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Immune signaling pathways in the endoparasitoid, *Pteromalus puparum*

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Abstract

Parasitoids serve as effective biocontrol agents for agricultural pests. However, they face constant challenges from host immune defense and numerous pathogens and must develop potent immune defense against these threats. Despite the recent advances in innate immunity, little is known about the immunological mechanisms of parasitoids. Here, we identified and characterized potential immune-related genes of the endoparasitoid, *Pteromalus puparum*, which act in regulating populations of some members of the Pieridae. We identified 216 immune-related genes based on interrogating the *P. puparum* genome and transcriptome databases. We categorized the cognate gene products into recognition molecules, signal moieties and effector proteins operating in four pathways, Toll, IMD, JAK/STAT, and JNK. Comparative analyses of immune-related genes from seven insect species indicate that recognition molecules and effector proteins are more expanded and diversified than signaling genes in these signal pathways. There are common 1:1 orthologs between the endoparasitoid *P.*

puparum and its relative, the ectoparasitoid *Nasonia vitripennis*. The developmental expression profiles of immune genes randomly selected from the transcriptome analysis were verified by a quantitative polymerase chain reaction. Our work provides comprehensive analyses of *P. puparum* immune genes, some of which may be exploited in advancing parasitoid-based biocontrol technologies.

KEYWORDS
bacteria, immunity, parasitoid wasp, *Pteromalus puparum*, transcriptome

1 | INTRODUCTION

Most vertebrates and invertebrates express innate immunity, composed of nonspecific, general responses to infections and invasions. Insects lack the antibody-based adaptive immune system known in vertebrates. They have evolved quite efficient innate immune mechanisms to identify and eliminate invaders (An et al., 2012; Beutler, 2004; Lemaitre & Hoffmann, 2007). While it is an oversimplification, their innate immunity functions are assorted into humoral and cellular immune responses, that operate against microbial and parasite challenges and participate in wound repair (Kleino et al., 2005). Insect immune system includes recognition molecules, signal moieties, and effector proteins. Their cognate genes constitute a signal transduction network, including the Toll, immune deficiency (IMD), c-Jun N-terminal kinase (JNK) and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways (Cao et al., 2015; Myllymäki & Rämet, 2014; Myllymäki, Valanne, & Rämet, 2014; Zhang et al., 2017).

The Toll and IMD pathways are two well-studied immune signaling pathways. Toll was first discovered in studies of *Drosophila* embryonic development and later found to act in immunity (Chowdhury et al., 2019; Ramirez, Muturi, Barletta, & Rooney, 2019). Usually, the Toll pathway is activated by Gram-positive bacteria and fungi. Gram-negative bacteria leads to activation of the IMD pathway. Both pathways are launched by pattern recognition receptors (PRRs) such as peptidoglycan recognition proteins (PGRPs) and β-glucan recognition proteins (β-GRPs), stimulated by microbial pathogen-associated molecular patterns (PAMPs) to mediate the induction of transcription factor nuclear factor kappa B (NF-κB). These reactions ultimately lead to melanization of encapsulated parasitoid eggs and bacteria-laden nodules, and synthesis of antimicrobial peptides (AMPs; Bulet & Stocklin, 2005; Engström, 1999; Y. Li, Xiang, Zhang, Huang, & Su, 2012; Zou, Shin, Alvarez, Kokoza, & Raikhell, 2010). Yajima et al. (2003) showed that eicosanoids, oxygenated products of some polyunsaturated fatty acids (Kim, Ahmed, Stanley, & An, 2018; Stanley & Kim, 2014) are necessary, but not independently sufficient, to activate the IMD pathway. The JAK/STAT pathway lead to antiviral immunity and the JNK pathway is induced by environmental stress and acts in wound healing and apoptosis (Igaki, 2009).

Of the approximately 6 or 700,000 estimated parasitoid wasp species, some of them are cultured and released in efficient biological control programs due to their capabilities in reducing populations of pest insect species on Earth (Boivin, Hance, & Brodeur, 2012; Burke & Strand, 2014; Lin et al., 2018). In a broad picture, parasitoid eggs are deposited on or into arthropod host species. The juvenile parasitoids develop on or in their hosts, ultimately killing the host. Immunity is the major axis of host/parasitoid interactions. Parasitoids protect themselves from host immunity with a wide range of biochemical factors, including parasitoid-associated virulence factors such as venom, polydnaviruses (PDVs), virus-like particles (VLPs), and teratocytes (Fang et al., 2011b; Strand, 2014; Strand & Burke, 2015). There is a lot of literature on each of these virulence factors that generally result in the suppression of host immunity to parasitoid eggs and young larvae. Parasitoids also live in nature, where every stage of their life cycles is subject to a wide range of

microbial infections and invasions. Although relatively little effort has been invested into parasitoid immune functions so far, a few research works have focused on AMPs and annotation of the immune genes in *Nasonia vitripennis* and some sister species (Gao & Zhu, 2010; Tian, Gao, Fang, Ye, & Zhu, 2010). Threats from predators, pathogens and hyperparasitoids could cause unpredicted impacts on parasitoid populations, which may lead to failures or reduced efficacy of biological control programs. Viewed from the perspective of fundamental ecological research or global pest management technologies, research into parasitoid immunology is valuable and necessary.

Here, we report on an endoparasitoid, *Pteromalus puparum*, a valuable parasitoid in managing the pupal stage of certain pierid species, especially the white butterfly, *Pieris rapae* (Cai, Ye, & Hu, 2004). Like all insects, this wasp is vulnerable to entomopathogens and predators whether inside or outside of its host. Research into the *P. puparum* immune system is necessary to understand its operational mechanisms and to improve large-scale rearing efficiencies needed to produce millions of parasitoids used in biological control programs.

We recently completed the whole-genome sequencing of *P. puparum* (unpublished results), which enables us to comprehensively identify immune genes in this species. We also had transcriptome data from various development stages, which provide detailed gene expression data on selected genes. Quantitative polymerase chain reaction (qPCR) analysis of immune responses to infections with a Gram-negative bacterium (*Escherichia coli*), a Gram-positive bacterium (*Micrococcus luteus*) and an insect-pathogenic fungus (*Beauveria bassiana*) at three time points (6, 24, 48 hr postinfection, PI) provided data on changes in gene expression profiles of immune-related genes. Comparative analysis of immune genes in several insect species helped determine the overall functional network of the immune components. Recently, we interrogated databases to identify immune related genes and gene families in six insect species, including *Drosophila melanogaster* (Hoffmann & Reichhart, 2002); *Anopheles gambiae* (Christophides et al., 2002), *Apis mellifera* (Evans et al., 2006), *Tribolium castaneum* (Zou et al., 2007), *Bombyx mori* (Tanaka et al., 2008), and *N. vitripennis* (Brucker, Funkhouser, Setia, Pauly, & Bordenstein, 2012; Sackton, Werren, & Clark, 2013; Werren et al., 2010). Comparing immune genes of *P. puparum* with these insects help us to find gene similarity or diversity. Here, we report an overview of immune genes of *P. puparum*.

