frontiers | Frontiers in Marine Science

ORIGINAL RESEARCH
published: 30 May 2022
doi: 10.3389/fmars.2022.825267

The Characteristics and Expression Profile of Peptidoglycan Recognition Protein 2 in the Accessory Nidamental Gland of the Bigfin Reef Squid During Bacterial Colonization

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Specialty section:
This article was submitted to Aquatic Physiology, a section of the journal Frontiers in Marine Science

Received: *30 November 2021*
Accepted: *02 May 2022*
Published: *30 May 2022*

Citation:
Li H-W, Kuo W-L, Chen C, Tseng Y-C, Chang C-F and Wu G-C (2022) The Characteristics and Expression Profile of Peptidoglycan Recognition Protein 2 in the Accessory Nidamental Gland of the Bigfin Reef Squid During Bacterial Colonization. Front. Mar. Sci. 9:825267. doi: 10.3389/fmars.2022.825267

Some cephalopods carry microorganisms in two specialized organs, the light organ and the accessory nidamental gland (ANG). For the light organ, comprehensive mechanisms have been described for winnowing (bacterial selection) and maintenance of the symbiotic luminescent bacterium *Vibrio fischeri* (*V. fischeri*). However, the mechanisms controlling bacterial selection and maintenance during bacterial colonization of the ANG are open biological issues with physiological significance. Our recent study on bigfin reef squid (*Sepioteuthis lessoniana*) already showed that the ANG bacterial community shifts gradually and exhibits decreased diversity throughout maturation. This study further describes a potential role of an innate immunity-involved molecule, peptidoglycan recognition proteins (PGRPs), in the ANG of bigfin reef squid during bacterial transmission and colonization. First, we found that four homologs of the PGRP family are expressed in the ANG of bigfin reef squid (*sl*PGRP2-5), but only *slPgrp2* transcript levels are highly correlated with ANG development and bacterial colonization. Besides, *slPgrp2* transcripts are mainly expressed in the epithelial cells of certain secondary tubules of ANG, and the expression levels are varied in the epithelial cells of other secondary tubules. This data reveals that *slPgrp2* transcripts may associate with the composition of bacterial consortium and its secretary factors. Moreover, recombinant *sl*PGRP2 had a negative effect of *Escherichia coli* (*E.coli*) which inhibited bacterial growth in culture. Therefore, our data suggest *slPgrp2* expression in the epithelial cells of secondary tubules in the ANG may have an essential role in the winnowing and maintenance of holobiont homeostasis in bigfin reef squid.

Keywords: PGRP, host immunity, symbiotic bacteria, ANG, cephalopod

INTRODUCTION

Animals often harbor large numbers of beneficial microorganisms on tissue surfaces, in digestive organs or in specialized organs. Some cephalopods have two specialized organs that house microbiota, including the light organ and accessory nidamental gland (ANG) (reviewed in Belcaid et al., 2019). Unlike the light organ, which is found in both sexes of bobtail squid (Sepiolidae), the ANG is found only in the females of most squid and cuttlefish (Decapodiformes) species; it is absent in some squid (Oegopsidae), all octopuses (Octopodiformes) and all nautiluses (Nautiloidea) (Lindgren et al., 2012). The ANG is thought to play an important role in the delivery of bacteria from the parent to the egg capsule by preventing fungal fouling (Kerwin et al., 2019). In contrast with the single bacterial population (*Vibrio fischeri*) hosted in the light organ of the bobtail squid (Ruby and Asato, 1993; Nyholm et al., 2002), a complex microbial consortium is hosted in the ANG of most squid and cuttlefish (Grigioni et al., 2000; Pichon et al., 2005; Collins et al., 2012; Kerwin and Nyholm, 2017; Yang et al., 2021). This bacterial consortium is populated by horizontal transmission from the environmental bacterial community (Kaufman et al., 1998; Li et al., 2019). Furthermore, the ANG bacterial community shifts gradually and decreases in diversity during maturation of the bigfin reef squid (Yang et al., 2021) and Hawaiian bobtail squid (Kerwin et al., 2021). Therefore, the establishment and maintenance of the complex consortium in the ANG appear to comprise an intricate process governed by host selection during the initial bacterial transmission and later bacterial colonization. However, a comprehensive understanding of bacterial selection mechanisms during colonization of the ANG is still lacking.

The animal immune system not only defends against microbial infection but also accommodates colonization by symbiotic microorganisms to maintain microbiota-host homeostasis (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Royet et al., 2011). In invertebrates, the innate immune system includes pattern recognition receptors that recognize motifs uniquely present in microorganisms and absent in the host. One family of such pattern recognition receptors is the peptidoglycan recognition proteins (PGRPs), which recognize structural components of bacterial cell walls such as peptidoglycan (most bacteria), lipoteichonic acid (Gram-positive bacteria), and lipopolysaccharide (Gram-negative bacteria) (Dziarski, 2004). PGRPs are conserved in vertebrates and many invertebrates, but no member of the PGRP family has been found in nematodes (Dziarski and Gupta, 2006; Montaño et al., 2011) or crustaceans (Liu et al., 2011; Bai et al., 2020). Unlike mammals (*Pglyrp1-4*) and fish (*pglyrp2, 5, and 6*), which have a few conserved PGRP members with a narrow range of functions, insects usually have many *Pgrp* genes and mainly involved in bacterial recognition and control, such as the 13 *Pgrp* genes in fruit fly (*Dorsophila melanogaster*) and 14 *Pgrp* genes in silkworms (*Bombyx mori*) (Dziarski and Gupta, 2006; Montaño et al., 2011). The diverse function of PGRPs is advantageous in invertebrates, due to the absence of adaptive immunity, in contrast to the moderate repertoire in vertebrates (Montaño et al., 2011). Vertebrates have evolved a large number of other pattern recognition receptors, such as C-type lectin receptors, nucleotide-binding oligomerization domain (NOD)-like receptors, RIG (retinoic acid-inducible gene)-I-like receptors, Toll-like receptors, scavenger receptors, and the mannose receptors (Royet et al., 2011). Moreover, vertebrates possess many secreted recognition molecules, such as complement, collectins, pentraxins and C-reactive protein (Royet et al., 2011).

Based on the structural characteristics, most PGRPs contain a C-terminal PGRP domain, which is homologous to bacteriophage type 2 amidase (Dziarski, 2004; Royet et al., 2011). Furthermore, PGRPs have been shown into three forms: secreted proteins (with a signal peptide), transmembrane receptors, and intracellular proteins (Royet et al., 2011). The diverse functions of PGRPs within a host's immune system can include both direct bactericidal effects and indirect effects, such as the induction of antimicrobial compound production and the modulation of inflammation and immune responses (Royet et al., 2011). Furthermore, PGRPs are known to be involved in the maintenance of beneficial and normal gut microbiota in mammals (Saha et al., 2010) and insects (Bischoff et al., 2006; Zaidman-Rémy et al., 2006). In the vent mussel (*Bathymodiolus septemdierum*), PGRPs have been suggested to regulate the homeostasis of gill symbionts (Ikuta et al., 2019). Moreover, PGRPs are thought to participate in the winnowing and maintenance of luminescent bacteria (*Vibrio fischeri*) in the light organ of the Hawaiian bobtail squid (*Euprymna scolopes*) (Nyholm and Graf, 2012; McAnulty and Nyholm, 2017). Therefore, PGRPs could serve essential biological roles in the control of commensal bacteria and in defense against pathogens.

