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# cDNA cloning and mRNA expression of a tandem-repeat galectin (PoGal2) from the pearl oyster, *Pinctada fucata*

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**ABSTRACT.** Galectins can recognize and specifically bind to β-galactoside residues, playing crucial roles in innate immune responses of vertebrates and invertebrates. We cloned the cDNA of a tandem-repeat galectin from the pearl oyster *Pinctada fucata* (designated as PoGal2). PoGal2 cDNA is 1347 bp long and consists of a 5'-untranslated region (UTR) of 3 bp, a 3'-UTR of 297 bp with one cytokine RNA instability motif (ATTTA), and an open reading frame of 1047 bp, encoding a polypeptide of 349 amino acids, with an estimated molecular mass of 38.1 kDa and a theoretical isoelectric point of 8.5. PoGal2 contains two carbohydrate recognition domains (CRDs); both have the conserved carbohydrate-binding motifs H-NPR and WG-EE. PoGal2 shares 50.6 and 50.9% identity with those of abalone (*Haliotis discus*) and the Manila clam (*Venerupis philippinarum*), respectively.

 

Phylogenetic analysis revealed that the tandem-repeat galectins formed two clades for the different species. Molluscan tandem-repeat galectins were clustered into a single clade, and nematode tandem-repeat galectins were clustered into another single clade. In both clades, CRD-N and CRD-C were divided into different groups. PoGal2 mRNA was constitutively expressed in all tissues analyzed, and the expression level of PoGal2 mRNA was found to be significantly up-regulated in digestive glands, gills and hemocytes after *Vibrio alginolyticus* stimulation/infection. Expression profile analysis showed that the expression level of PoGal2 mRNA was significantly up-regulated at 8, 12 and 24 h after *V. alginolyticus* infection. These results suggest that PoGal2 is a constitutive and inducible acute-phase protein involved in the innate immune response of pearl oysters.

**Key words:** *Pinctada fucata*; Pearl oyster; Lectin; β-galactoside; Innate immunity

## INTRODUCTION

Pearl oyster *Pinctada fucata* is distributed along the coastal area of South China and is the most important bivalve mollusk for seawater pearl production in China. However, since the mid-1990, pearl oyster has suffered serious diseases caused mainly by bacteria (Lau et al., 2006), a rickettsia-like organism (Wu and Pan, 1999), parasites (Hine and Thorne, 2000; Spiers et al., 2008), and virus (Suzuki et al., 1998; Kitamura et al., 2000, 2002), which could be related to the dramatic decline in the South China seawater pearl production. In order to control disease and enhance the yields and quality of seawater pearls, it is necessary to understand the innate immune defense mechanisms of pearl oyster, which lacks an adaptive immune system. Recently, we characterized some immune-related genes of pearl oyster, such as the inhibitor of NF-κB (IκB) (Zhang et al., 2009a), a clip-domain serine protease (Zhang et al., 2009b), a putative lipopolysaccharide-induced TNF-α factor (LITAF) (Zhang et al., 2009c), and interferon-gamma-inducible lysosomal thiol reductase (GILT) (Zhang et al., 2010), and demonstrated that they played an important role in the innate immune responses of pearl oyster.

Invertebrates lack adaptive immunity and rely exclusively on the innate immune response to defend themselves against pathogens (Kim et al., 2008). Recognition of non-self material by the innate immune system is mediated by pattern recognition receptors (PRRs), which recognize and bind to different molecules on the surfaces of invading microorganisms (Song et al., 2010). Galectin is one of PRRs that can recognize and specifically bind to β-galactoside (Tasumi and Vasta, 2007) and then regulate a variety of biological processes in vertebrates, such as cell adhesion (Barondes et al., 1994a,b; van den Brule et al., 1995; Friedrichs et al., 2007; Alge-Priglinger et al., 2009), development (de Boer et al., 2009; Kim et al., 2009), cancer progression (Cludts et al., 2009; Canesin et al., 2010), cytokine secretion (Rabinovich et al., 1999; Filer et al., 2009), apoptosis (Sturm et al., 2004; Koh et al., 2009), and immune regulation (Ferraz et al., 2008; Mengshol et al., 2010).

