthetic dinoflagellates (zooxanthellae), allowing them to proliferate and reproduce even in oligotrophic conditions (Rosic et al., 2014). Symbiotic zooxanthellae are harbored in the stony coral's endodermal cells which supply their hosts with organic carbon and oxygen. The organic carbon and oxygen generated from photosynthesis are served as the main source energy of entire coral reef ecosystems (Kavousi et al., 2015; Shinzato et al., 2014). In return, the coral host supplies carbon dioxide and indispensable inorganic nutrition for the symbiotic zooxanthellae and the protection against other grazers or predators (Hoegh-Guldberg et al., 2007). However, despite the apparent significance of this symbiosis not only to the host but also to the entire coral reef ecosystems, many mechanisms underlying its establishment and maintenance remain to be explored.

It has been suggested that the first step in establishing the symbiosis is the chemical recognition of the coral host and symbiont commonly mediated by lectin and glycan molecules (Iguchi et al., 2011; Kita et al., 2015; Wood-Charlson et al., 2006). For example, the lectin in the octocoral *Sinularia lochmodes* (SLL-2) and coral *Acropora millepora* (Millectin) binds with D-galactose and mannose in zooxanthella surface, respectively (Jimbo et al., 2013; Kvennefors et al., 2008). Specifically, the lectin in the coral *Ctenactis echinata* has been shown to bind with lactose, melibiose, and D-galactose, which results to the transformation of zooxanthellae from a flagellated motile form to a nonmotile coccoid form (equivalent to the symbiotic stage), and even suppress its growth (Jimbo et al., 2010). In *S. lochmodes* SLL-2, the transformation of zooxanthellae after its binding was attenuated by the presence of

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glycosidases or N-acetyl-D-galactosamine (Jimbo et al., 2013). Furthermore, trehalose has also been shown to function as a chemical attractant in the establishment of symbiosis in the coral *Fungia scutaria* (Hagedorn et al., 2015). Therefore, the recognition of the zooxanthellae by host coral as mediated by lectin plays a significant role in the establishment and maintenance of symbiosis, and thus, is indispensable.

Lectins are generally important as pattern recognition receptors (PRRs) in invertebrates, which allows them to discriminate non-self from self through the binding to pathogen-associated molecular patterns (PAMPs) including glucan, mannose, LPS among others (Arason, 1996; Hanington et al., 2010; Marques and Barracco, 2000; Yang et al., 2015). These lectins are therefore involved in the invertebrates' innate immune responses, and act as the only defense against invading microorganisms (Loker et al., 2004; Wang and Wang, 2013). In arthropods, lectins are thought to be involved in suppressing inflammations due to viral and bacterial infections by inducing the production of antibiotics to prevent their proliferation (Lecchini et al., 2014; Wang et al., 2014). In mollusks, lectins can serve as opsonin to mediate the phagocytosis and encapsulation of invading pathogenic microorganisms (Yang et al., 2011). It has also been shown in cnidarians that lectins have agglutination activity against various bacteria but can be inhibited by some saccharides and glycoproteins (Imamichi and Yokoyama, 2010; Kvennefors et al., 2008). Coral lectins, like Millectin and tachylectin, have also been observed to participate in the immunity, suggesting the potential of coral lectins recognizing not only the symbiotic zooxanthellae but also the other pathogenic microorganisms in the endosymbiont. This potential function of coral lectins however remains to be further understood.

Pocillopora damicornis, a coral mainly distributed in the tropical/subtropical area of the Indian and Pacific Oceans, belongs to Pocilloporidae. In the wake of global warming, increased sea surface temperature has resulted to the expulsion of the symbiotic zooxanthellae and the bleaching of stony corals. Because coral lectins play significant roles in the recognition and stabilization of zooxanthellae, understanding of their function would pave a way to further understand the underlying mechanisms in the establishment and maintenance of coral-zooxanthella symbiosis, such as in bleaching events. In this study, we cloned the RBL gene in the coral *P. damicornis* (here as PdRBL-1), and used to investigate the affinity of PdRBL-1 to PAMPs and zooxanthellae. Then, we determined the effect of PAMPs on the zooxanthella recognition of PdRBL-1 and changes in its expression after heat stress. Results of this study will contribute to the further understanding of possible mechanisms underlying coral bleaching due to heat stress and the potential onset of pathogenic infections.