The bigfin reef squid (also called oval squid, *Sepioteuthis lessoniana*) is closely associated with coral reef habitats, and lays jelly-like egg strings in strip-like structures such as seagrasses, sea fans, coral rubble, and reefs (Norman, 2003). The development of the ANGs has been studied in female bigfin reef squid (Li et al., 2019) and other coleoid cephapods (Lum-Kong, 1992; Kaufman et al., 1998; Kerwin et al., 2021). During the ANG development, the ANG gradually transfers bacteria from the outer epithelial cell layer, invaginating bacteria from the marine environments into the ANG and forming tubule structures (contains primary tubules in the outer layer and secondary tubules in the inner layer of the ANG) for bacterial flora (Lum-Kong, 1992; Kaufman et al., 1998; Li et al., 2019; Kerwin et al., 2021). During the female maturation, four developmental stages of ANGs were categorized on the basis of morphological and histological characteristics in bigfin reef squid (**Figure 1**): juvenile squid with a colorless ANG without visible bacterial flora (stage 1), immature squid with a colorless ANG with bacterial flora (stage 2), maturing squid with a white/light-orange ANG with large numbers of bacterial flora (stage 3), and mature squid with a pigmented ANG with large numbers of bacterial flora (stage 4) (Li et al., 2019). Furthermore, our previously study suggest that robust transferrin expression in the outer layer of the ANG plays an important role in microbe selection by the squid during bacterial transmission (Li et al., 2019). However, the regulatory mechanism of squid's immune

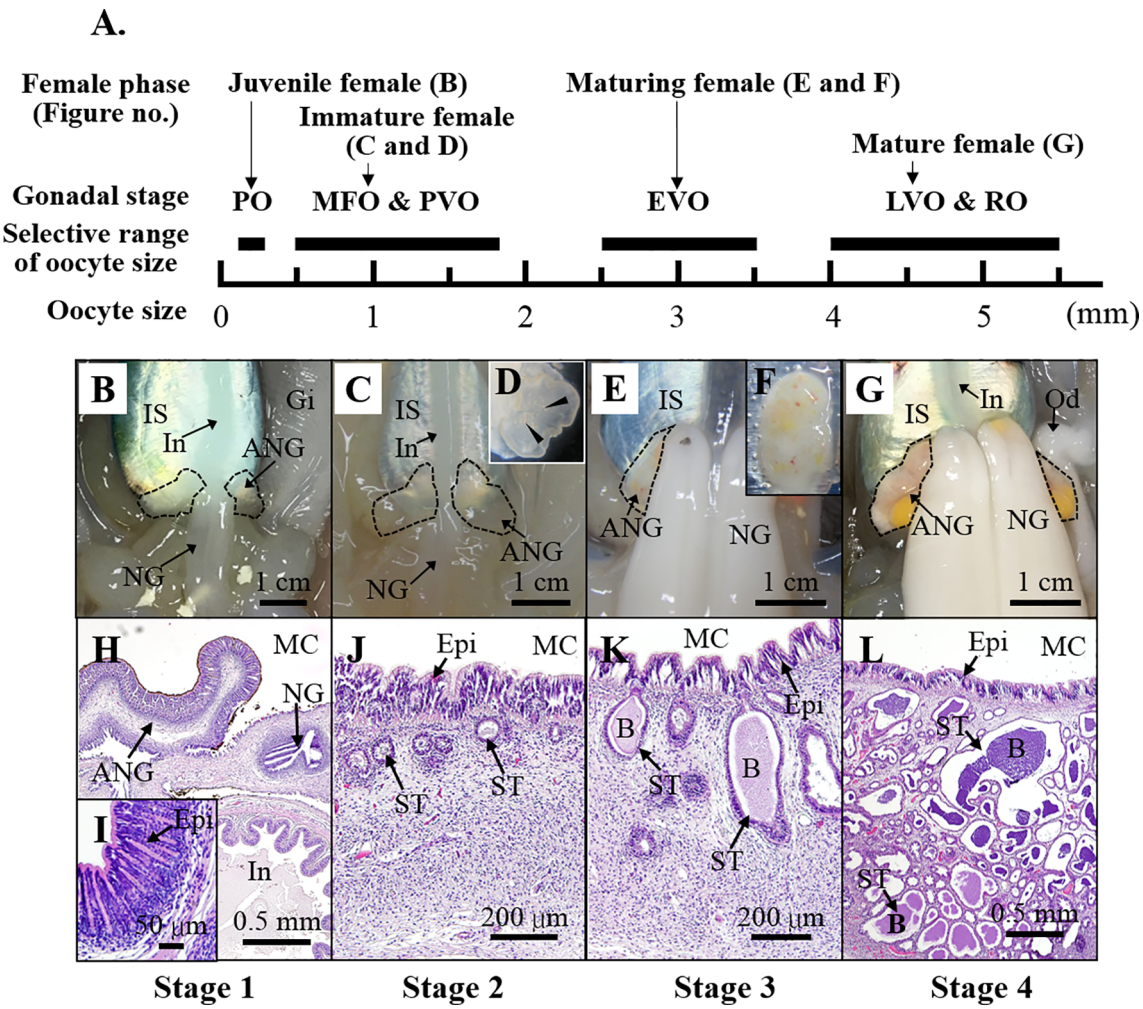

FIGURE 1 | The characteristics of ANG during female squid maturation. **(A)** The profiles of female growth, including the juvenile female (primary oocyte stage), immature female (multiple follicular oocyte stage and previtellogenic oocyte stage), maturing female (early vitellogenic oocyte stage), and mature female (late vitellogenic oocyte stage and ripe oocytes). **(B–G)** Morphological changes during ANG growth. **(B)** juvenile squid with a colorless ANG (stage 1); **(C, D)** immature squid with a colorless ANG several white spots (stage 2); **(E, F)** maturing squid with a white/light-orange ANG (stage 3); **(G)** mature squid with a pigmented ANG (stage 4). **(H–L)** Histological changes during ANG growth. **(H, I)** juvenile squid with a colorless ANG without visible bacterial flora (stage 1); **(J)** immature squid with a colorless ANG with bacterial flora (stage 2); **(K)** maturing squid with a white/light-orange ANG with large numbers of bacterial flora (stage 3); **(L)** mature squid with a pigmented ANG with large numbers of bacterial flora (stage 4). PO, primary oocyte; MFO, multiple follicular oocyte; PVO, previtellogenic oocyte; EVO, early vitellogenic oocyte; LVO, late vitellogenic oocyte stage; RO, ripe oocyte; IS, ink sac; In, intestine; Gi, gill; NG, nidamental gland; ANG, accessory nidamental gland; Od, oviduct; MC, mantle cavity; Epi, epithelial cell; ST, secondary tubule; B, bacteria.

system in the ANG to maintain holobiont homeostasis during the female maturation in bigfin reef squid remains unknown.

In Hawaiian bobtail squid, five homologs of the PGRP family (*Pgrp1-5*) were reported to be expressed in the light organ (Goodson et al., 2005) and hemocytes (Collins et al., 2012). PGRP family genes have also been identified in other cephalopods as well (reviewed in Castillo et al., 2015). Therefore, this study especially aims to determine possible physiological significances of bigfin reef squid's PGRPs in the ANG. We further characterize the potential involvement of bacterial transmission and colonization in the ANG to maintain holobiont homeostasis during female maturation in bigfin reef squid.

MATERIALS AND METHODS

Squid Collection

Bigfin reef squids were purchased from a fisherman on Heping Island, Keelung city, Taiwan. The squids were collected by hand jigging from a boat off the northeast coast of Taiwan. The squids were maintained in a seawater system on the boat, then transferred to a seawater tank until tissue sampling (less than 2 hr). The squids were anesthetized in seawater containing 5% ethanol at room temperature and then decapitated after tissue sampling. All procedures and investigations were approved by the National Taiwan Ocean University Institutional Animal Care

and Use Committee and were performed in accordance with standard guidelines.

Tissue Histology and Gram Staining

The reproductive phase of female was determined based on ovarian stage by oocyte size and morphological characteristic (mature eggs in oviduct), as described previously (Chen et al., 2018). The oocyte size was examined by hematoxylin and eosin (H&E) staining and stereomicroscope for small ovary (oocyte size smaller than 1.5 mm) and others (oocyte size lager than 1.5 mm), respectively. The oocyte stage was determined based on the oocyte size, in accordance with our previous study (Chen et al., 2018). The oocyte stage and relative oocyte diameter as follow: primary oocyte stage (35-300 μm), multiple follicular oocyte stage (0.3-1.2 mm), previtellogenic oocyte stage (1.2-1.8 mm), early vitellogenic oocyte stage (1.8-3 mm), late vitellogenic oocyte stage (3-5 mm), and ripe oocyte stage (5-5.5 mm). The developmental status of most ANG (36 of 38 ANGs) was examined by H&E staining, as described previously (Chen et al., 2018). The ANGs were fixed with 4% paraformaldehyde in PBS at 4°C for 16 hr, then dehydrated in methanol and stored at -20°C. Dehydrated ANGs were transferred to xylene and then embedded in paraffin. Sections (5 μm in thickness) were rehydrated with PBS, treated with HistoVT One (Nacalai Tesque), and stained with hematoxylin and eosin. The ANG stage was determined based on morphological characteristics and confirmed by histology, in accordance with our previous study (Li et al., 2019). Four developmental stages of ANGs were used for RNA analysis in this study (**Figure 1**): stage 1 (n = 4), stage 2 (n = 14), stage 3 (n = 12), and stage 4 (n = 9). The characteristics of sampled squids (n = 39) was shown in **Supplemental Table 1 (Table S1)**.

