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ORIGINAL ARTICLE

Screening of immune-related genes against bacterial infection in *Ostrinia furnacalis* (Lepidoptera: Crambidae)

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Key words. Transcriptomic sequencing, immunity, differentially expressed genes, insect, agricultural pest

Abstract. *Ostrinia furnacalis* Guenée is an important pest of maize. The current use of microbial agents to control *O. furnacalis* have been relatively successful. However, upon infestation with microorganisms, *O. furnacalis* initiates an innate immune response to defend itself against foreign invaders. Therefore, understanding the immune mechanisms in *O. furnacalis* is important to ensure a more efficient use of microbial agents for the control of this pest. In the present study, a gram-positive bacterium, *Staphylococcus aureus*, and a gram-negative bacterium, *Escherichia coli*, were used to induce an immune response in *O. furnacalis*. Transcriptomic sequencing was then used to screen for genes that were significantly differentially expressed after microbial induction and sequence comparison and phylogenetic analysis to identify immune-related genes the expression of which was significantly induced. The results show a large number of genes were differentially expressed in *O. furnacalis* after microbial induction, the majority of which were immune-related genes. The expression of some immune-related genes such as those encoding antimicrobial peptides, peptidoglycan recognition proteins and serine protease inhibitors were strongly induced. Overall, our study increases the understanding of the mechanism of the innate immune response in *O. furnacalis* and provides a theoretical basis for better biological control using microbial agents.

INTRODUCTION

Ostrinia furnacalis Guenée (Lepidoptera: Crambidae), commonly known as Asian corn borer, is a major pest of maize production in China, leading to 10–30% loss of maize yield and 50% or more when abundant and seriously threatens the quality and yield of maize (Wang et al., 2000). At present, chemical control is the main method for controlling *O. furnacalis*. However, the long-term use of chemical pesticides could result in *O. furnacalis* developing pesticide resistance along with an increase in environmental pollution (Mu & Wang, 1988; Wang & Wang, 2019; Zhi et al., 2021). As an environmentally friendly method, biological control can play a key role in controlling *O. furnacalis* in a sustainable manner (Wu et al., 2018).

Currently, the widely used biocontrol agents for the control of *O. furnacalis* include *Beauveria bassiana* and *Bacillus thuringiensis* (Ma et al., 2008; Feng et al., 2017). In addition to biological agents used for the control of *O. furnacalis*, it is frequently reported infected with pathogenic microorganisms in field surveys (He et al., 2002; Li et al., 2009; Duan et al., 2014). Although these microorganisms can kill Asian corn borer it has an immune response that enable it to resist and kill the invading pathogenic microorganisms. However, the detailed molecular mechanism of the interaction between *O. furnacalis* and pathogenic bacteria has not yet been clarified and elucidating this mechanism can help to better exploit the lethal effect of pathogenic microorganisms by restraining the natural immune response of *O. furnacalis*. Therefore, the screening and identification of immune-related genes are important for understanding the natural immune system in *O. furnacalis* and improving the efficiency of biocontrol techniques.

Insects defend themselves against pathogen infestation by immune responses and the recognition of pathogens is the first step in initiating this response (Kanost et al., 2004; Vogel et al., 2011). Insects recognize pathogens by means of pattern recognition proteins (PRPs) (Hughes, 2012; Wang et al., 2019). The classes of insect PRPs identified to date and the corresponding pathogen pattern molecules include peptidoglycan recognition proteins, β-1,3-glucan recognition protein, C-type lectins, fibrinogen-related proteins, thioester-containing proteins and macrophage scav-

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enger receptors (Yu et al., 2002; Lemaitre & Hoffmann, 2007; Ragan et al., 2009; Sumathipala & Jiang, 2010; Bang et al., 2013, 2022). As a member of the PRP family, PGRP only recognizes molecules on the surface of pathogenic organisms and then selectively activates pathways such as the Toll pathway, immune deficiency (IMD) pathway, JAK-STAT pathway and reactive oxygen species metabolism or blackening reaction to kill the pathogens (Jiang et al., 2010; Huang et al., 2015; Wang et al., 2016; Lu et al., 2019; Hou et al., 2020; Wei et al., 2020).