2 | MATERIALS AND METHODS

2.1 | Insect feeding

The *P. puparum* and host *P. rapae* colonies were reared under a 14 L:10D photoperiod at 25 ± 1°C (Yang, Lin, et al., 2017). The wasps were maintained in finger-type holders and fed with 20% (v/v) honey solution. *P. rapae* was fed with fresh cabbages. Pupal hosts were used for parasitism. The parasitized larvae were maintained as just described. Newly emerged adult wasps were collected immediately for colony maintenance.

2.2 | Identification of immune-related genes

Known immune-related gene sequences from *D. melanogaster*, *T. castaneum*, *A. gambiae*, *B. mori*, and *A. mellifera* were used as queries to search for homologs in the *P. puparum* genome using local BLAST (*E*-value: $1e^{-5}$). Identified genes were confirmed manually by the nonredundant nucleotide database using NCBI online BLASTP. The genomic location information was predicted through the alignment of mRNA-genome by the NCBI spideyweb (http://www.ncbi.nlm.nih.gov/spidey/spideyweb.cgi). The signal peptides and transmembrane regions were determined using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM program v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The mitochondrial regions predicted were detected by the MITOPROT Server (https://ihg.gsf.de/ihg/mitoprot.html). Domain analysis of the immune-related proteins was done by PROSITE (http://au.expasy.org/prosite/), Pfam (http://www/sanger.ac.uk/Software/Pfam/), and SMART (http://smart.embl-heidelberg.de/).

2.3 | Sequence alignment and phylogenetic analysis

Immune genes were aligned with genes of the same families from other insects. All of the sequence alignments were performed by ClustalX 2.0 (Thompson, 1997). All of the sequences alignments used for phylogenetic analyses are listed in Figure S1. The neighbor-joining method was used to construct phylogenetic trees by MEGA5.0 (Tamura et al., 2011) with 1,000 bootstrap replicates.

2.4 | Sample collection, complementary DNA synthesis, and qPCR analysis

RNA samples were prepared for cDNAs synthesis as described (Yang, Lin, et al., 2017). In brief, wasps in different developmental stages were collected. The samples include embryos, mixed 1st, 2nd, 3rd larval instars, early pupal females and males, and adult female and male wasps. The Gram-negative bacterium *E. coil*, Gram-positive bacterium *M. luteus*, and fungus *B. bassiana* were used for immune challenges. Immune challenge method was performed following R. J. Wang et al. (2017). Briefly, we anesthetized *P. puparum* female adults with carbon dioxide for 30 s. Acupuncture needles with microbes were used to penetrate the abdominal cuticle of the wasps. Then we collected wasps 6, 24, and 48 hr post-prick with different microbes to analyze gene expression profiles. All sample groups were individually washed and placed in centrifuge tubes prefilled with Trizol reagent (Sangon). RNA samples were obtained following the manufacturer's instructions. Genomic DNA contamination was cleared with RNase-free DNase. The concentration of total RNA was estimated by measuring the absorbance at 260 nm.

PrimeScript™ One-Step RT-PCR Kits (Takara, Japan) was used in cDNAs synthesis. We designed all the primers on the Primer 3 web site (Untergasser et al., 2012; Table S1), and had them synthesized commercially (Sangon, China). The qPCR reaction procedure was performed in 25 μl volumes containing 2 μl cDNA. The qPCR procedure was carried on a BIO-RAD CFX96™ Real-Time System in following the SYBR Green Supermix Kits (Takara) protocols. The thermocycler was programmed at 95°C for 30 s for enzyme activating, followed by 95°C for 5 s with 40 cycles, in the final step 60°C for 34 s for annealing. We checked dissociation curves at the end of PCR reactions. *P. puparum actin* 1 was set as a reference gene in the immune challenge experiments. The 18S rRNA was the reference gene to verify development expression profiles. The expression level of *P. puparum actin 1* is much stable among different microbial infections. The transcript level of 18S rRNA gene showed good stability at differently developmental stages of *P. puparum* (Yang, Lin, et al., 2017). The $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) was used to calculate relative accumulations of mRNA. Statistical analysis of the expression profiles of development was conducted by one-way analysis of variance (ANOVA). Two-way ANOVA was used in the expression profiles of immune infected cases.

2.5 | Analyses of RNA-seq data

Samples from RNA-seq libraries were previously generated from six wasp life periods in our lab. We used the same samples for qPCR verification of genes randomly selected from transcriptome analysis. The expected number of fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) values were calculated by TopHat and Cufflinks (Trapnell et al., 2012).

3 | RESULTS AND DISCUSSION

3.1 | Overview of immune genes

We identified 216 immune-related genes in *P. puparum* (Tables S2 and S3). All of the predicted immune-related amino acid sequences are summarized in Table S4. These genes are classified into three functional groups pattern recognition proteins, signal transduction molecules and effector proteins. Pattern recognition proteins stimulate downstream signals in four immune signal pathways, Toll, IMD, JNK, and JAK-STAT. These pathways

lead to the activation of immune effectors, prophenoloxidases (PPOs), thioester-containing proteins (TEPs), AMPs, and enzymes involved in detoxification of reactive oxygen species (ROS). Comparing *P. puparum* data with those of six other insect species, the total number of immune-related genes (216) in *P. puparum* is smaller than those of *D. melanogaster* and *A. gambiae*, but more than that of *A. mellifera* (Table S3). Our analysis of gene numbers among seven insect species, which are mostly signaling molecules, show these genes are conserved and present as single-copy orthologs. Both *P. puparum* and *N. vitripennis* are polyparasitism wasps in the family of Pteromalidae. *P. puparum* immune genes are present in 1:1 orthology to genes in *N. vitripennis*.

In the following section, these immune-related gene families in three functional groups (pattern recognition proteins, signal transduction molecules involved in four immune pathways, and effector proteins) were described independently. The details of each gene family may include sequence alignment, phylogenetic tree, RNA-seq, and qPCR data.