The bacterial distribution was examined with ASK® Rapid Gram Stain Kit (Tonyar Biotech. Inc., Taoyuan, Taiwan). Gram staining was performed according to the manufacturer's protocol. Rehydrated sections (5 μm in thickness) were stained with crystal violet and Lugol's solution, then stained with safranin and counterstained with tartrazine solution (HT3028, Sigma). The bacterial species were divided into two large groups according to color, violet Gram-positive bacteria and pink Gram-negative bacteria.

Total RNA Extraction and cDNA Synthesis

Tissues were homogenized in Trizol reagent (Invitrogen). Extraction of nucleic acids and protein was performed according to the manufacturer's protocol. The nucleoprotein complexes were mixed with chloroform to respectively separate the DNA, RNA, and protein into a lower phenol-chloroform phase, an upper aqueous phase, and an interphase. Isopropanol was used to precipitate the total RNA from the aqueous phase. The quality and quantity of isolated total RNA were determined by gel electrophoresis and NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific), respectively. First-strand cDNA was synthesized from 1 μg total RNA with oligo(dT)12-18 primers (Promega) using Superscript III reverse transcriptase (Invitrogen). The resultant cDNA was used for gene cloning and RNA analysis.

Cloning of the Bigfin Reef Squid *Pgrp* Genes

The ANG (stage 2) from an immature female squid was used to isolate total RNA. The cDNA synthesis, cDNA library construction, and illumine sequencing were done by Welgene, Inc. Paired-end sequencing (150bp) was performed on a HiSeq 2000 sequencer (Illumina). A *de novo* transcriptome assembly was performed using CLC Genome Workbench 8.0 and used in our previous study (Li et al., 2019). This transcriptomic database was used to identify a fragment of the target gene in this study. A local blast on CLC Genome workbench was used to detect homologs of *Pgrp* in bigfin reef squid. The sequences of *Pgrp* genes were confirmed by cloning. The cloning primers were listed in **Table 1**. Full-length (containing open reading frame) cDNA sequences of *slPgrp2* (GenBank accession no. MZ781965), *slPgrp3* (GenBank accession no. MZ781966), *slPgrp4* (GenBank accession no. MZ781967), and *slPgrp5* (GenBank accession no. MZ781968) were obtained in this study.

Sequence Alignment and Phylogenetic Analysis

For the phylogenetic analysis, a subset of PGRP sequences from various taxa was retrieved from GenBank and compared with the PGRPs identified from the bigfin reef squid. The consensus PGRP domain of PGRPs was determined by SMART sequence analysis (http://smart.embl-heidelberg.de/) and used for analysis. Alignments were performed using MUSCLE in MEGA11. The phylogenetic tree was constructed using the neighbor-joining method in MEGA11. The number at each node represents the bootstrap probability (% from 1000 replicates). The accession numbers of the sequences used for analysis are listed in **Table S2**.

RNA Analysis

Quantitative real-time PCR (qPCR) analysis was modified from our previous study (Chen et al., 2018). *Elongation factor 1 alpha* (*Ef1a*; GenBank accession no. MG924746) was used as an internal control to normalize the gene expression levels.

TABLE 1 | Oligonucleotides for specific primers.

Gene	Orientation	Sequence	Analysis
Pgrp2	Sense	5'-CATTTCGTCGTTTATTGAGGA-3'	Cloning
	Antisense	5'-CTCTATACCAATTTATACAATGTCA -3'	Cloning
Pgrp3	Sense	5'-CACGTGTCACTCCTACATT -3'	Cloning
	Antisense	5'-CTCAACCGAATGCATGTTTTT-3'	Cloning
Pgrp4	Sense	5'-GAGCGAAGGAAGGAAGGA -3'	Cloning
	Antisense	5'-GGGGACAGTTTTACTTATGCATT-3'	Cloning
Pgrp5	Sense	5'-GACGGCAACAGTGAATCAGGATCT -3'	Cloning
	Antisense	5'-GTGCGGTGGTAGTCGGTGTT-3'	Cloning
Pgrp2	Sense	5'-GGCACTATCTGCTGTTCAGAAT-3'	qPCR
	Antisense	5'-GCACATCCCTGTGTCCAAATA-3'	qPCR
Pgrp3	Sense	5'-GGTGTCCAGAAGGGTTACAT -3'	qPCR
	Antisense	5'-GCCATTGTTTGATACGCTCATA-3'	qPCR
Pgrp4	Sense	5'-TGCCTGTGGTGTTGAGTTAG -3'	qPCR
	Antisense	5'-GCCATGTTTGAATGAGAGCATAC-3'	qPCR
Pgrp5	Sense	5'-GCACAACCAAGTCAGCATGTA -3'	qPCR
	Antisense	5'-GTTTCCATCTCCACCAACCAAA-3'	qPCR
Ef1a	Sense	5'-CCAGGTGACAATGTTGGTTTC-3'	qPCR
	Antisense	5'-GTCTCTTTGGGTGGGTTATTCT-3'	qPCR

Specific qPCR primers for target genes and amplicon sizes are listed in **Table 1**. Gene expression quantification for standards, samples, and controls was conducted simultaneously by qPCR (CFX Connect™ Real-Time PCR Detection System; Bio-Rad Laboratories) with the SYBR Green Master Mix (Bio-Rad Laboratories). The thermal cycling conditions were as follows: one cycle of 95°C for 10 min, then 40 cycles of 95°C for 15 sec, and 60°C for 1 min. The PCR specificity was confirmed by a single melting curve in unknown samples and template-contained controls. No signal was detected by qPCR in non-template controls. The reaction efficiency of different genes was evaluated by serially diluted standards. The data were analyzed according to the $2^{-\Delta\Delta}$Ct method. The relative expression values of target genes in all samples were normalized to *Ef1a*, and the highest value of the target genes was defined as 100%.

In situ Hybridization

Digoxigenin-11-UTP (DIG)-labeled antisense and sense probes were synthesized using cDNA fragments of *Pgrp2* (nucleotides -28-654, 682bp). The DIG-labeled antisense RNA probe was used to detect the localization of *Pgrp2*. A DIG-labeled sense RNA probe was used as a control for non-specific signals. Whole mount *in situ* hybridization (ISH) was performed as described in our previous study (Guo et al., 2021). Fixed ANGs were dissected into small pieces by vibratome sectioning (0.2 mm in thickness). Then, the sections were rehydrated, and processed for ISH. The ANG samples were incubated with DIG-labeled RNA probes at 60°C overnight. mRNA expression was detected with ANG-pre-adsorbed alkaline phosphatase-conjugated sheep anti-DIG antibody (11093274910, Roche) and visualized using the NBT/BCIP Detection System. The samples were then fixed with 4% paraformaldehyde and dehydrated in ethanol. The dehydrated samples were transferred from ethanol to infiltration buffer, and then embedded in plastic (Technovit 3040). The sections (5 μm in thickness) were rehydrated in water and counterstained with Fast red to label the cell nucleus.

Turbidity Assay

The ORF of *slPgrp2* was cloned into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression plasmid, pET-28a$^{(+)}$ DNA (69684-3CN, Novagen). The plasmid was transformed into ECOS™21 BL21 (DE3) competent cells (Yestern Biotechnology). The *E. coli* were grown at 37°C, and the recombinant *sl*PGRP2 (r*sl*PGRP2) was induced by the addition of 1 mM of IPTG, followed by culture at 37°C for 5 hr in LB medium. The growth rate of *E. coli* was evaluated by spectrophotometer (absorbance at 600 nm). *E.coli* carrying an IPTG-inducible orange-spotted grouper (*Epinephelus coioides*) *dmrt1* (*ecdmrt1*) expression cassette were used as reference control.