Some studies demonstrated that galectin of bivalves could target glycans on the surfaces of bacteria and parasites (Rabinovich and Gruppi, 2005) and play a crucial role in innate im-

 

mune responses of bivalves (Sato et al., 2009). A tandem-repeat galectin, McGal from Manila clam *Ruditapes philippinarum*, has also been proven to bind to the surface of *Perkinsus olseni* as PRR, and it is up-regulated after challenge with *P. olseni* or *Vibrio tapetis* (Kim et al., 2008). Another tandem-repeat galectin, BgGal from freshwater snail *Biomphalaria glabrata*, selectively recognizes the schistosome-related sugar lacNAc and strongly binds to hemocytes and the tegument of *Schistosoma mansoni* sporocysts in a sugar-inhibitable fashion (Yoshino et al., 2008). A multidomain galectin of eastern oyster *Crassostrea virginica* (CvGal) is responsible for recognizing the protozoan parasite *Perkinsus marinus* (Tasumi and Vasta, 2007). Another multidomain galectin, AiGal from bay scallop *Argopecten irradians*, was also characterized and shown to be involved in innate immune responses (Song et al., 2010). However, the evolution and functions of galectin in mollusks are still incipient compared with those in vertebrates. In our previous study, a multidomain galectin (PoGal1) was characterized and demonstrated to be involved in innate immune response of the pearl oyster *P. fucata* (Zhang et al., 2011). In the present study, a tandem-repeat galectin from the pearl oyster *P. fucata* (designated as PoGal2) was isolated and its tissue distribution and temporal expression profile after bacterial challenge were also investigated. This study will hopefully provide insight into the evolution of galectins, as well as their important functions in the innate immune responses of pearl oyster.

## MATERIAL AND METHODS

### Pearl oyster and immune challenge

The pearl oyster *P. fucata* (body weight 18.2-22.5 g) was obtained from the pearl oyster culture base of the South China Sea Fisheries Research Institute in Xincun village, Hainan Province, China, and maintained at 25-27°C in tanks with recirculating seawater for one week before experiments. The pearl oysters were fed twice daily with *Tetraselmis suecica* and *Isochrysis galbana* in the whole experiment process. The pearl oysters were injected into the adductor muscle with 100 μL phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4) as the control group. The bacterial challenge group was injected with 100 μL *Vibrio alginolyticus* resuspended in PBS to $OD_{600}$ = 0.4 (1 OD = $5 \times 10^8$ bacteria/mL) into the adductor muscles of each pearl oyster. At each time (0, 2, 4, 8, 12, 24, and 36 h), digestive gland was collected from the control group and the bacterial challenge group and stored in liquid nitrogen until use. For tissue distribution analysis, unchallenged pearl oyster's digestive gland, gonad, hemocytes, gills, mantle, adductor muscle, and intestine were collected as the unchallenged group and stored in liquid nitrogen until use. The same tissues were also collected and stored from the bacterial challenge group at 8 h post-injection. Pearl oysters of each group were divided into three replicates with equal amounts and fed in three tanks. Five pearl oysters were randomly sampled from each group at each time, and corresponding tissues were mixed in equal amounts as one sample.

### cDNA library construction and expressed sequence tag (EST) analysis

A cDNA library was constructed from the whole body of a pearl oyster challenged by *V. alginolyticus*, using the ZAP-cDNA synthesis kit and the ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Random sequencing of the library us-

 

ing T3 primer yielded 6741 successful sequencing reactions. BLAST analysis of all ESTs revealed that an EST of 483 bp (pmpca0_008512) was similar to the galectins of abalone *Haliotis discus* (ABN54798) and Manila clam *Venerupis philippinarum* (ACA09732). The corresponding colony was picked up and resequenced to obtain the complete sequence of the PoGal2.

## Sequence analysis of PoGal2

PoGal2 amino acid sequence was predicted using the DNATool version 6.0 software. The percentage of similarity and identity of the known galectin sequences was calculated using the MatGAT program (Campanella et al., 2003) with default parameters. The protein domain was predicted with the simple modular architecture research tool (SMART) program (Schultz et al., 1998; Letunic et al., 2006). The protein sequence of PoGal2 was compared to its counterpart sequences currently available in GenBank using the BLAST program (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov). Multiple alignment of PoGal2 was carried out with the ClustalW program (http://www.ebi.ac.uk/clustalw/). The phylogenetic tree was constructed with the MEGA program version 4 (Tamura et al., 2007) based on amino acid sequence alignment. The phylogenetic tree was tested for reliability using 1000 bootstrap replications.

## Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of PoGal2

The expression pattern of PoGal2 in digestive gland, gonad, hemocytes, gills, mantle, adductor muscle, and intestine from the unchallenged group and the bacterial challenged group at 8 h post-injection was detected by quantitative RT-PCR. Temporal expression level in digestive gland after bacterial challenge was also detected by quantitative RT-PCR. Total RNA samples were extracted using the RNeasy Mini Kit (QIAGEN) according to manufacturer instructions, and treated with DNase I (QIAGEN) to remove contaminating DNA. Subsequently, the first-strand cDNA was synthesized based on manufacturer instructions of the PrimeScript™ RT reagent Kit (Perfect Real Time) (TaKaRa) using total RNA as template. The cDNA mix was diluted to 1:5 and stored at -80°C for subsequent quantitative RT-PCR. Two PoGal2 gene-specific primers, PoGal2-F (5'-AATCCGCCAGTACCCCTAAC-3') and PoGal2-R (5'-AGGCGCATGTCACAGTGTAG-3'), were designed to amplify a product of 164 bp. The β-actin gene was used as an internal control to verify the real-time quantitative RT-PCR and adjust the cDNA templates. Two β-actin gene-specific primers, β-actin-F (5'-GCCGAAAGAGAAATCGTCAG-3') and β-actin-R (5'-TGGCTGGAATAGGGATTCTG-3'), were designed to amplify a fragment of 183 bp.

Quantitative RT-PCR was performed in a total volume of 20 µL containing 10 µL 2X SYBR Green Real-time PCR Master Mix (TaKaRa DRR041A), 1 µL cDNA, 0.16 µM of each primer and 8.2 µL double-distilled water. The quantitative RT-PCR program consisted of a denaturation step at 96°C for 2 min, followed by 40 amplification cycles of 15 s, denaturation at 96°C, 15 s annealing at 56.5°C, and 30 s extension at 72°C. Fluorescence readings were performed at the end of each cycle. To analyze PoGal2 mRNA expression level, the comparative CT method ($2^{-\Delta\Delta CT}$ method) was used. The CT for the

 

target amplified PoGal2 and the CT for the internal control β-actin were determined for each sample. Differences in the CT for the target and the internal control, called ΔCT, were calculated to normalize the differences in the amount of total cDNA added to each reaction and the efficiency of the RT-PCR. The control group was used as the reference sample, called the calibrator. The ΔCT for each sample was subtracted from the ΔCT of the calibrator, the difference was called ΔΔCT. PoGal2 mRNA expression level could be calculated by $2^{-\Delta\Delta CT}$, and the value stood for an n-fold difference relative to the calibrator.

### Statistical analysis

Statistical analysis was carried out with the GraphPad Prism 5.0 software. The normality of the distribution and homogeneity of variances of data were examined by Shapiro-Wilks and Cochran tests, and the data were then analyzed by one-way analysis of variance (ANOVA) with default parameters to identify differences between groups. Differences were considered to be statistically significant when P values were lower than 0.05.

## RESULTS

### cDNA cloning and characterization of PoGal2

Based on the identified EST, the full-length of PoGal2 cDNA was obtained by resequencing from the cDNA library. The PoGal2 was 1347 bp long and was deposited in GenBank with accession No. HQ014601. The complete sequence of PoGal2 cDNA consisted of a 5'-untranslated region (UTR) of 3 bp, an open reading frame (ORF) of 1047 bp encoding a polypeptide of 349 amino acids with an estimated molecular mass of 38.1 kDa and a theoretical isoelectric point of 8.5, and a 3'-UTR of 297 bp with a 13-bp poly(A) tail and one cytokine RNA instability motif (ATTTA), and a typical canonical polyadenylation signal sequence (AATAAA). The SignalP software analysis indicated no typical signal peptide in PoGal2. The SMART analysis showed that PoGal2 contained two carbohydrate recognition domains, CRD-N (residues 13 to 147) and CRD-C (residues 210 to 348), which indicated that PoGal2 was a tandem-repeat galectin (Figure 1).

### Homology and phylogenetic analysis of PoGal2

The MatGat software analysis showed that PoGal2 had the highest identity (50.9%) and similarity (63.2%) with abalone galectin, and shared 50.6% identity and 64.7% similarity with the Manila clam galectin (Kim et al., 2008). The two PoGal2 CRDs shared 36.9% identity and 58.3% similarity with each other. Multiple alignment of galectins revealed that each CRD of PoGal2 contained the conserved galectin sequence motif (H-NPR, WG-EE; Figure 2).