The insect humoral immune response consists mainly of two signalling pathways, Toll and IMD, which induce antimicrobial peptide (AMP) production, and coagulation and melanisation reactions involving a series of enzymes such as polyphenol oxidase. AMPs are an important component of the natural immune system of insects, which are effective against both gram-positive and gram-negative bacteria (Chu et al., 2013). Based on their amino acid composition and antimicrobial activity AMPs can be broadly classified into five major groups: defensins, cecropins, lysozymes, proline-rich proteins and glycine-rich proteins (Brogden, 2005). To date studies on AMPs and their immune signalling pathways have mainly focused on *Drosophila melanogaster*, *Bombyx mori* and *Manduca sexta* (Yu et al., 2002; Gobert et al., 2003; Kaneko et al., 2006; Lemaitre & Hoffmann, 2007).

Recent studies on the innate immune mechanism in *O. furnacalis* induced by microorganisms have focused on lysozymes and attacins. Wang et al. (2009) cloned and expressed a C-type lysozyme from *O. furnacalis*, which exhibits inductive activity against both gram-positive and gram-negative bacteria. Zhang et al. (2013) screened and identified lysozymes and attacins from the transcriptome of *O. furnacalis* and analysed their expression profiles after microbial induction. However, there are few studies on other immune-related genes in *O. furnacalis* induced by microorganisms.

In the present study, we selected the gram-positive bacterium, *Staphylococcus aureus* and gram-negative bacterium, *Escherichia coli,* to infect *O. furnacalis*. The transcriptomes of *O. furnacalis* larvae induced by different microorganisms were then sequenced and the data subjected to a differential expression analysis. The immune-related genes that were significantly expressed were screened out and those known to play a highly specific role in the immune defence were identified. Then a phylogenetic analysis of two such genes was carried out. This study provides a basis for further analysis of immune genes and immune responses related to the interaction between immune effectors and pathogens, and provides basic information for the development of microbial formulations for the biological control of *O. furnacalis*.

METHODS AND MATERIALS

Experimental insect

O. furnacalis used in this study was reared on artificial food in a manually-controlled climate chamber at 26 ± 1°C, a relative humidity of 80 ± 5% and a photoperiod of 16L : 8D, as described by Song et al. (1999).

A gram-positive bacterium, *S. aureus* and gram-negative bacterium, *E. coli*, were used in the immune challenge experiment. Both bacteria were obtained from the General Microbiological Culture Collection Centre in China. *S. aureus* and *E. coli* were cultured overnight in liquid medium at 37°C under shaking culture conditions of 200 rpm. Subsequently, both strains were collected by centrifugation at 2500 × *g* for 5 min at 4°C and washed with sterile 1 × phosphate-buffered saline (PBS) solution. Finally, both strains were sterilized at 121°C for 20 min.

Sample collection and treatment

Fourth-instar larvae of *O. furnacalis* of comparable size were selected based on their head widths. Four treatment groups were established: larvae injected with *S. aureus* (SA group), *E. coli* (EC group), PBS solution (PBS group) and not treated (CK group). Five microliters of the bacterial solution (3×10^6 cells/mL) and PBS solution were injected into each larva. The injected larvae were disinfected with 70% alcohol and collected after 24 h of rearing in the climate chamber. The bacterial solution was injected into the abdomen of *O. furnacalis*, with batches of 15 larvae injected in each treatment and replicated three times.

RNA extraction and transcriptomic sequencing

The total RNA in *O. furnacalis* in each treatment was extracted using the Trizol method, according to the manufacturer's instructions (Life Technologies). RNA quality was determined using 1% agarose gel electrophoresis and the electrophoretic bands observed using a gel imager. The concentration and purity of the extracted RNA were determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was verified using an Agilent 2100 system. Finally, the qualified RNAs were sequenced using the PacBio SequeII sequencing platform at Genepioneer Biotechnologies.

Data processing and gene identification

Raw data were processed using SMRTlink software to obtain clean data, which were then assembled using Trinity software to obtain unigenes (Grabherr et al., 2011). Unigenes were identified using several databases, including Non-redundancy (NR) (Deng et al., 2006), Swiss-Prot (Apweiler et al., 2004), Clusters of Orthologous Groups (COG) (Tatusov et al., 2000), euKaryotic Orthologous Groups (KOG) (Koonin et al., 2004), Gene Ontology (GO) (Ashburner et al., 2000), Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa et al., 2004) and Protein family (Pfam) (Finn et al., 2014).