Data Analysis

Data were shown as mean ± standard deviation (SD). The Shapiro-Wilk test was used to test the normal distribution (variance > 0.5 indicates normality). The Games-Howell test was used to check the homogeneity of different groups. Student's *t*-test was used to check the significance of difference between two groups. With One-way ANOVA followed by a Games-Howell test (homogeneity of variance < 0.05) was used to check the significance of differences among three or more groups. Statistical Package for the Social Sciences (SPSS) software was used. In all cases, $P < 0.05$ indicated significant difference.

RESULTS

Molecular Identification and Phylogenetic Analysis of Bigfin Reef Squid *slPGRPs*

Both vertebrates and invertebrates, PGRPs have a highly conserved C-terminal region of the PGRP domain. Therefore, the deduced amino sequences of the PGRP domain sequence from *Pgrp* genes (*Pgrp1-5*) in bobtail squid were used as a reference to search for *Pgrp* genes in bigfin reef squid. Four PGRP domain-containing transcripts were found in a draft transcriptome database for the ANG of bigfin reef squid. The sequences of these four genes were determined by cDNA cloning. The deduced amino acid sequences of the genes showed conservation of the PGRP domain (Amidase 2). A phylogenetic analysis of the PGRP domains revealed that bigfin reef squid PGRP domain-contained genes cluster with PGRPs in cephalopods (**Figure 2A**). One deduced amino sequence of gene (named *slPgrp2*) was located in the same group as PGRP1 and PGRP2 of Hawaiian bobtail squid, but it was not grouped specifically with either branch (**Figure 2A**). The predicted protein structure of this gene includes a signal peptide that is expected to cause its secretion (**Figure 2B**). This characteristic (secretion) was observed for PGRP2 but not PGRP1 of the Hawaiian bobtail squid (Goodson et al., 2005). Three deduced amino sequence of genes (named *slPgrp3*, *slPgrp4*, and *slPgrp5*) belong to the same branch as PGRP3, PGRP4, and PGRP5 in Hawaiian bobtail squid (**Figure 2A**). Similar to the genes in Hawaiian bobtail squid (Collins et al., 2012), the predicted protein structures of these genes included a glycophosphatidylinositol (GPI)-anchoring site in *sl*PGRP3, a transmembrane receptor domain in *sl*PGRP4, and a secretion signal in *sl*PGRP5 (**Figure 2B**).

Expression Profiles of *slPgrp* Genes During Female Growth

The *slPgrp* family (*slPgrp2*, *slPgrp3*, *slPrgp4*, and *slPgrp5*) transcript levels were then analyzed in various tissues of mature females. According to the qPCR results, *slPgrp2* was predominantly expressed in the hepatopancreas, but it was also detected in the ANG and ovary (**Figure S1**). Only slight or no expression of *slPgrp2* was found in other tissues (**Figure S1**). Compared to the gene expression level of *slPgrp2* (highest transcription level in hepatopancreas, 3.4E-02), *slPgrp4* (highest transcription level in hemolymph-isolated hemocytes, 0.127), and *slPgrp5* (highest transcription level in hemocytes, 4.11) in various tissues, *slPgrp3* showed weak or no expression in various tissues (highest transcription level in gills, 1.19E-06) (**Figure S1**). *slPgrp4* and *slPgrp5* were predominantly expressed in hemocytes and exhibited slight expression in other tissues (**Figure S1**). Based on the weak levels of gene expression in ANG, we did not analyze the *slPgrp3* expression.

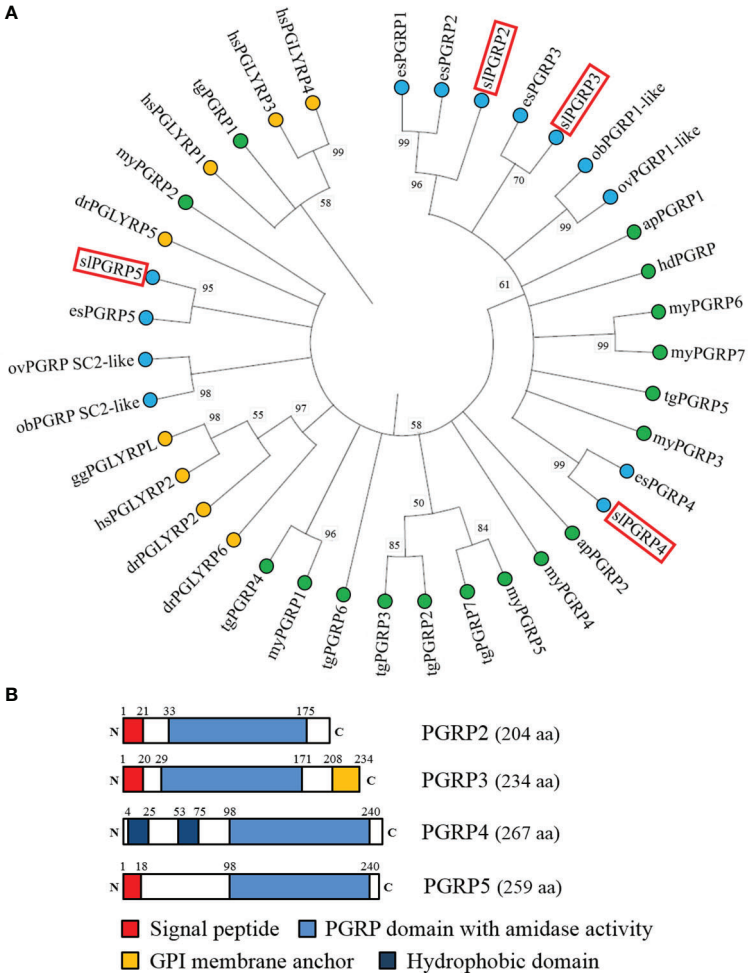

FIGURE 2 | Phylogenetic analysis of PGRP family proteins from various taxa and domain structure of PGRPs in the bigfin reef squid. **(A)** The phylogenetic analysis of PGRPs in various species. The PGRP domain (with amidase) was used for analysis. The number at each node represents the bootstrap probability (% of 1000 replicates). Branches correspond to values of 50% and higher. The PGRPs of bigfin reef squid (*sl*PGRP2, *sl*PGRP3, *sl*PGRP4, and *sl*PGRP5) are indicated by red boxes. The PGRPs from vertebrates, bivalves and gastropoda, and cephalopods are highlighted in yellow, green and blue circles, respectively. The gene names and accession numbers are listed in **Table S2**. **(B)** The predicted structure of *sl*PGRPs in the bigfin reef squid. Red denotes the signal peptide. Blue denotes PGRP domain with amidase activity. Yellow denotes glycosylphosphatidylinositol (GPI) membrane anchor. Indigo denotes hydrophobic domain (predicted transmembrane domain).

We investigated whether the expression of *slPgrp* family (*slPgrp2*, *slPgrp4*, and *slPgrp5*) is correlated to the growth of female bigfin reef squid. Based on histological characteristics of the ovary, gene transcript levels were analyzed at the primary oocyte stage of immature females (stage 2 of ANG) and vitellogenic oocyte stage of mature females (stage 4 of ANG). Results from qPCR showed that ovarian *slPgrp2* expression levels were high in the immature females, and the expression level was significantly decreased in the mature females (**Figure 3A**). The expression level of *slPgrp2* was low in the ANG of immature females, but the expression level was significantly increased in the mature females (**Figure 3A**). No differences in *slPgrp2* expression were found between age groups in the hepatopancreas, gills, mantle, and NG (**Figure 3A**). qPCR results also showed that *slPgrp4* expression level in gills, NG, and ANG were high in immature females and that the expression levels were significantly decreased in the mature females (**Figure 3B**). No differences in *slPgrp4* expression were found between age groups in the hepatopancreas, mantle, and ovary (**Figure 3B**). Furthermore, *slPgrp5* expression level in hepatopancreas was low in the immature females and significantly increased in the mature females (**Figure 3C**). The *slPgrp5* expression level in ANG was high in the immature females and significantly decreased in mature females (**Figure 3C**). No differences in *slPgrp5* expression were identified between age groups in the gills, mantle, ovary, and NG (**Figure 3C**). Thus, among *slPgrp* genes expressed in female-specific tissues (ovary, NG, and ANG), only the expression of *slPgrp2* in the ANG was positively correlated with growth of female bigfin reef squid.