To evaluate the molecular evolutionary relationships of individual PoGal2 CRD, an unrooted phylogenetic tree was constructed by the NJ method, based on 36 CRDs of 15 galectins (Figure 3). The multidomain galectins were clustered into a single clade, and this clade was divided into four groups, respectively, on the basis of the different CRD position in multidomain galectins (Figure 3). The tandem-repeat galectins were divided

into two clades based on the different species. The tandem-repeat galectins of the mollusk were clustered into a single clade, which was divided into two groups based on the different position in tandem-repeat galectins, and the tandem-repeat galectins of the nematodes were also clustered into a single clade, which was also divided into two groups based on the different position in tandem-repeat galectins (Figure 3). These results suggested that the tandem-repeat galectin evolved through gene replication.

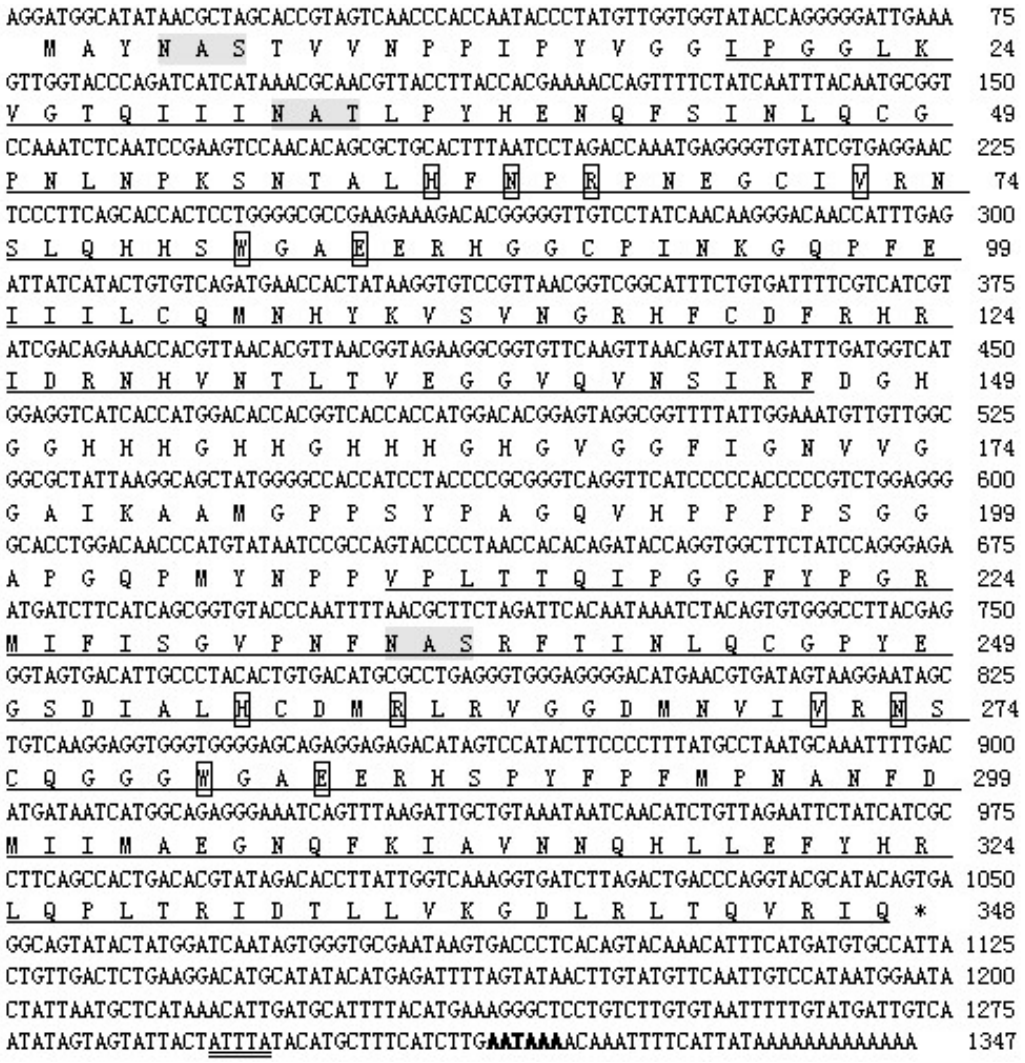


**Figure 1.** Nucleotide sequence of cDNA and deduced amino acid sequence of PoGal2. Numbers on the right indicate the nucleotide and amino acid positions, respectively. Potential *N*-glycosylation sites are indicated in gray. Carbohydrate recognition domains identified by the SMART program are underlined. Asterisk marks the stop codon. Polyadenylation signal sequence is marked in bold. Cytokine RNA instability motifs are double underlined. Conserved amino acid residues in each CRD that are responsible for the galactosyl-binding activity are boxed.