Analysis of differentially expressed genes (DEGs)

The fragments per kilobase of transcript per million mapped reads (FPKM) method was used to calculate the level of expression of the genes (Audic & Claverie, 1997). This method eliminates the influence of gene length and sequencing differences when calculating the effects of gene expression and is commonly used for estimating the level of gene expression in transcriptomic data analysis. The DEGs were obtained using DESeq2 software with a threshold of a fold change > 2 and false discovery rate < 0.05 (Anders & Huber, 2010).

Phylogenetic analysis

The phylogenetic tree was constructed based on the reported amino acid sequences of Lepidoptera. The sequences were aligned using MAFFT (https://www.ebi.AC.UK/Tools/MSA/MAF/) and then imported into MEGA5.0 software. The phylogenetic tree was constructed using the neighbour-joining algorithm,

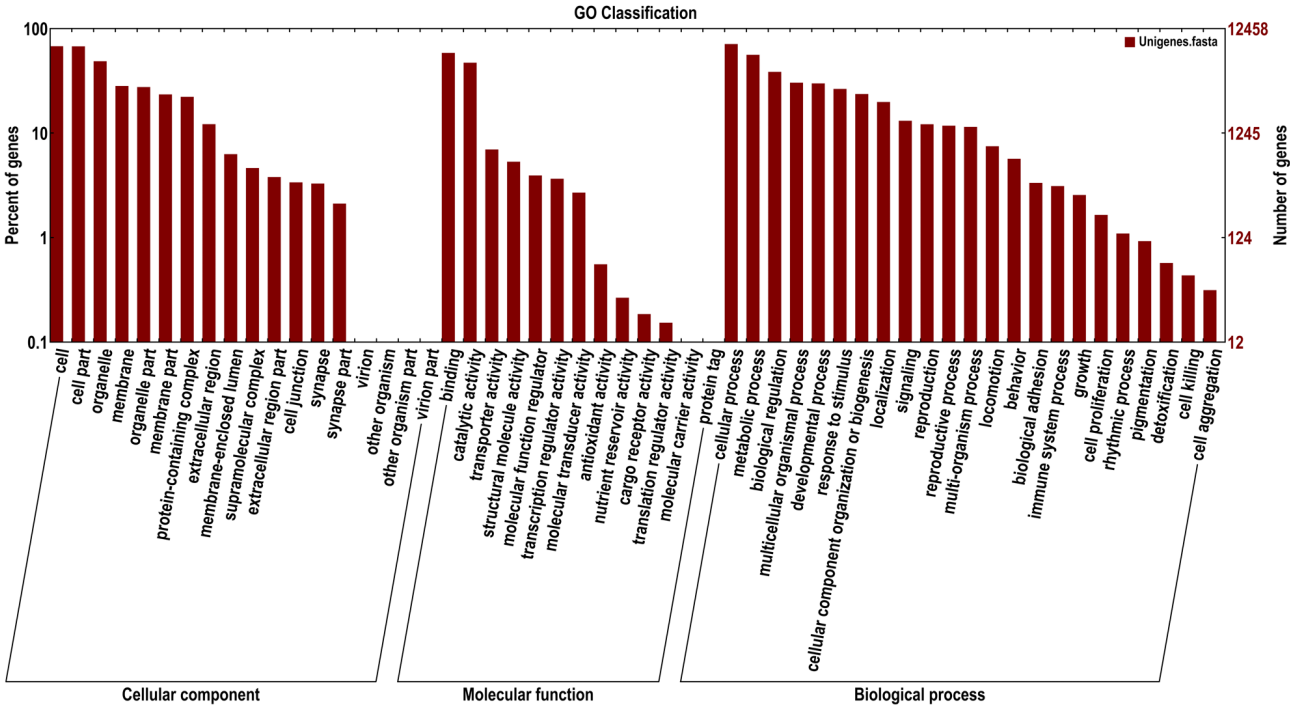

Fig. 1. Gene ontology (GO) of *Ostrinia furnacalis*. Categories of cellular components, molecular function and biological processes were analysed. The X-axis represents different functional categories and the Y-axis the numbers and percentage of genes.

and the confidence of each branch was tested using the bootstrap method for 1000 trials.