3.2 | Gene diversity in pattern recognition proteins

3.2.1 | Peptidoglycan recognition proteins

Peptidoglycan recognition proteins (PGRPs) detect peptidoglycans (PGNs), components of nearly all bacterial cell walls (except members of the genus Mycoplasma), but not in insects (Dziarski & Gupta, 2006). Two basic classes of PGRPs (PGRP-S and PGRP-L) are generally recognized, based on gene structure and amino acid chain lengths (S. Wang & Beerntsen, 2015). For *D. melanogaster*, PGRP-S can recognize Gram-positive bacteria by interacting with Lys-type PGN and PGRP-L detects Gram-negative bacteria through binding DAP-type PGNs (R. J. Wang et al., 2017). There is also some overlap response between Gram-negative and Gram-positive bacteria. *Drosophila* PGRP-SD can bind to Gram-negative bacteria (Leone et al., 2008; Q. Wang, Ren, Liu, Xia, & Chen, 2019). We identified nine *PGRP* genes in *P. puparum* (Table S2) and assorted them into four *PGRP-Ls* and five *PGRP-Ss* (three with transmembrane regions and four with signal peptides). PpPGRP-L2 and PpPGRP-S3 have neither a signal peptide nor a transmembrane region (Figure S2). They were named according to the results of the blast and the phylogenetic analysis. A typical PGRP domain is about 160 amino acids in length, usually located at the C-terminal sequence. We aligned the PpPGRPs with PGRPs from *D. melanogaster* and *A. mellifera* (Figure 1), showing the sequence conservation among them. All PpPGRPs feature three α-helix and seven β-sheets. PpPGRP-S1 and PpPGRP-S2 contain five conserved amino acids (His, Tyr, His, Thr, Cys), which are essential for amidase activity (Y. P. Li et al., 2015). An Arg residue is conserved in PpPGRPs, except PpPGRP-S3. Arg residue in this position interacts with DAP-type PGNs (Lim et al., 2006) and we infer that these PpPGRPs operate against Gram-negative bacteria. PpPGRPs have close orthology to PGRPs in *N. vitripennis* (Figure S3). L-type PpPGRPs formed in one clade. PpPGRP- L1, L3, and L4 are located on one scaffold (Figure 2), and clustered with corresponding *N. vitripennis* genes. Our speculation is these arose from one or more gene duplication events.

Most of the *PpPGRPs* were expressed at relatively low levels in embryos and then increased their expression levels in larvae subsequently with apparent peaks in pupae and mature adults (Table S5). We also analyzed the expression profiles of *PpPGRPs* at the indicated time points after pathogen challenge. The induction in the expression profiles of *PpPGRP-L* type genes is moderate (data not shown). Figure 3 shows the relative expressions levels of four *PpPGRP-S* type genes were induced post pathogen immune challenge compared to PBS challenged. They reached a peak 24 hr post *B. bassiana* or *M. luteus* infection. The expression profiles of *PpPGRP-S2* were also slightly elevated post *E. coil* challenged. It is reported that PGRP-S can usually recognize Gram-positive bacteria and promote the Toll pathway. Because *M. luteus* is a type of Gram-positive bacterium, it may induce high expression levels of *PGRP-S* genes of *P. puparum* after immune challenge. In addition, *B. bassiana* is a kind of entomopathogenic fungus. Previous studies verified that *PGRP-S* could be unregulated by fungi challenges in some insects (Dai et al., 2015; Xiong et al., 2015). After *B. bassiana* infection, the expression of *P. puparum PGRP-S* may be induced strongly. However, in this manuscript, we only detected the expression profiles of *PGRP-Ss*. How these genes promoting the immune responses of *P. puparum* should be further investigated.

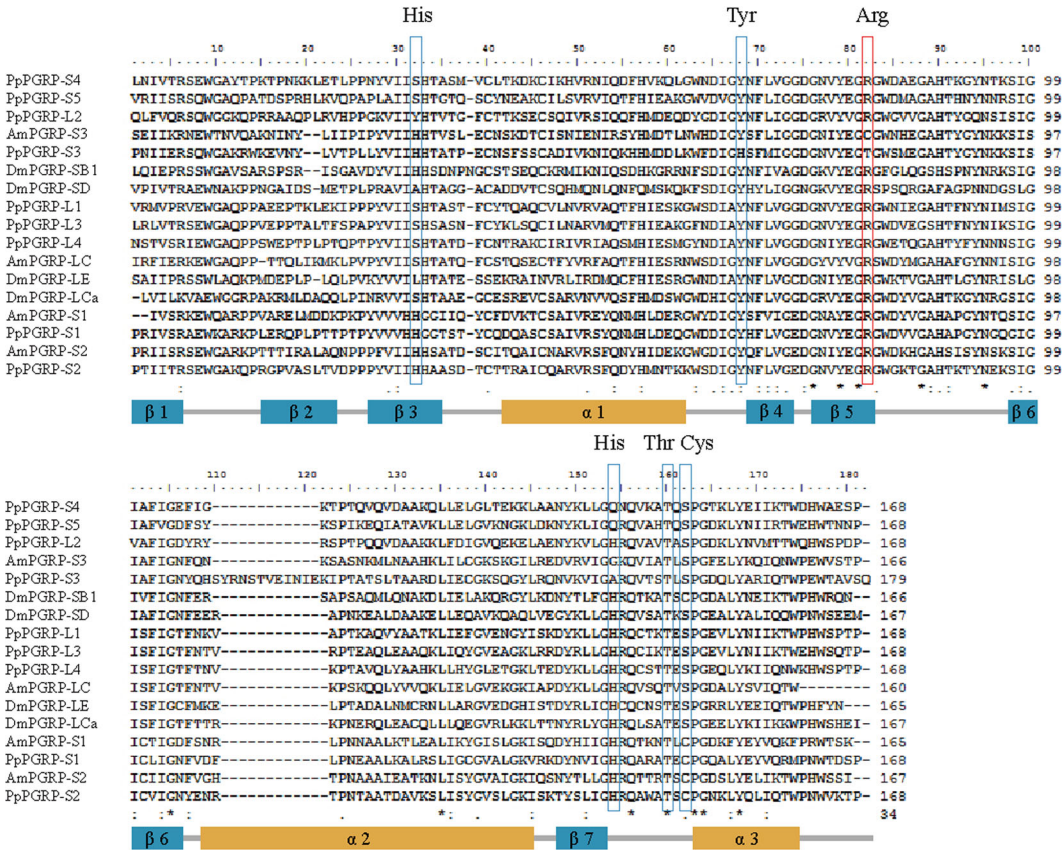

FIGURE 1 Multiple sequence alignment of the PGRP domains from *Pteromalus puparum*, *Apis mellifera*, *Drosophila melanogaster*. Identical amino acids were marked by asterisks whereas conservative substitutions were marked by dots. Blue indicates the residues required for Zn2+-binding/amidase activity. Red indicates amino acids required for recognition/binding of diaminopimelic acid (DAP)-type peptidoglycans (PGNs). Regions of protein secondary structural elements are marked under the sequences. PGRP, peptidoglycan recognition proteins

3.2.2 | β-1,3-glucan recognition proteins

β-1,3-Glucan recognition proteins (βGRP), is also known as Gram-negative binding protein (GNBP) in some insects (Hughes, 2012). βGRPs occur in invertebrates and act against bacteria and fungi by binding to bacterial glucanases (Ma & Kanost, 2000; Ochiai & Ashida, 1988). There are two *βGRPs* in *P. puparum* (Table S2). Both feature a signal peptide with seven exons. βGRP1 (PpβGRP1) has a conserved β-1,3-glucan-recognition domain and a glucanase-like domain (Figure S2). PpβGRP2 lacks the N-terminal β-1,3-glucan-recognition domain, which is responsible for high affinity to 1,3-β-glucan. The C-terminal glucanase-like domain is large (~350 residues) but lacks key residues in the active sites (Ma & Kanost, 2000; Ochiai & Ashida, 1988).