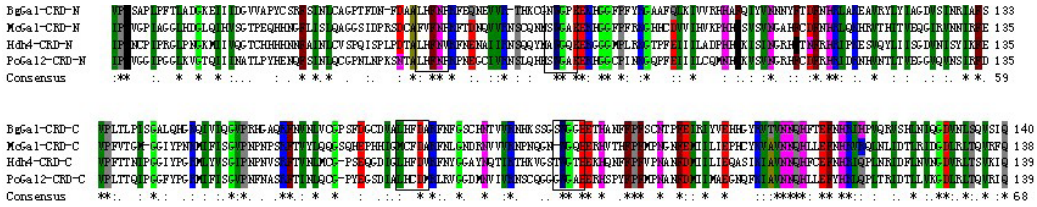


**Figure 2.** Multiple alignment of the deduced amino acid sequence of PoGal2 and other galectins. The black boxes indicate the galectin sequence motif (H-NPR, WG-EE). Residues shared by several galectins are indicated with the following symbols: asterisks = identical residue in all sequences; colons = conserved substitutions according to similar properties of the amino acids; periods = semi-conserved substitution. The galectins used are as follows: BgGal (*Biomphalaria glabrata*, EF534720), McGal (*Ruditapes philippinarum*, ACA09732), Hdh4 (*Haliotis discus* hannai, ABN54798).