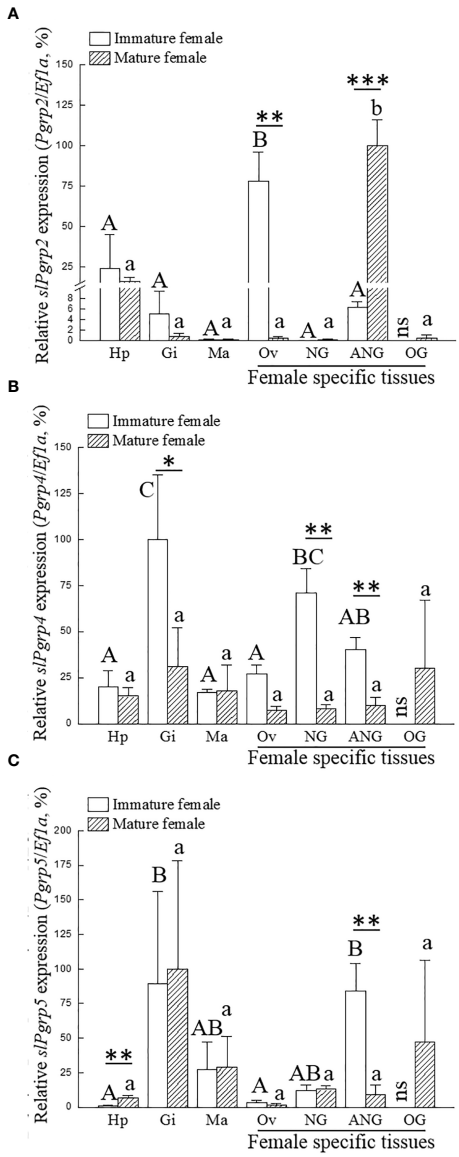

FIGURE 3 | Expression of *slPgrp* transcripts in various tissues. The gonadal stage of female squid was distinguished by histology. Immature (n = 3; primary oocyte stage) and mature (n = 3; vitellogenic oocyte stage) females were used to analyze gene expression in different tissues. Three *slPgrp* genes were selected for analysis, including *slPgrp2* **(A)**, *slPgrp4* **(B)**, and *slPgrp5* **(C)**. Different capital letters indicate significant differences between tissues in immature female ($P < 0.05$). Different lower-case letters indicate significant differences between tissues in mature female ($P < 0.05$). Asterisks indicate significant difference between the immature and mature females (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, respectively). Hp, hepatopancreas, Gi, gill; Ma, mantle (muscle); Ov, ovary; NG, nidamental gland; ANG, accessory nidamental gland; OG, oviductal gland; ns, no sample.

Expression Profiles of *slPgrp2* During ANG Growth

We next sought to clarify the correlation between *slPgrp* (*slPgrp2*, *slPgrp4*, and *slPgrp5*) transcript levels and distributions in the ANG. The relative portion of outer and inner layer of ANG was identified as **Figure 4A**. The outer layer of ANG was composed by outer epithelial cell layer and intermediate filament layer (**Figures 4B, C**). The inner layer of ANG was composed by inner epithelial layer (**Figure 4D**). From the mature female squid, the relative portion of outer and inner layers of ANGs (stage 4) were isolated under a stereomicroscope and analyzed separately, as in our previous study (Li et al., 2019). Histological result showed that the outer layer of the ANG was completely removed from the ANG in outer layer-isolated ANG in our previous study (Li et al., 2019). Thus, the relative portion of outer layer of the ANG contained outer epithelial cell layer with a small portion of inner layer of the ANG for RNA analysis. qPCR results showed that the outer layer had lower *slPgrp2* expression than the inner layer of ANG in mature females (**Figure 4E**). In contrast, the outer layer had higher *slPgrp4* and *slPgrp5* expression than the inner layer (**Figure 4E**). Next, gene transcript levels were analyzed at different ANG stages. The histological features and bacterial distributions of different ANG stages in female squids are shown in **Figure 1**. According to qPCR, *slPGRP2* expression levels were low at stage 1, stage 2, and stage 3 of ANG development, but the expression levels were increased significantly at stage 4 of ANG development (**Figure 5**). Thus, the data showed that *slPgrp2* expression in the inner layer of ANG is correlated with bacterial colonization and pigmentation during ANG growth.

Localization of *slPgrp2* Expression

To analyze the distribution of *slPgrp2* in the maturing ANG, ISH for *slPgrp2* was performed on ANGs of different stages. In the stage 2 ANG, *slPgrp2* transcripts were detected in the epithelial cells of primary tubules, but no signal was observed in the epithelial cells of secondary tubules (**Figures 6A, B**). No signal was observed from the control sense probe in stage 2 ANGs (**Figure 6C**). At stage 4, sl*Pgrp2* transcripts were mainly expressed in the epithelial cells of certain secondary tubules of the ANG, but slight or no signal was observed in primary tubules and other secondary tubules at stage 4 of ANG development (**Figures 6D, E**). No signal was observed from the control sense probe for *slPgrp2* at stage 4 of ANG development (**Figure 6F**). Thus, *slPgrp2* transcripts showed differential expression levels in the epithelial cells of different secondary tubules of ANG.

slPgrp2 Expression in Different Areas of ANG

Next, we examined transcript levels and distributions of *slPgrp2* in different secondary tubules at stage 4 of ANG development. *slPgrp2* transcripts were differentially distributed in different regions of the ANG (**Figures 7A–C**). No signal was observed from the control sense probe for *slPgrp2* in serial sections (**Figure 7D**). Thus, *slPgrp2* expression was varied in the epithelial cells of different secondary tubules in the ANG. According to the HE staining (**Figure 7E**) and Gram staining (**Figures 7F–H**) results, different area of ANG showed the difference on colors and bacterial density. To analyze *slPgrp2* expression in relation to different region of secondary tubules in

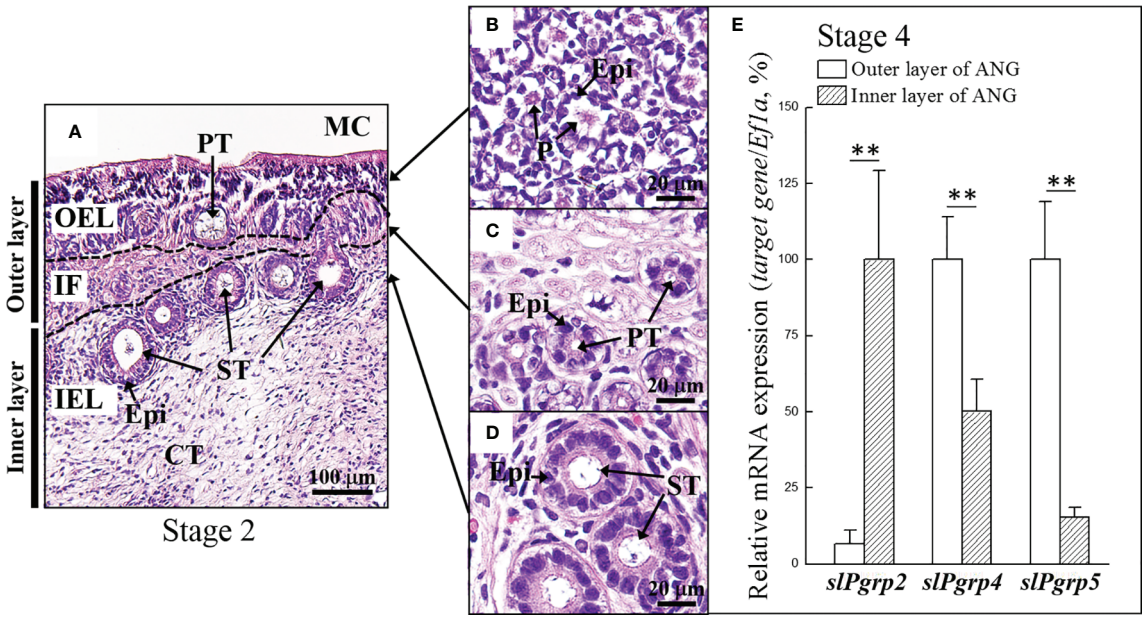

FIGURE 4 | Expression of *slPgrp* transcripts in different layers of ANG. **(A)** The definition of outer and inner layer of ANG. **(B, C)** The outer layer of ANG was composed by outer epithelial cell layer and intermediate fiber layer, respectively. **(D)** The inner layer of ANG was composed by inner epithelial cell layer. The relative portion of outer and inner layer of ANGs from mature female squid (stage 4, n = 4) were isolated under a stereomicroscope and analyzed separately. **(E)** Expression levels of *slPgrp2*, *slPgrp4*, and *slPgrp5* were analyzed by qPCR. $^{**}P < 0.01$ comparing the outer and inner layer of ANGs. OEL, outer epithelial cell layer; IF, intermediate fiber layer; IEL, inner epithelial cell layer; MC, mantle cavity; CT, connective tissue; Epi, epithelial cell; PT, primary tubule; ST, secondary tubule; P, pore.