3.2.3 | C-type lectins

C-type lectins (CTLs) recognize a diverse range of ligands and are defined by the presence of at least one C-type lectin-like domain (CTLD). They are Ca^{2+} dependent and function largely outside the cells (Shi et al., 2014; Xia, You, Rao, & Yu, 2018). Invertebrate CTLs could mediate several immune responses including encapsulation (P. Wang et al., 2017), bacterial reorganization and clearance (Tanji, Ohashi-Kobayashi, & Natori, 2006), and activation of

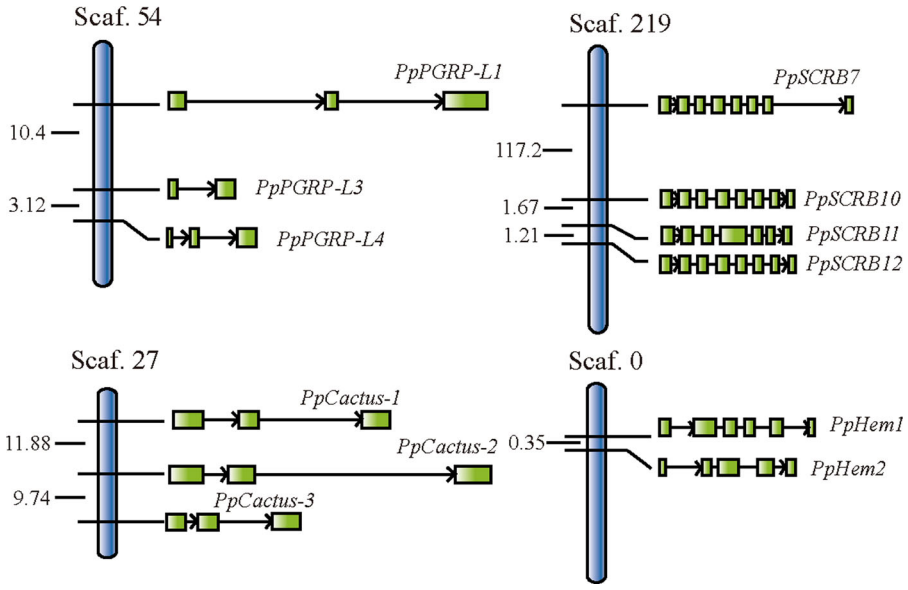

FIGURE 2 Structure and location of *Pteromalus puparum* immune-related genes on scaffolds. Scaffold number is shown at the top of each bar. The distance of two adjacent genes (kilobases, kb) is presented on the left of the bar. Gene names and their exon-intron structures are shown on the right. The arrows indicate the transcription orientations. The exons are shown with blue boxes.The total length of the blue boxes presents the gene size on scaffolds

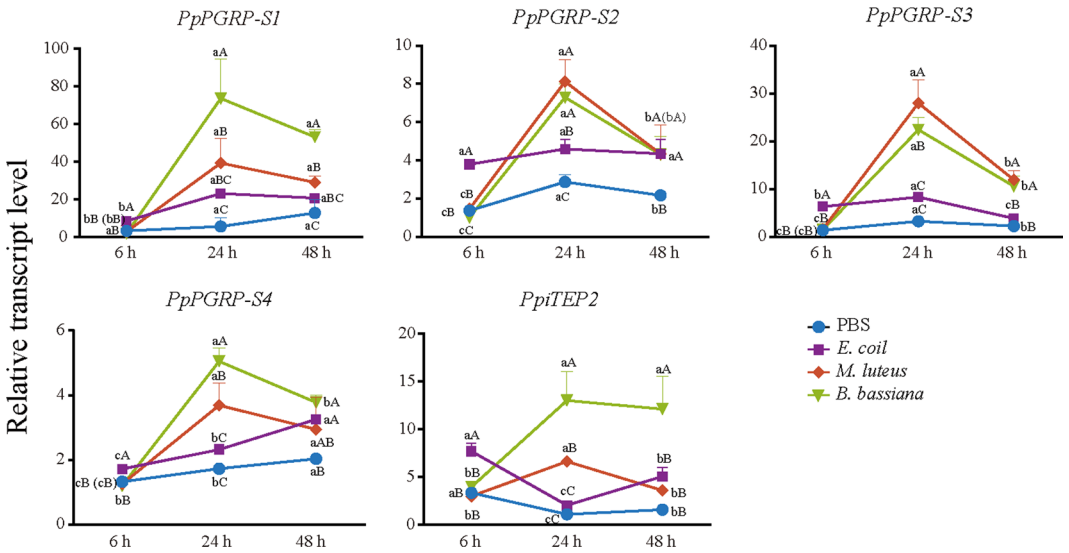

FIGURE 3 Expression levels of immune-related genes following different immune challenge. Expression levels of immune-related genes following the infection of Gram-negative (*Escherichia coli*), Gram-positive (*Micrococcus luteus*) bacterium or the entomopathogenic fungus (*Beauveria bassiana*) were analyzed using quantitative polymerase chain reaction. Time points along the *x*-axis represent the hours postinfection. Error bars represent the means ± standard deviations from three biological replicates. *Pteromalus puparum actin 1* was used as the reference gene. Two-way analysis of variance was used to determine the combined effects of infection and time by Duncan's new multiple range method. The different lowercase letters (a–c) represent the significant difference at the different time points after infection with the same pathogen ($p < .05$), and the capital letters (A~D) indicate the significant difference at the same time points after different pathogenic infection ($p < .05$)

PPO (J. L. Wang et al., 2014). There are 26 *CTLs* in *P. puparum*, more than those of *A. mellifera*, *T. castaneum*, *B. mori*, *A. gambiae*, but less than those of *N. vitripennis* and *D. melanogaster* (Table S3). The lengths of the *P. puparum* CTL (PpCTL) genes range from 157 to 4,150 amino acid residues and the exons range from 2 to 36 (Table S2). Most PpCTLs feature one CTLD. PpCTL8 and PpCTL10 have three CTLDs and PpCTL16, PpCTL22, and PpCTL14 are dual CTLD proteins. Previous studies demonstrated *B. mori* multibinding protein named BmMBP with two CTLDs in tandem. Both CTLDs were different in binding affinity and BmMBP could activate cellular immune activities following infections with several microbial species (Watanabe et al., 2006). We infer PpCTLs with dual or triple CTLD domains may have diverse functions in immunity. Analysis of CTLs from *D. melanogaster*, *B. mori*, *A. mellifera*, and *P. puparum* showed all the genes are divided into two groups (Figure 4). Group A CTLs formed eight branches with orthologous homologies. Group B genes from three species were separated into three clusters to be species-specific.