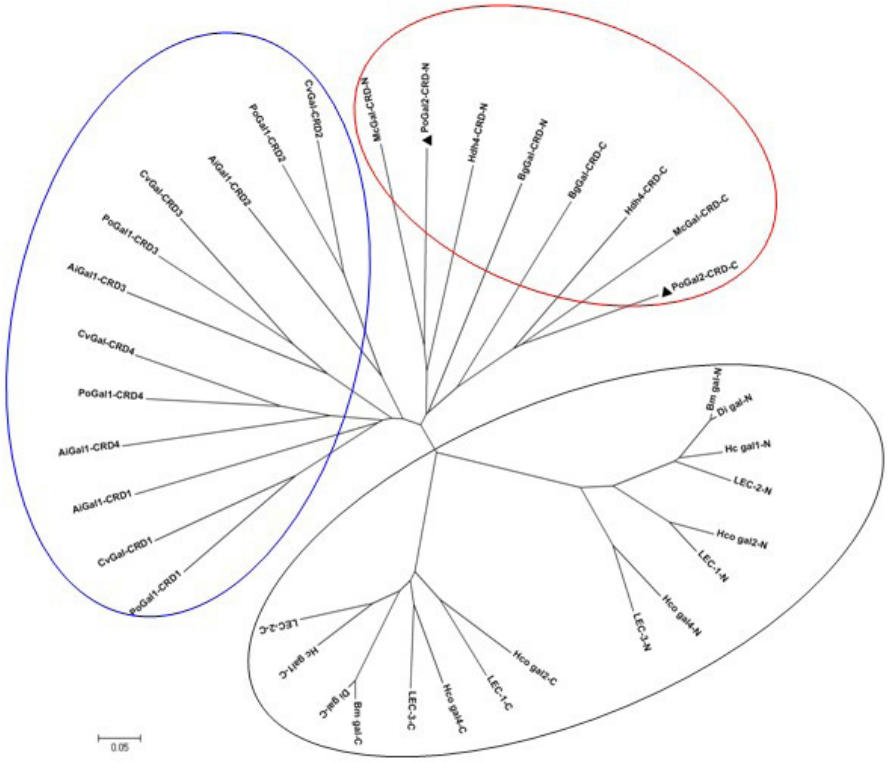


**Figure 3.** Phylogenetic relationship of invertebrate galectins. The unrooted phylogenetic tree was constructed by the neighbor-joining (NJ) method in the MEGA 4.0 software. Bootstrap support values for the NJ tree are shown at the nodes (of 1000 replicates). The GenBank number for the galectin sequences are as follows: CvGal (*Crassostrea virginica*, ABG75998), AiGal1 (*Argopecten irradians*, FJ469998), Bm_gal (*Brugia malayi*, AAF37721), Di_gal (*Dirofilaria immitis*, AAF37720), Hc_gal1 (*Haemonchus contortus*, AAD11972), Hco_gal2 (*H. contortus*, AAB88823), Hco_gal4 (*H. contortus*, AAF63404), LEC-1 (*Caenorhabditis elegans*, AAB87718), LEC-2 (*C. elegans*, BAB11968), LEC-3 (*C. elegans*, BAB11969), Hdh4 (*Haliotis discus*, ABN54798), McGal (*Venerupis philippinarum*, ACA09732), BgGal (*Biomphalaria glabrata*, EF534270), PoGal1 (*Pinctada fucata*, ACO36044).

## PoGal2 mRNA expression pattern in different tissues

Quantitative RT-PCR analysis was performed to investigate the expression pattern of PoGal2 mRNA with the β-actin as an internal control. The results showed that PoGal2 mRNA was constitutively expressed in all tissues analyzed, including digestive gland, gonad, hemocytes, gills, mantle, adductor muscle, and intestine, both in the unchallenged group and in the bacterial challenged group at 8 h post-injection. As shown in Figure 4, the expression level of PoGal2 mRNA of the unchallenged group in digestive gland was higher than in other tissues, and lower in hemocytes and adductor muscle.

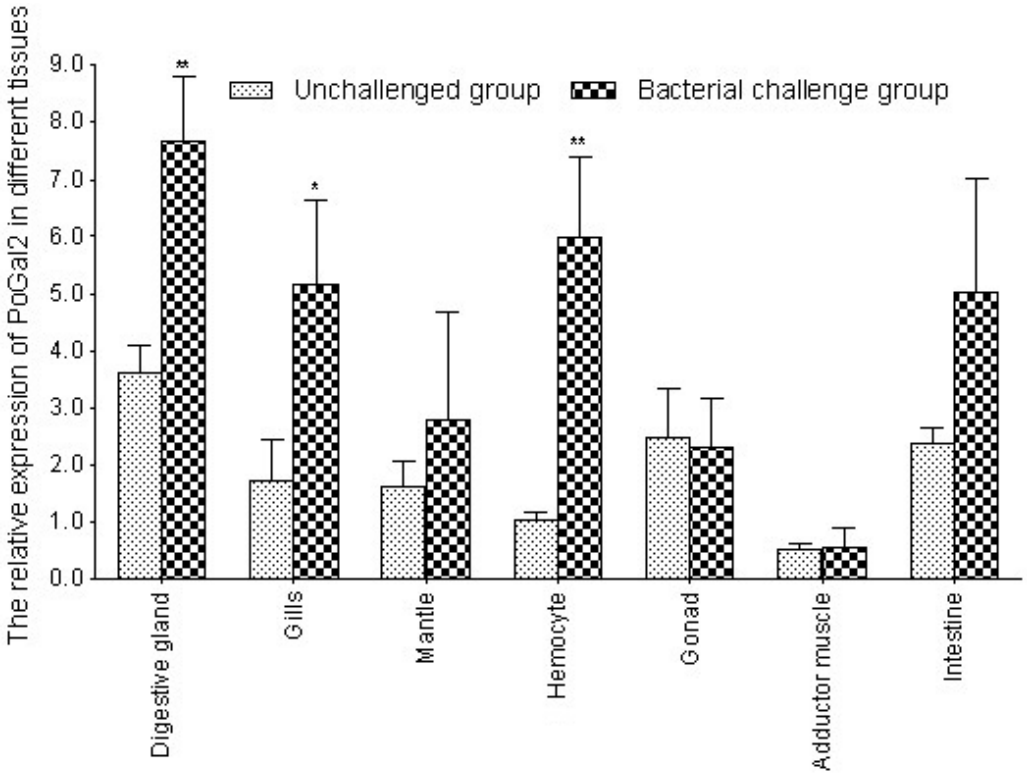


**Figure 4.** PoGal2 mRNA expression pattern in different tissues of adult pearl oyster. The healthy pearl oyster as the control group and pearl oyster injected with *Vibrio alginolyticus* as the bacterial challenge group. Real-time quantitative RT-PCR was carried out with RNA samples from digestive gland, gills, mantle, adductor muscle, gonad, intestine, and hemocytes. The β-actin gene was used as an internal control to calibrate the cDNA template for all the samples. Vertical bars represent the means ± SE (N = 3) for each tissue. Significant difference from control is indicated with an asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

Compared with the unchallenged group, in the bacterial challenge group at 8 h post-injection, the expression of PoGal2 mRNA in digestive gland, gills and hemocytes was significantly up-regulated ($P < 0.05$), but not significantly different in mantle, gonad, intestine, and adductor muscle. After bacterial challenge at 8 h, the expression level of PoGal2 mRNA in hemocytes was 5.8-fold higher than that in the unchallenged group.