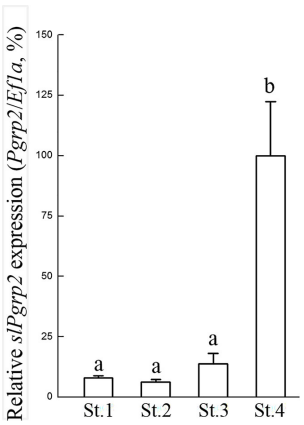

FIGURE 5 | Gene expression profile of *slPgrp2* during ANG growth. According to histology, four different stages of ANGs were used for gene expression analysis, including stage 1 (n = 4), stage 2 (n = 14), stage 3 (n = 12), and stage 4 (n = 9). The expression profile of *slPgrp2* during ANG development was analyzed by qPCR. Different lower-case letters indicate significant differences between stages of ANGs ($P < 0.05$). St, stage.

the ANG, the different regions were identified by color, isolated under stereomicroscopy and analyzed separately. Eight regions were isolated from two ANGs (stage 4), including three white regions, one red region, two orange regions, and two yellow regions (**Figure 7I**). qPCR analysis showed differential *slPgrp2* transcripts associated with different regions (**Figure 7J**). Furthermore, three mature female squid (stage 4) were used to examine the correlation between region colors and *slPgrp2* transcript levels. Unexpectedly, the expression levels of *slPgrp2* showed no apparent correlations with the different region colors (**Figure 7K**). These data confirmed our conclusion made from ISH results that *slPgrp2* transcripts are differentially expressed in different secondary tubules of ANG. Importantly, these data also do not rule out the possibility that *slPgrp2* expression in the epithelial cells of secondary tubules in the ANG might correspond to microenvironment characteristics, such as region colors and it bacterial community.

The Effect of Recombinant *sl*PGRP2 on *E. coli* Growth

To analyze the potential bactericidal activity of *sl*PGRP2, we evaluated the effect of r*sl*PGRP2 expression on *E.coli* growth in culture. For the bacterial culture study, *E.coli* carrying an IPTG-inducible *slPgrp2* expression cassette were treated with or without IPTG. According to turbidity measurements, cell density was increased in cultures of the control (without IPTG treatment) during the experimental period. In contrast, cell density was significantly lower in cultures with the expression of r*sl*PGRP2 (with IPTG treatment) compared with the control (without IPTG treatment) (**Figure 8**). *E.coli* carrying an IPTG-inducible *ecdmrt1* expression cassette were used as a reference control. Turbidity assay

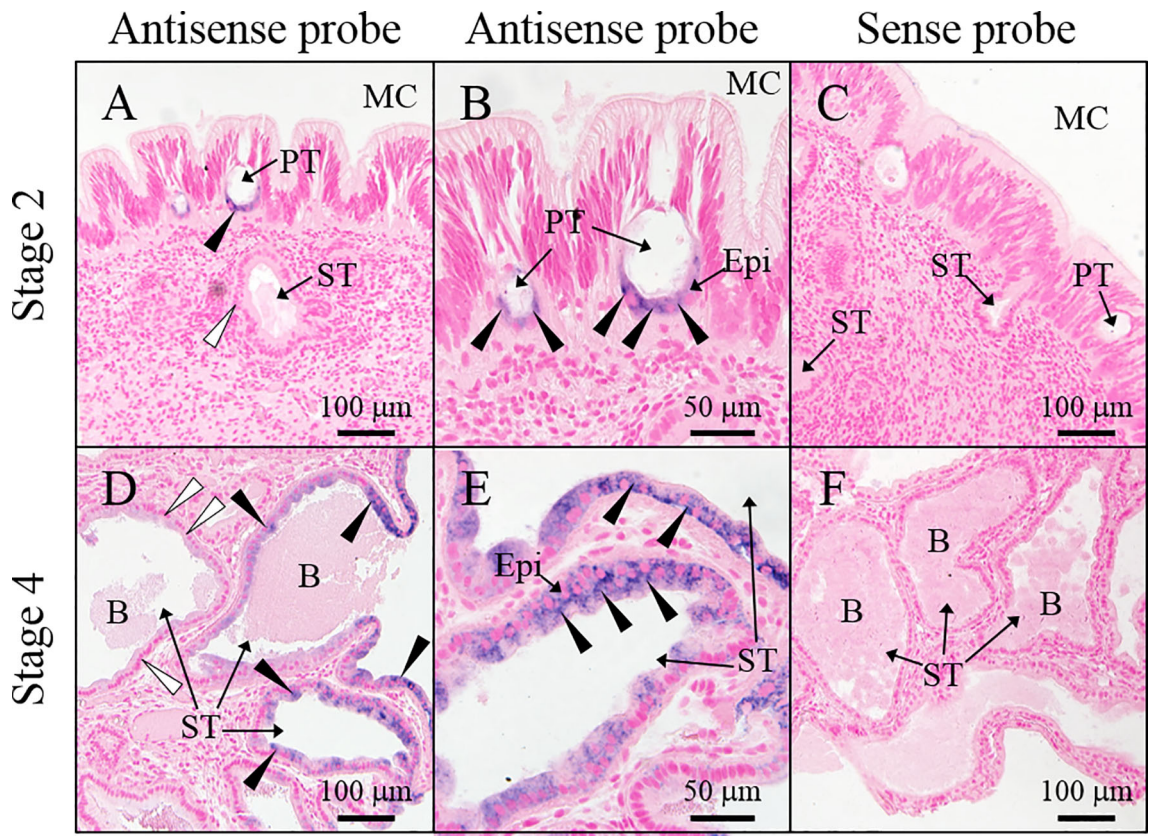

FIGURE 6 | Location of *slPgrp2* transcripts in ANG. **(A, B)** *slPgrp2* transcripts were detected in epithelial cells of primary tubules in the ANG using an antisense probe in immature female squid (stage 2). **(C)** Background staining was evaluated using a sense probe for *slPgrp2* in immature female squid (stage 2). **(D, E)** *slPgrp2* transcripts were detected with an antisense probe in epithelial cells of secondary tubules of ANG in mature female squid (stage 4). **(F)** Background staining was evaluated using a sense probe for *slPgrp2* in mature female squid (stage 4). Black arrowhead denotes *slPgrp2* signal, and white arrowhead denotes areas without *slPgrp2* signal in epithelial cells. B, bacteria; Epi, epithelial cells; MC, mantle cavity; PT, primary tubule; ST, secondary tubule.

results showed that cell density was significantly lower in cultures with the expression of *rec*Dmrt1 (with TPTG treatment; OD_{600} = 1.71 ± 0.01) compared to the control (without IPTG treatment; OD_{600} = 1.13 ± 0.01) after 3hrs of IPTG treatment. Although the negative effect of *E.coli* growth was observed in cultures of both IPTG-inducible *ecdmrt1* and *slPgrp2* expression cassette. Our data also showed that cell density was significantly higher in cultures with the expression of *rec*Dmrt1 (OD_{600} = 1.13 ± 0.01) compared with the expression of *rsl*PGRP2 (OD_{600} = 0.7 ± 0.02) after 3hrs of IPTG treatment. These data suggest that *sl*PGRP2 has negatively effect in bacterial growth.