RESULTS

Transcriptomic data analysis

An average of 23,265,313 reads with a GC content of 49.5% were obtained for the CK group (no treatment), 24,571,445 reads with a 49% GC content for the EC group, 25,881,618 reads with a 47.9% GC content for the PBS and 26,199,582 reads with a 46.1% GC content for the SA group. In total, 166,714 transcripts and 56,551 unigenes were assembled. The N50 of transcripts and unigenes were 2,189 and 1,893, respectively, and the integrity of their assembly was high. The highest percentage of transcripts was in the 500–1000 bp (28.59%) range and that of unigenes in the 300–500 bp (40.42%) range (Table 1).

Table 1. Summary of the quality of the sequencing data for samples of *Ostrinia furnacalis*.

Sample	Read Sum	GC (%)	Q20%	Q30%
CK1	20630594	49.87	97.41	92.86
CK2	28463757	50.62	97.59	93.18
CK3	20701587	48.09	97.66	93.41
Ec1	27679461	48.69	97.67	93.39
Ec2	25859685	49.1	97.48	93.04
Ec3	20175190	45.99	97.41	92.89
PBS1	24961063	47.46	97.54	93.07
PBS2	27399683	48.75	97.7	93.45
PBS3	25284108	47.44	97.55	93.12
Sa1	27700232	46.21	97.66	93.34
Sa2	26348489	45.42	97.6	93.24
Sa3	24550026	46.56	97.41	92.86

Gene identification

The genetic sequences were compared using Blast software. In total, 23,607, 13,433, 5,946, 12,983, 12,458, 22,733 and 13,275 genes were identified using the Nr, Swiss-prot, COG, KOG, GO, KEGG and Pfam databases, respectively (Table 2).

GO identification includes three important functional categories: cellular components, molecular functions and biological processes. In the present study, the unigenes were mainly in cells in the cellular component (8,435 genes, 21.09%), molecular function (7,284 genes, 45.16%) and biological process categories (8,851 genes, 19.14%) (Fig. 1).

The COG database is used for the orthologous classification of unigenes. This classification revealed that among the 25 groups, 1,752 genes were classified as only having a "general function prediction", with the majority of the remaining genes classified as "replication, recombination and repair" (948 genes). "amino acid transport and metabolism" (613 genes) and "carbohydrate transport and metabolism" (536 genes) (Fig. 2).

The KEGG pathway revealed that the majority of the unigenes were associated with carbohydrate metabolism

Table 2. Unigenes identified in *Ostrinia furnacalis*.

Values	Number of unigenes	Percentage (%)
Nr	23607	98.29
Swiss-Prot	13433	55.93
PFAM	13275	55.27
KOG	12983	54.05
GO	12568	51.87
COG	5946	24.76
Total	24017	100