## Temporal expression profile of PoGal2 mRNA after *V. alginolyticus* challenge

After *V. alginolyticus* challenge, the temporal expression pattern of PoGal2 mRNA in digestive gland was detected by quantitative RT-PCR. The expression of PoGal2 mRNA was significantly up-regulated ($P < 0.05$) in digestive gland after artificial infection with *V. alginolyticus*. After 8 h of *V. alginolyticus* stimulation, the expression level of PoGal2 mRNA was 4.0-fold higher than that in the control group, and the expression level of PoGal2 mRNA was also significantly up-regulated at 12 and 24 h after bacterial stimulation (Figure 5).

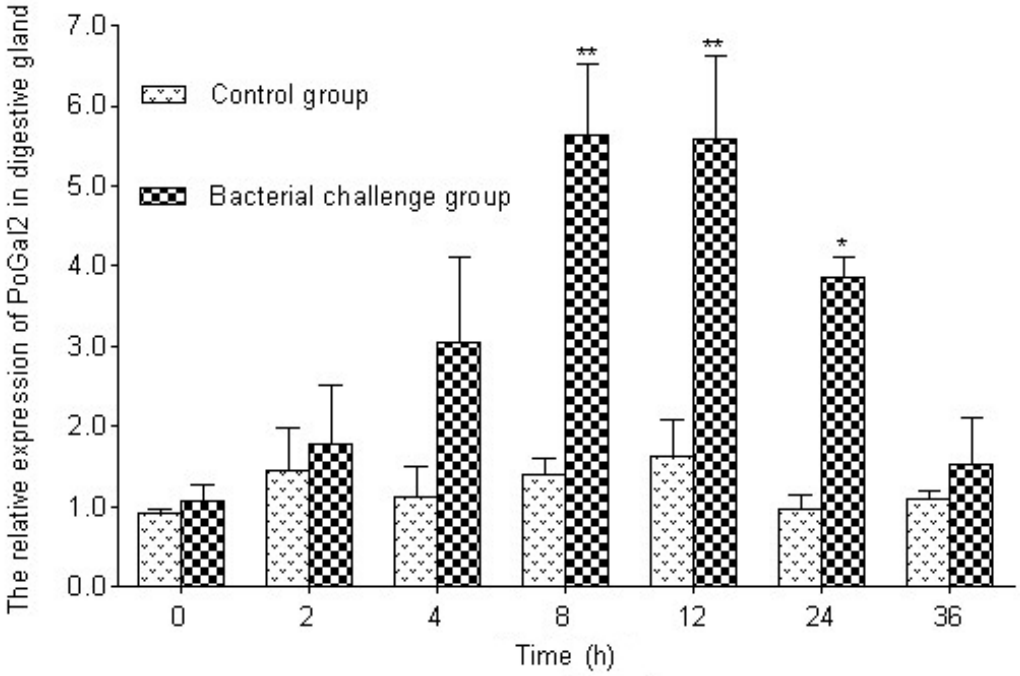


**Figure 5.** PoGal2 expression level in digestive gland after the challenge of *Vibrio alginolyticus* was detected by real-time quantitative RT-PCR. PoGal2 mRNA expression in *V. alginolyticus*-treated samples was normalized to that in the control group. The β-actin gene was used as an internal control to calibrate the cDNA template for all the samples. Vertical bars represent the means ± SE (N = 3) for each time. Significant difference from control is indicated with an asterisk at $P < 0.05$ and two asterisks at $P < 0.01$.