2. Materials and methods

2.1. Coral collection and heat stress treatment

Coral *P. damicornis* colonies were collected from the coral reef in Wenchang, Hainan Province, China, and transferred and cultured in flow-through aquaria (ca. 100 L) filled with filtered seawater (26 °C) at Hainan University. Cultures were illuminated with four fluorescent bulbs (Philips T5HO Activiva Active 54 W) in a 12 h/12 h light-dark cycle for one month to acclimatize in laboratory conditions.

Thirty coral nubbins were prepared from the clones of the same *P. damicornis* isolate. Twelve coral nubbins were incubated at 32 °C, which we referred hereafter as the heat stress group, while another twelve coral nubbins remained at 26 °C and used as the control group. Subsequently, six nubbins were sampled in each group at 12 and 24 h of incubation, while nubbins at 0 h were considered as the blank group (no treatment). Each sample was stored in liquid nitrogen immediately for RNA extraction.

2.2. RNA extraction, cDNA synthesis and qRT-PCR analysis of PdRBL-1 mRNA expression

Total RNA was extracted from coral nubbins using Trizol reagent (Invitrogen) following the manufacturer's protocol. The cDNA library was then constructed using Promega M-MLV kit, and diluted to 1:40 for the subsequent experiments. The qRT-PCR and $2^{-\Delta\Delta Ct}$ method were employed to determine and estimate the mRNA expression levels of PdRBL-1 in coral nubbins under different temperature conditions as described in Zhou et al. (2012). All the primers used were listed in Table 1 and the fragment of elongation factor (PdEF) was used as the endogenous control. Three replicates were tested for each sample or nubbin.

2.3. Gene amplification and sequence characterization

Transcriptome libraries of the stony coral *P. damicornis* after ammonium stress were previously constructed and sequenced, and transcript assembly yielded 77,199 coral-derived transcripts (Yuan et al., 2016). Based on BLAST results, one transcript (No. TR53818|c0_g1_i1) was homologous to the RBLs identified previously in other animals.

The whole-length cDNA sequence of PdRBL-1 was then obtained via rapid amplification of cDNA ends (RACE) method (Wang et al., 2012). The Expert Protein Analysis System was applied in the amino acid analysis (http://www.expasy.org/). The protein domain was predicted with SMART software (http://smart.embl-heidelberg.de/). Multiple sequence alignment of PdRBL-1 with the RBLs from other species (downloaded from GenBank) was employed by the ClustalW software.

2.4. Preparation of purified recombinant PdRBL-1 protein

The cDNA fragment encoding the mature peptide of PdPBL-1 was cloned and inserted into pEASY-E1 expression vector (TransGen, China). After transformation and screening for positive inserts using PCR, plasmids with correct inserts were isolated and transformed into *E. coli* BL21 (DE3)-Transetta (TransGen, China). Then, the positive transformants were isolated and protein expression was induced by adding IPTG at the final concentration of 0.5 mmol L^{-1}. Finally, recombinant PdRBL-1 protein (here as rPdRBL-1) was purified by a Ni^{2+} chelating Sepharose column (Sangon Biotech).

2.5. PAMP binding assay based on surface plasmon resonance

The binding activity of rPdRBL-1 to PAMPs was tested with surface plasmon resonance (SPR) method following the previous study (Xu et al., 2016). Analysis of ligand binding kinetics was performed at 24 °C on a BIAcore T200 SPR instrument (GE Healthcare). His-tag antibody was first immobilized onto CM5 chip, then 1.0 mg mL^{-1} PAMPs was injected including LPS (lipopolysaccharide. *E. coli* 0111:B4), Lipid A, LTA (*Staphylococcus aureus*), β-Glucan (*Euglena gracilis*), Mannose (*Saccharomyces cerevisiae*) and Poly (I:C). After 5 min of dissociation, rPdRBL-1 and bound analytes were washed with glycine-HCl. Data was analyzed with BIAcore T200 evaluation software.

2.6. Zooxanthella binding assay of rPdRBL-1

The recognition of rPdRBL-1 to zooxanthellae was tested via western blotting. Briefly, live zooxanthellae were first isolated from *P. damicornis* as described in Shaw et al. (2012), and diluted with