DISCUSSION

According to this systematic study, we found four *slPgrps* in ANG transcriptome database. We analyzed the mRNA expression profile of these *slPgrps* in various tissues and different female phases, only the *slPgrp2* expression profile was correlated to the ANG development. We further characterized the spatial and temporal expression profiles of *sl*Pgrp2 in ANG during the female maturation. We provide evidence that *slPgrp2*: 1) gene expression profile is correlated with ANG growth in the female squid; 2) localizes on epithelial cells of the ANG, including the outer epithelial cell layer (primary tubules) and the inner epithelial cell layer (secondary tubules); 3) has high variability in expression levels in the different inner epithelial cell layer (secondary tubule). However, the color of different regions (pigmentation), which represent bacteria-synthesized carotenoids, did not correlated with the *slPgrp2* expression levels. These data suggest that the epithelial cell-synthesized *sl*PGRP2 may be involved in bacterial selection during bacterial colonization in the female bigfin reef squid.

Both the *slPgrp2* and *slPgrp5* showed high similarity in predicted protein structures. The apparent roles for *slPgrp2* are striking in contrast to *slPgrp5*. Unlike *slPgrp5*, which was mainly expressed in the outer epithelial cell layer, *slPgrp2* was dominantly expressed in the inner epithelial cell layer. Thus, this study is beginning to reveal the role of different *slPgrps* in ANG growth.

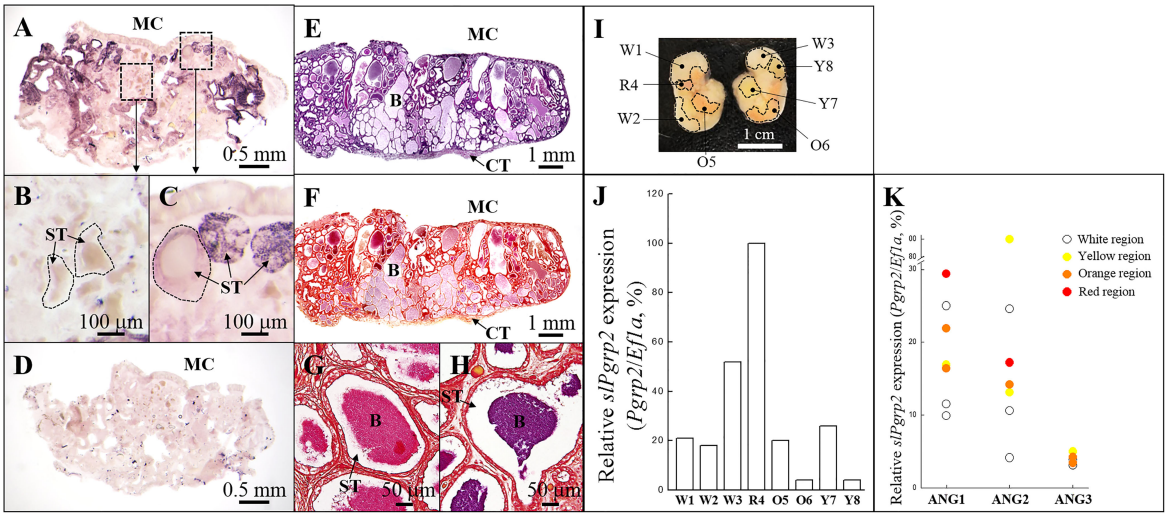

FIGURE 7 | Expression of *slPgrp2* transcripts in different areas of ANG in the mature female squid. **(A–C)** *slPgrp2* mRNA expression of ANG was detected by an antisense probe in mature female squid (stage 4). **(D)** Background staining was evaluated using a sense probe for *slPgrp2* in serial sections. **(E, F)** Histological characteristics and Gram staining of ANG in mature female squid, respectively. **(G, H)** Gram staining in different secondary tubules of ANG. **(I)** Different regions in ANG were distinguished by color and isolated under a stereomicroscope. Each color region was analyzed separately. **(J)** Expression of *slPgrp2* mRNA was analyzed by qPCR in different colors regions, as shown in **(I)**. **(K)** Four different colors of bacterial regions were detected and used for correlation with gene expression pattern: white, yellow, orange, and red. *slPgrp2* mRNA expression showed high variability in areas with different regions, even between regions with the same color. Dash line in **(B, C)** denotes the border of secondary tubule. B, bacteria; MC, mantle cavity; ST, secondary tubule; W, white; R, red; Y, yellow; O, orange.

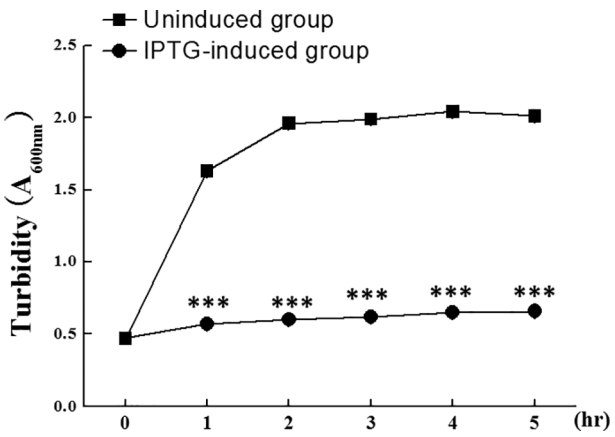

FIGURE 8 | The effect of rs*l*PGRP2 in bacterial growth. Recombinant *sl*PGRP2 (rs*l*PGRP2) was induced by the addition of IPTG. The potential effect of rs*l*PGRP2 in bacterial growth was analyzed by growth rate of *E. coli* using turbidity assay (spectrophotometer absorbance at 600 nm). *E.coli* carrying an IPTG-inducible *slVasa* expression cassette were used as reference control to show that the vector did not negatively affect E. coli growth. Samples were collected at 1-hr intervals after IPTG addition in IPTG-induced group. *E.coli* without IPTG treatment (uninduced group) was used as a reference. The three asterisks indicate Student's t-test ($P < 0.001$).

Bacterial Community of ANG in Cephalopods

The ANG houses a bacterial consortium that is horizontally transmitted from the environmental bacterial community (Kaufman et al., 1998; Li et al., 2019). In the present (**Figure 1**) and our previous studies (Li et al., 2019), our data show that the amount of bacterial flora in ANG varied during ANG growth. Furthermore, our recent study showed that the ANG bacterial community shifts gradually and decreases in diversity during maturation of the bigfin reef squid (Yang et al., 2021). In the Hawaiian bobtail squid, the microbiota of ANG undergoes a reduction of complexity during squid maturation and development of ANG (Kerwin et al., 2021). Such changes in bacterial diversity in the ANG during maturation stages could be due to host selection.

The core bacterial taxa found in cephalopod ANGs are Gram-negative *Alphaproteobacteria*, but the compositions of bacterial communities vary among cephalopod species (Grigioni et al., 2000; Pichon et al., 2005; Collins et al., 2012; Kerwin and Nyholm, 2017; Kerwin et al., 2021; Yang et al., 2021). Notably, the ANG bacterial community in Hawaiian bobtail squids collected from the field was conserved across hosts and was not affected by maintenance in artificial conditions (Kerwin et al., 2017). We suspect that the bacteria consortium in ANG is strongly selected by the host and varies according to species.

The Importance of Symbiotic Bacteria in Squid ANG for Offspring

Marine environments contain many microorganisms, some of which are lethal. Animals that lay eggs without subsequent parental care must have some mechanisms to protect developing embryos from infection and biofouling. In some crustaceans, parents deposit bacteria on eggs, and these bacteria secrete antimicrobial compounds to inhibit biofouling

(Gil-Turnes et al., 1989; Gil-Turnes and Fenical, 1992). Similarly, bacteria from the ANG are transmitted to the egg jelly coat in most squid and cuttlefish (Kerwin and Nyholm, 2017; Huang et al., 2018). In the Hawaiian bobtail squid, the bacteria composition of the jelly coat shows a high similarity to that in the ANG (Kerwin and Nyholm, 2017). Furthermore, eggs treated with antibiotics (suppress the bacteria deposited on the eggs) were susceptible to heavy biofouling and very low hatching rates compared to untreated eggs in Hawaiian bobtail squid (Kerwin et al., 2019). The growth of some fungi and yeast was inhibited by extracts from the symbiotic bacteria isolated from ANG and jelly coat (Kerwin et al., 2019; Suria et al., 2020). Therefore, delivery of ANG bacteria to the eggs play an important role in promoting embryo survival by preventing fungal fouling of the egg capsule for most squid and cuttlefish.