## DISCUSSION

Galectins can bind to a wide array of glycoconjugates, and then mediate cell-cell and cell-extracellular matrix interactions, and are therefore involved in diverse biological functions in vertebrates (Kim et al., 2008; Sato et al., 2009). In the Phylum Mollusca, the presence of galectins has also been demonstrated (Suzuki and Mori, 1989; Wilson et al., 1992), and the molecular mass of isolated lectins in some species was consistent with galectins possessing single- or dual-CRDs (Mitra and Sarkar, 1988; Ozeki, 1998). Recently, galectin cDNAs were cloned and characterized from eastern oyster (Tasumi and Vasta, 2007), Pacific oyster (Yamaura et al., 2008), Manila clam (Kim et al., 2008), abalone (EF392832), freshwater snail (Yo-

shino et al., 2008), bay scallop (Song et al., 2010), and pearl oyster (Zhang et al., 2011). In addition, an EST for galectin homologues has also been identified from Pacific oyster (Rafferty and Powell, 2002). To further understand the evolution and functions of galectins of bivalves, a tandem-repeat galectin cDNA was characterized from pearl oyster *P. fucata* in this study.

Sequence analysis indicated that PoGal2 consisted of two canonical galectin CRDs connected by linkers with 62 amino acid residues, which were longer than those of Manila clam (Kim et al., 2008) and snail (Yoshino et al., 2008). So far, the galectins with two CRDs were only isolated from Manila clam (Kim et al., 2008), abalone (ABN54798) and freshwater snail (Yoshino et al., 2008), which were typical tandem-repeat galectins (Leffler et al., 2004; Vasta et al., 2004). In the phylogenetic tree, all CRDs of PoGal2, McGal, BgGal, and Hdh4 formed a single clade, and then this clade was divided into two groups on the basis of individual CRD position. On the other hand, all CRDs of nematodes also formed a single clade, and moreover, CRD-N and CRD-C were also divided into two different groups in this clade. These results suggest that the tandem-repeat galectins might have originated by repeated duplication of a single galectin (Tasumi and Vasta, 2007; Song et al., 2010).

Previous studies demonstrated that galectins might be secreted via a "nonclassical" secretory pathway, involving protein targeting to the inner plasma membrane followed by membrane blebbing and secretion (Nickel, 2003). As with other galectins, PoGal2 also did not contain a typical signal sequence. Multiple alignment of PoGal2 CRDs with other known galectin CRDs showed that PoGal2 contained many evolutionarily conserved structural features of CRD in each CRD of PoGal2, such as the typical motifs H-NPR and WG-EE, which form sugar-binding sites either by hydrogen bonds or van der Waal forces (Hirabayashi et al., 2002), suggesting that PoGal2 may have similar carbohydrate-binding properties as the superfamily of galectins.

Our previous studies demonstrated that the expression level of LITAF and GILT of pearl oyster was significantly up-regulated after 8-h challenge (Zhang et al., 2009a,b,c, 2010). Therefore, in this study, the 8-h time was selected for quantitative RT-PCR tissue analysis. PoGal2 mRNA was also constitutively expressed in all tissues analyzed in healthy pearl oyster (unchallenged group). After bacterial challenge at 8 h, the expression level of PoGal2 mRNA was significantly up-regulated in digestive gland, gills and hemocytes. The results suggest that PoGal2 may be involved in the innate immune responses of pearl oyster. Similarly, CvGal mRNA of eastern oyster was universally expressed in hemocytes, palps, gills, mantle, midgut, and rectal tissues (Tasumi and Vasta, 2007). AiGal mRNA of bay scallop was constitutively expressed in all tissues analyzed, with the highest expression level in hepatopancreas (Song et al., 2010). In Manila clam, McGal mRNA was also detected in gills, mantle, foot, palps, heart, and hemocytes (Kim et al., 2008).

The digestive gland of mollusk was thought to be an important immune organ, which could secrete various enzymes to hydrolyze microorganisms and be involved in digestive and defense functions (Tisca and Mosca, 2004). Thus, we selected the digestive gland to study the expression profile of PoGal2 after the immune simulation. After bacterial challenge, the expression of PoGal2 mRNA was significantly up-regulated 4.0-fold at 8 h, and the expression was also significantly up-regulated at 12 and 24 h. In bay scallop, the expression of AiGal mRNA was also significantly up-regulated after *Vibrio anguillarum* or *Micrococcus luteus* stimulation, but not significantly altered after *Pichia pastoris* stimulation (Song et al., 2010). These results suggest that PoGal2 was constitutively expressed under normal conditions and its expression was up-regulated after *V. alginolyticus* stimulation. PoGal2 may participate in the innate immune response in pearl oyster.

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