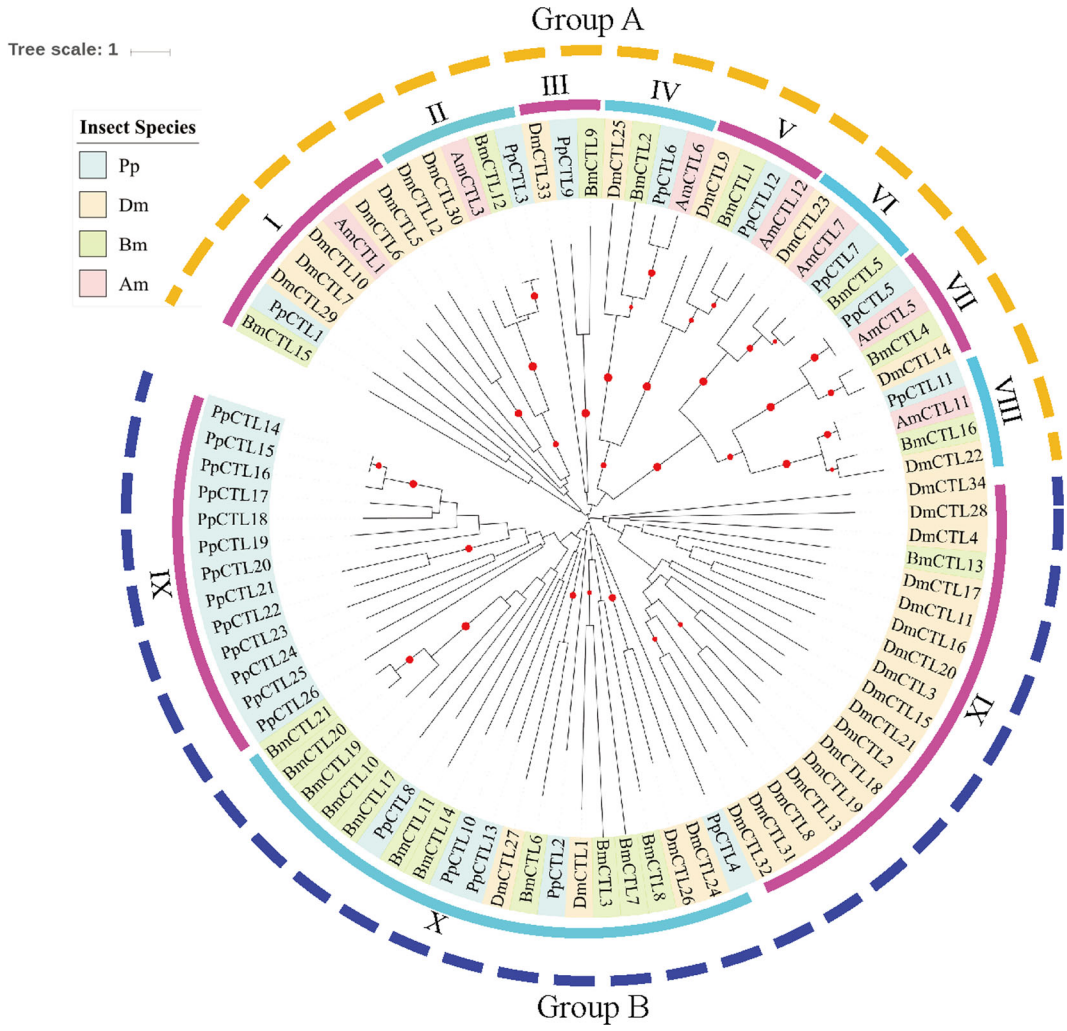

FIGURE 4 Phylogenetic analysis of CTL genes. The CTL domain of amino acid sequences of *Pteromalus puparum* (Pp), *Apis mellifera* (Am), *Bombyx mori* (Bm) and *Drosophila melanogaster* (Dm) was aligned. Phylogenetic tree was constructed by the neighbor-joining method, using the program Mega 5.10. Red spots at the nodes denote bootstrap values greater than 500 from 1000 trials. CTL, C-type lectins

3.2.4 | Scavenger receptors B

Scavenger receptors B (SCRB) comprise a large superfamily of proteins. In insects, they interact with numerous ligands including self-ligands such as lipoproteins or apoptotic cells (Peiser & Gordon, 2001) and microbial ligands (bacteria and fungi; Areschoug & Gordon, 2009; Philips, 2005). They may participate in the maintenance of homeostasis and in pathogen clearance (Canton, Neculai, & Grinstein, 2013). We identified 12 genes encoding SCRBs (Table S2). Eleven of them have a CD36 domain with two transmembrane domains located at both termini of the CD36 region. The sequence lengths are about 500–600 amino acids with seven to ten exons. Phylogenetic analysis with the indicated species showed SCRBs are conserved in amino acid sequences (Figure S4). The PpSCRBs were clustered with orthologous genes from five insect species with high bootstrap values. The tree is separated into nine clades, with Group II encompassing four PpSCRBs. These four are located at the same scaffold (Figure 2).

3.2.5 | Fibrinogen-related proteins

While the numbers of *P. puparum* recognition proteins are similar to other species, *P. puparum* has one *FREP*, compared to 61 *FREPs* in *A. gambiae* (Table S3). Genome sequences of 12 *Drosophila* species show each species has from 14 to 43 *FREPs* (X. Wang, Zhao, & Christensen, 2005), which act in recognition and defense against microbes. Fibrinogen-related proteins (FREPs) may also be involved in fibrin coagulation (Hanington & Zhang, 2011; Oren, Escande, Paz, Fishelson, & Rinkevich, 2008). The functions of FREPs in *P. puparum* have not been investigated.