Control of Commensal Microbiota in Squid by PGRPs

PGRPs are conserved from insects to mammals and have diverse functions in antimicrobial defense as well as recognition and maintenance of symbiotic organisms (Royet et al., 2011). Unlike insects, which have many *Pgrp* genes, cephalopods and vertebrates have fewer *Pgrp* genes (Goodson et al., 2005; Collins et al., 2012). Only four *Pgrp* genes were found in the ANG of bigfin reef squid, including the secreted *slPgrp2* and *slPgrp5*, GPI-anchored *slPgrp3*, and the *slPgrp*4 surface receptor (**Figure 2**). In bigfin reef squid, *slPgrp2* mRNA are predominantly expressed in the ovary and ANG, while *slPgrp4* and *slPgrp5* mRNA are predominantly expressed in hemocytes (**Figure 2** and **Supplemental Figure 1**). Furthermore, only the *slPgrp2* gene expression levels of epithelial cells are correlated to the ANG growth (**Figures 4**–**6**). In Hawaiian bobtail squid, PGRPs participate in the winnowing and maintenance of the symbiotic luminescent bacterium *V. fischeri* within the light organ (Nyholm and Graf, 2012; McAnulty and Nyholm, 2017; Nyholm and McFall-Ngai, 2021). Both PGRP1 and PGRP2 play integral roles in the recognition of symbiotic bacteria during *V. fischeri* colonization of the Hawaiian bobtail squid light organ (Troll et al., 2009; Troll et al., 2010). After *V. fischeri* enters the light organ duct and then colonizes the crypts, the PGRP1 level decreases dramatically and ciliated epithelial cells undergo apoptosis to promote the loss of appendages during the morphogenesis of light organ (Troll et al., 2009). Furthermore, epithelial cell-synthesized PGRP2 is secreted into the lumen of the crypts, where it degrades peptidoglycan fragments secreted by *V. fischeri* (Troll et al., 2010). *Pgrp5* mRNA is dominantly expressed in hemocytes of Hawaiian bobtail squid (Collins et al., 2012). Notably, the expression of *Pgrp5* is decreased in hemocytes of Hawaiian bobtail squids upon removal of symbiotic bacteria (Collins et al., 2012). Thus, the colonization of the light organ influence *Pgrp* gene expression in the Hawaiian bobtail squid (Collins et al., 2012; Nyholm and McFall-Ngai, 2021). Taken together, these observations and ours suggest that *slPgrp2* may participate in bacterial colonization during the ANG growth in female bigfin reef squid.

PGRP2 in both bigfin reef squid and Hawaiian bobtail squid share 67% identity and 88% similarity at the amino acid level. PGRP2 showed bactericidal activity in Hawaiian bobtail squid (Troll et al., 2010). PGRP2 also prevents the inflammatory effects of hydrolyzed peptidoglycan fragments released from symbiotic bacteria in the light organ (Troll et al., 2010). Bactericidal PGRPs are also found in other species, such as some bivalves (Kong et al., 2018; Huang et al., 2019; Yang et al., 2019), teleosts (Li et al., 2007; Kim et al., 2010; Li et al., 2020) and mammals (Lu et al., 2006). In zebrafish, knockdown of *pglyrp5* and *pglyrp6* expression during embryogenesis results in increased sensitivity to infections with pathogenic bacteria and poor survival (Chang and Nie, 2008; Li et al., 2007). Moreover, *Pgrp*-deficient mice (*Pglyrp1*$^{-/-}$, *Pglyrp2*$^{-/-}$, *Pglyrp3*$^{-/-}$, and *Pglyrp4*$^{-/-}$) are more sensitive to colitis than wild-type mice due to aberrations in the interferon-γ regulating pathway (Saha et al., 2010). Thus, mammalian PGRPs are important regulators of commensal microbiota and intestinal homeostasis, protecting the host from tissue damage and colitis. Our results speculate that inner epithelial cell layer-expressed *slPgrp2* may participate in bacterial selection during the bacterial colonization of ANG. We suspect that *sl*PGRPs may have a similar essential role in the defense against bacteria and further participate in the winnowing (bacterial selection) and maintenance of symbiotic bacteria in the bigfin reef squid ANG in comparison to Hawaiian bobtail squid ANG or light organ. In bigfin reef squid, *slPgrp2* was highly expressed in ovary. In fish, *pglyrp2* transcripts is expressed in the developing oocytes (Li et al., 2007). Moreover, high expression level of Pglyrp2 protein is found in mature eggs and developing embryos (Li et al., 2007). Fish Pglyrp2 proteins showed bactericidal activity (Li et al., 2007). Therefore, *slPgrp2* may have essential role in the defense against bacteria during the early stage of embryonic development in bigfin reef squid. Furthermore, the potential roles of other homologs, *slPgrp4* and *slPgrp5* in hemocytes, should be examined with regard to the integral bacterial colonization of the ANG.

CONCLUSION AND FUTURE DIRECTIONS

Although the mechanisms of colonization in the ANG association are not yet as well understood as those of the light organ, this study in ANG are able to provide a new insight of mechanisms of colonization and maintenance in complex consortia. In this study, we examined the temporal and spatial profiles of *slPgrp2* transcripts. Unlike PGRP2, which is broadly distributed in the surface area (epithelial cell layer) of different organs in Hawaiian bobtail squid (Troll et al., 2010), we found epithelial cell-expressed *slPgrp2* shows differentially expression levels in different regions of ANG. This data reveals that *slPgrp2* transcripts may associate with the composition of bacterial consortium and it secretary factors. Further studies are needed to determine which bacteria species can trigger *slPgrp2* synthesis and cause the secretion of *sl*PGRP2 into secondary tubules of the ANG. A specific antibody for *sl*PGRP2 is required to better explore the regulatory machinery underlying the interactions of microbiomes with host immunity. The eukaryotic cell-expressed

r*sl*PGRP2 is required to examine the bactericidal activity (against *E. coli* or other bacterial isolates from ANG of bigfin reef squid).

Recently, a study of the whole genome of Hawaiian bobtail squid provides new insight on organ-specific regulatory mechanism in light organ and ANG (Belcaid et al., 2019). The transcripts of light organ have high similarity with the eye and the ANG have high number of ANG-specific transcripts. These analyses show that the light organ and ANG are likely to have evolved *via* different mechanisms. Future studies are need to perform the ANG transcriptome analysis of bigfin reef squid that will make a better understanding of the regulatory mechanism of complex bacterial consortium.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by National Taiwan Ocean University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

H-WL and G-CW collected the samples. H-WL conducted the molecular cloning, RNA analysis, turbidity assay and WB experiments. W-LK conducted the ISH study. CC conducted the phylogenetic analysis. Y-CT gave comments. C-FC gave the comments and participated in compiling the main manuscript. G-CW designed and conducted experiments, analyzed the data, and participated in compiling the main manuscript. All authors reviewed and approved the manuscript.

FUNDING

This work was partly supported by grants from the Ministry of Science and Technology [MOST 104-2923-B-019-001-MY4] and by the Center of Excellence for the Oceans, National Taiwan Ocean University, from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) of Taiwan.

ACKNOWLEDGMENTS

We thank Dr. Marcus Calkins at Academia Sinica for reading and correcting of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.825267/full#supplementary-material

Supplementary Figure 1 | Tissue distributions of *slPgrp* gene expression in mature female squid. Mature female squid was used for gene expression analysis in various tissues. Four *Pgrp* genes were identified from the ANG transcriptomic database and selected for gene expression analysis, including *slPgrp2* **(A)**, *slPgrp3* **(C)**, *slPgrp4* **(B)**, and *slPgrp5* **(C)**. The relative gene expression level of each *Pgrp* is also shown in the tissue with highest expression. Ma, mantle (muscle); Te, tentacle; Ol, optic lobe; Br, brain; St, stomach; Hp, hepatopancreas; Gi, gill; He, heart; NG, nidamental gland; ANG, accessory nidamental gland; Od, oviduct; Og, oviductal gland; Hm, hemocytes.

Supplementary Table 1 | The characteristics of sampled female squids.

Supplementary Table 2 | List of gene names and accession numbers for phylogenetic analysis.

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