3.3 | Signal transduction pathways

3.3.1 | The Toll signal pathway

The Toll pathway acts in embryonic development and immunity (Anderson, 2000; Valanne, Wang, & Rämet, 2011). Once triggered, Spätzle-processing enzyme cleaves extracellular inactive Späzle into active Spätzle, which binds to a Toll receptor, causing receptors to dimerize (Figure 5a). We identified five Spätzle genes in

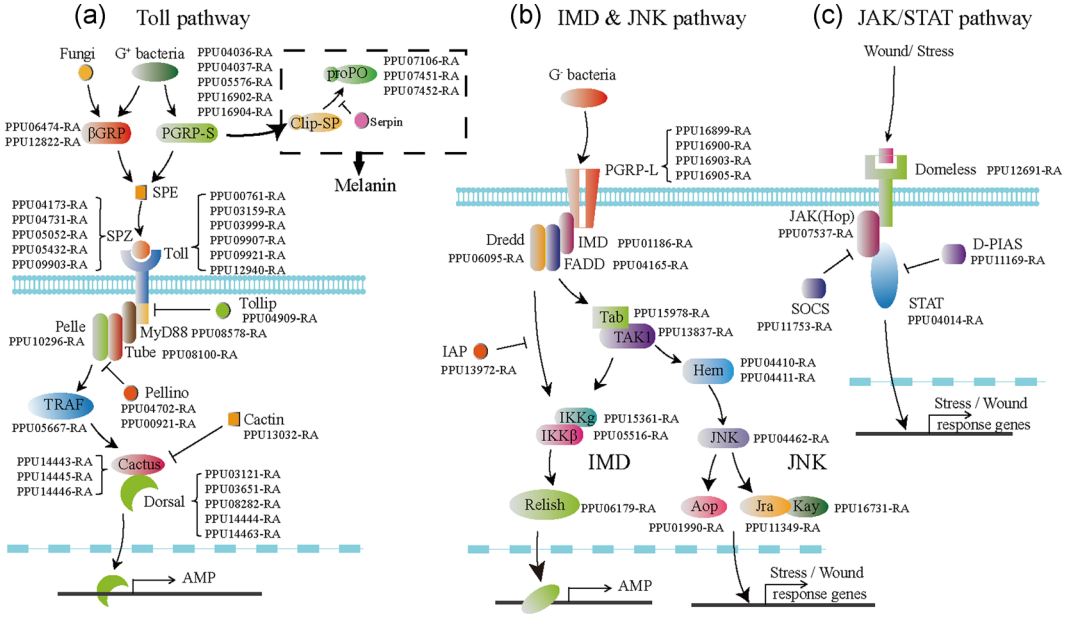

FIGURE 5 Putative signaling pathways and melanotic processes in *Pteromalus puparum*. (a) The Toll pathway; (b) The IMD & JNK pathway; (c) The JAK–STAT pathway. This diagram is modified from Cao et al. (2015) and Zou et al. (2007) and drawn by the softwares of Adobe illustrator and Photoshop

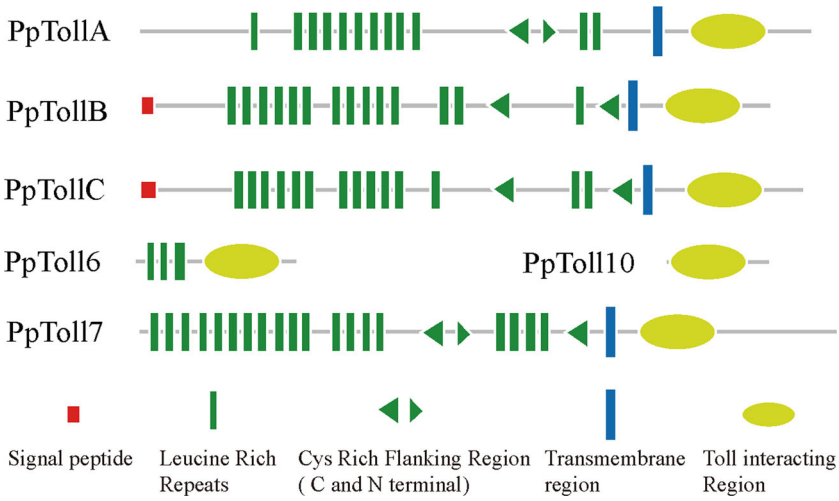

FIGURE 6 Domain organizations of six *Pteromalus puparum* Toll genes

P. puparum and named them according to the sequence similarity with Spätzle genes from other insect species. qPCR analysis showed that none of them were significantly upregulated following an immune challenge (data not shown). However, *P. puparum* Spätzle4 (*PpSpz4*) is specifically expressed in embryos at levels substantially higher compared to other *PpSpz*s (Table S5). We infer that PpSpz4 participates in regulating embryonic development. Our phylogenetic tree divided the PpSpzs into five parts, all well clustered with other insects Spzs, slightly different from the distribution of *N. vitripennis* Spzs (Figure S5).

There are six Tolls in *P. puparum* (Figure 6). Tolls are characterized by several extracellular leucine-rich repeat (LRR) domains and an intracellular Toll/IL-1 receptor (TIR) domain. All *P. puparum* Tolls (PpTolls) contain LRRs, a transmembrane domain and a TIR domain, except PpToll6, which does not possess a transmembrane domain, and PpToll 10, which does not possess an LRR and a transmembrane domain (Figure 6). Phylogenetic analysis shows six PpTolls belong to four of six subgroups (Figure 7). Corresponding to the high *PpSpz4* expression recorded by RNA-seq, we found a similar expression pattern in *PpTollB* (Table S5).

Downstream from Toll, MyD88, Tube, and Pelle are recruited sequentially via the interaction of death domain (Valanne et al., 2011). In the current model (Hoffmann & Reichhart, 2002; Valanne et al., 2011) of Toll actions, Pelle associates with TRAF, to interact with Cactus and release Dorsal/Dif (Figure 5a). Compared with the one Cactus in *B. mori*, *D. melanogaster*, *A. aegypti*, and *T. castaneum*, three Cactus were found in *A. mellifera*, *P. puparum*, and *N. vitripennis*. The three *P. puparum* Cactus genes share similar sequence length and scaffold_27 location with the same orientation and exon-intron structures (Figure 2). We speculate the Cactus gene expansions are the consequence of gene duplication. *P. puparum* and *N. vitripennis* have five Dorsal genes, more than those of *A. mellifera* and *A. gambiae*. Pellino inhibits the activity of Pelle (Yamamoto-Hino & Goto, 2016). Two Pellinos are present in *P. puparum* and *N. vitripennis* (Table S3). Other known insect genomes have only one Pellino gene. Both *P. puparum* Pellinos contained typical pellino domains, confirmed by NCBI blast results. Transcriptomic data showed *P. puparum* Pellino2 gene was specifically expressed in embryo and had very low FPKM values in other development stages (Table S5). Thus, we cloned the overall length of Pellino2 gene by PCR. The sequence of Pellino2 obtained is the same as it predicted in genome annotation. We constructed the phylogenetic tree of Pellinos (Figure S6). *P. puparum* Pellino1 clustered with Pellinos from other insect species. *P. puparum* Pellino2 and orthologous *N. vitripennis* Pellino formed a clade some distance from other Pellinos including those in Hymenoptera insects.

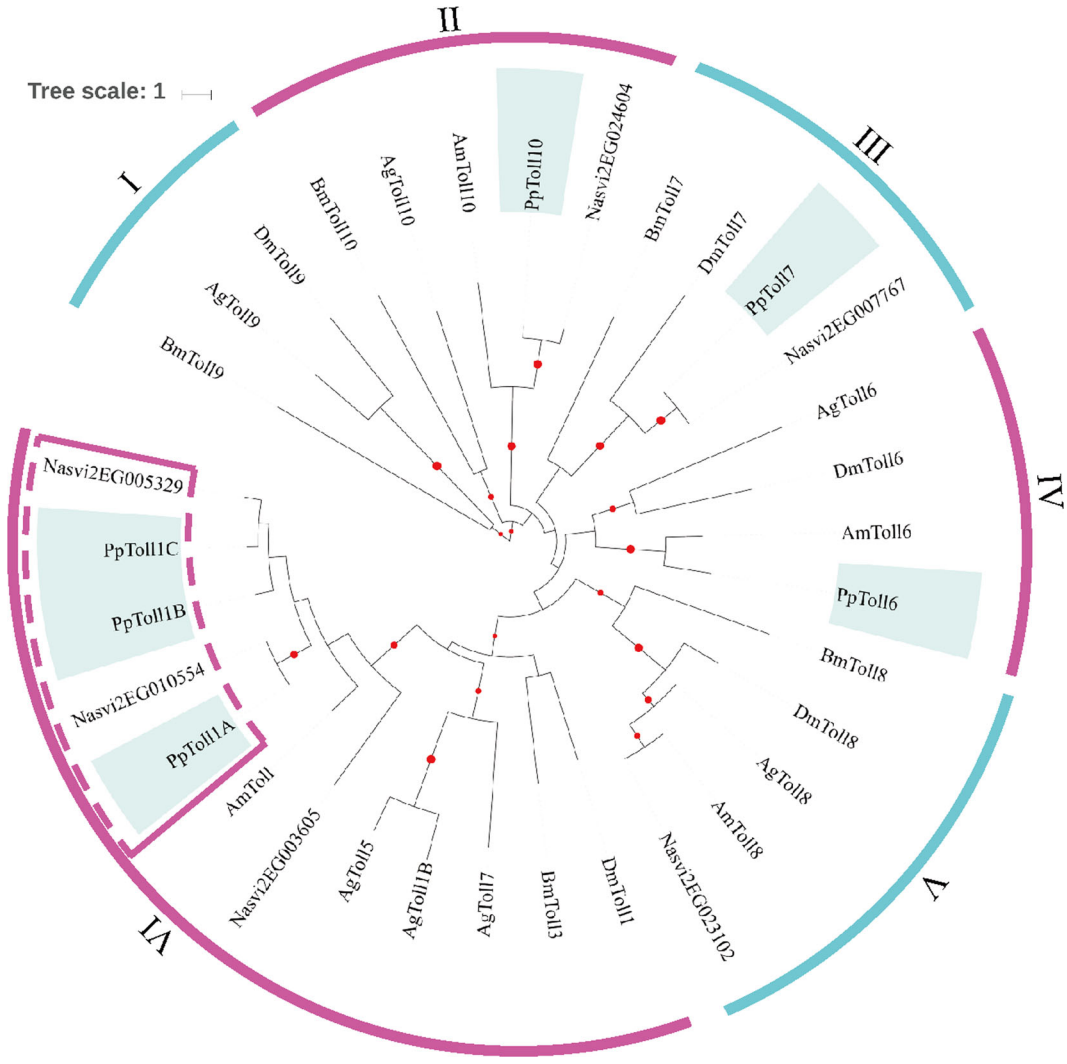

FIGURE 7 Phylogenetic analysis of Toll genes. The Toll domain of amino acid sequences of *Pteromalus puparum* (Pp), *Nasonia vitripennis*, *Apis mellifera* (Am), *Bombyx mori* (Bm), *Anopheles gambiae* (Ag) and *Drosophila melanogaster* (Dm) were aligned. Phylogenetic tree was constructed by Neighbor-joining method, using the program MEGA 5.10. Red spots at the nodes denote bootstrap values greater than 500 from 1000 trials

3.3.2 | IMD and JNK pathway

The IMD pathway is initiated by PGRP-L (Figure 5b). We identified all eight categories of signal transduction genes, each present in 1:1 orthologs to other insects. IMD was activated first, then recruit FAS-associated death domain (FADD), Dredd (caspase 8) complex. Further, an IKK complex activated by upstream TAK1 and Tab2 and Dredd works together to phosphorylate Relish, which enters the nucleus to modulate gene expression (Cao et al., 2015). TAK1 also functions as JNK kinase-activating kinase and activates downstream signal proteins in the JNK pathway following immune stimulation (Kleino & Silverman, 2014; Silverman et al., 2003). The components in JNK pathway include dual specificity mitogen-activated protein kinase kinase (*Hem*), *JNK*, *Kay*, and Jun-related antigen (*Jra*). Comparing to one *Hem* in *D. melanogaster*, *B. mori*, *A. gambiae*, *A. mellifera*, we found two *Hem* genes in *P. puparum* genome. They are adjacently located in scaffold_0 with the same orientation (Figure 2).

3.3.3 | JAK–STAT pathway

The JAK–STAT signal pathway acts in viral infections and tissue damage (Agaisse & Perrimon, 2004; Myllymäki & Rämet, 2014). Hemocytes release cytokines, unpaired-1, -2, and -3, that bind to the cell-surface receptor, domeless to cause receptor dimerization and subsequent activation of Hopscotch (Hop; Figure 5c). Activated Hop recruits STAT as its ligand and finally translocates STAT to the nucleus to activate gene transcription (Baeg, 2005; Xia et al., 2015). We found all of three transduction molecules in *P. puparum*, along with regulators, suppressor of cytokine signaling (*SOCS*), and inhibitor of activated STAT (*D-PIAS*) are also identified.

3.4 | Gene for immune-responsive effectors

Effectors participate in phenoloxidase-dependent melanization, elimination of infectious microorganisms, apoptosis, and other immune-related mechanisms (Landete, 2016). AMPs are short and show lineage specificity and diversity among insect orders. We identified 47 *AMPs* in *P. puparum* according to the sequence repertoires of *N. vitripennis* (Tian et al., 2010). Here, we discuss other effectors, including reactive oxygen species (ROS), PPOs, and thioester-containing proteins (TEPs).

3.4.1 | Antioxidant enzymes

ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radical, is generated in the mitochondria of plants (Apel & Hirt, 2004) and animals (Turrens, 2003) as a product of aerobic metabolism. These species leak from mitochondria and react with proteins, lipids and nucleic acids, creating what is called "oxidative stress." Cells protect themselves from oxidative stress by activating genes encoding enzymes, such as superoxide dismutases (SODs), catalases (CATs), and peroxidases that metabolize the ROS into harmless products (Schieber & Chandel, 2014). There are four *SOD* genes in *P. puparum* (Table S2; Perry, Shin, Getzoff, & Tainer, 2010). Cytoplasmic Cu/Zn-SOD (SOD1) and extracellular Cu/Zn-SOD (SOD3) are cytoplasmic enzymes that require zinc and copper for transport and metabolism (Ni et al., 2007; Parker, Parker, & Keller, 2004). Mn-SOD (SOD2) has four manganese binding sites, a Mn-SOD region, and a mitochondrial targeting sequence (Curtis et al., 2007). PpSOD1a, PpSOD1b, and PpSOD3 feature Cu/Zn SOD signature regions and all the amino acids needed for copper/zinc binding and dismutation (Figure S7).

Peroxidases contain heme-containing peroxidases (*HPXs*) and nonheme peroxidases, and the latter can be assorted into two groups: glutathione peroxidases (*GPXs*) and thioredoxin peroxidases (*TPXs*; Missirlis et al., 2003; J. M. Wang et al., 2019). There are two *GPXs*, five *TPXs*, and nine *HPXs* in *P. puparum*, similar to other insects, such as *A. mellifera*, *D. melanogaster*, and *T. castaneum* (Table S3). The numbers of *CAT* encoding genes differ among insect species. There are two in *D. melanogaster*, four in *T. castaneum*, and seven in *B. mori*, with one *CAT* in *P. puparum*, *N. vitripennis*, *A. mellifera*, and *A. gambiae*.

3.4.2 | Prophenoloxidases

The prophenoloxidases (PPO)-activating system is induced by a cascade of serine proteases to convert the inactive PPO into active PO (Binggeli, Neyen, Poidevin, & Lemaitre, 2014; Lu et al., 2014). PO catalyzes melanin synthesis during nodulation, encapsulation, and wound healing (Lu et al., 2014). We recorded three *PPO* candidate genes. PPOs have a copper ion that interacts with hemolymph oxygen (Cristino et al., 2010). *P. puparum PO1* and *PPO1* show significant differential expression in larval stages and *PO3* is expressed in the pupal stage (Table S5).

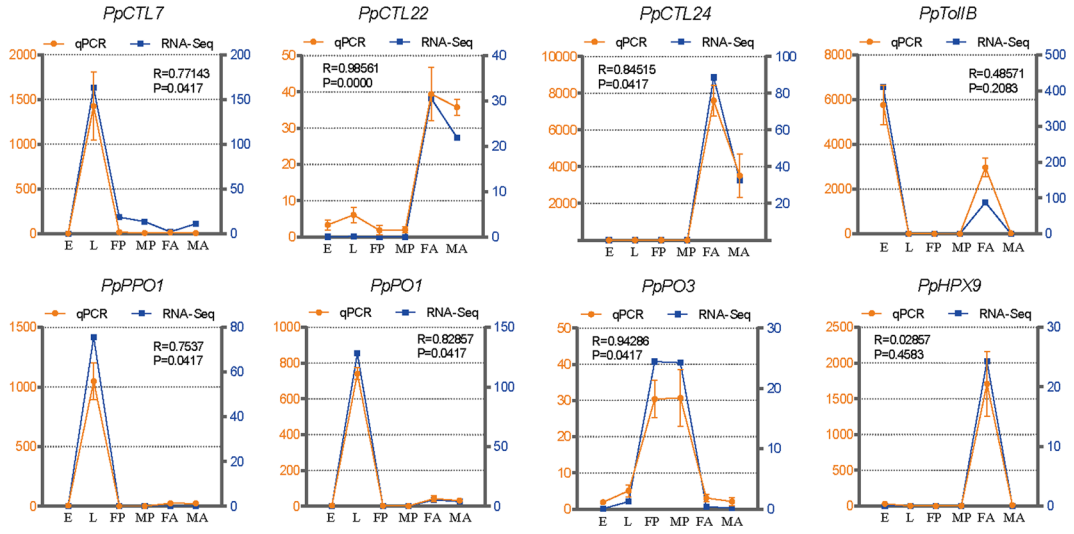

FIGURE 8 Comparison of the relative expression profiles of immune genes by qPCR and RNA-seq. Left vertical axis coordinate is relative expression level of qPCR (orange); right vertical axis coordinate is FPKM of RNA-Seq (blue). R-values are the correlation coefficients between qRT-PCR and RNA-seq. *p*-Values show the significance of the correlation coefficients. qPCR, quantitative polymerase chain reaction

3.4.3 | Thioester-containing proteins

Three *TEPs* have been identified in *P. puparum*. We classified these into one α_2-macroglobulin (*PpA2M*) and two insect TEPs (*PpiTEPs*) based on a phylogenetic tree (Yang, Mei, et al., 2017). A2Ms are universal protease inhibitors, and iTEPs may act in immunity (Wu, Noonin, Jiravanichpaisal, Soderhall, & Soderhall, 2012). The *A. gambiae* TEP1 serves as opsonins to promote phagocytosis of bacteria (Yassine, Kamareddine, & Osta, 2012). It also can act as a recognition molecule that attaches to the surface of parasites and accelerates melanization (Blandin, Marois, & Levashina, 2008). In *D. melanogaster*, *iTEP2*, *iTEP3*, and *iTEP6* were induced following immune challenge. They also remove bacteria and fungi via phagocytosis (Stroschein-Stevenson, Foley, O'Farrell, & Johnson, 2006). We determined expression profiles of *PpiTEPs* after infection. The expression level of *PpiTEP2* was unregulated after bacterial or fungal infection compare to PBS infection (Figure 3). PpiTEP2 contains a common thioester motif (GCGEQ), whereas the corresponding position in the sequences of PpiTEP1 is replaced by DCGEQ (Yang, Mei, et al., 2017). Because thioester motif is an important part of thioester bond, allowing the formation of a covalent bond to bind microbial surfaces, we infer that the PpiTEP2 act in immunity.

We also verified the expression profiles of selected immune-related genes at different developmental stages of *P. puparum* and calculated the correlation coefficients between qPCR and RNA-seq results (Figure 8). The Spearman's correlations of four genes (>0.8) are extremely strong and two (from 0.6 to 0.79) are strong. *p*-Value of the correlation analyses of these genes was significant ($p < .05$). The *R*-value and *p*-value of the remaining two genes is not very good. This may be due to significantly low FPKM values (even were approximately equal to 0) of these genes at their severally developmental stages. The Spearman Rank of qPCR and FPKM values did not match very well. However, the curves of the expression patterns drawn from the RNA-seq and qPCR data showed high fitting degree, via the observation from Figure 8. These results indicated that the RNA-seq data is reliable and supported the parallel between RNA-seq and qPCR data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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