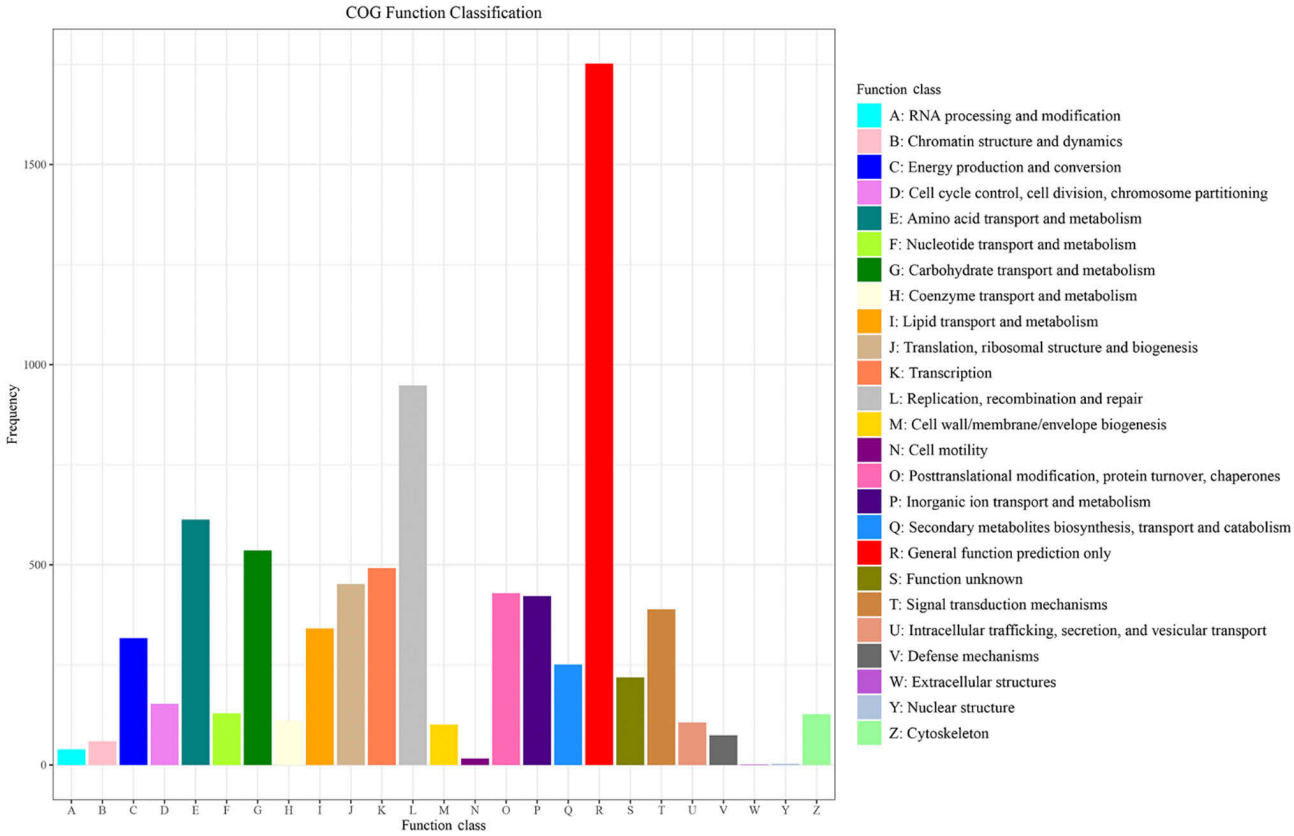

Fig. 2. Clusters of orthologous groups (COG) in *Ostrinia furnacalis*. The genes were divided into 25 functional groups. The X-axis represents the different functional categories and Y-axis the frequency of genes.

Table 3. The immune-related genes in *Ostrinia furnacalis* that differed in expression after infection with *Escherichia coli*.

Gene ID	Identification	Regulation
TRINITY_DN791	attacin [*Ostrinia furnacalis*]	up
TRINITY_DN3414	proline-rich protein [*Galleria mellonella*]	up
TRINITY_DN3414	proline-rich protein [*Galleria mellonella*]	up
TRINITY_DN11788	cecropin A [*Hyphantria cunea*]	up
TRINITY_DN459	hemolin [*Ostrinia furnacalis*]	up
TRINITY_DN508	gloverin-like [*Pieris rapae*]	up
TRINITY_DN2950	antimicrobial peptide cecropin 6 [*Manduca sexta*]	up
TRINITY_DN6436	peptidoglycan-recognition protein LB-like [*Bicyclus anynana*]	up
TRINITY_DN4799	antimicrobial peptide cecropin 6 [*Manduca sexta*]	up
TRINITY_DN410	gloverin-like [*Pieris rapae*]	up
TRINITY_DN6827	attacin [*Ostrinia furnacalis*]	up
TRINITY_DN2313	cecropin-like [*Pieris rapae*]	up
TRINITY_DN10601	peptidoglycan recognition protein [*Ostrinia furnacalis*]	up
TRINITY_DN4279	PREDICTED: beta-1,3-glucan-binding protein [*Amyelois transitella*]	up
TRINITY_DN15537	PREDICTED: gloverin-like [*Amyelois transitella*]	up
TRINITY_DN2877	beta-1,3-glucan-binding protein-like [*Spodoptera litura*]	up
TRINITY_DN4458	beta1-3 glucan recognition protein [*Ostrinia furnacalis*]	up
TRINITY_DN791	attacin [*Ostrinia furnacalis*]	up
TRINITY_DN6405	MKRN2 opposite strand protein [*Spodoptera litura*]	up
TRINITY_DN11125	modular serine protease-like [*Spodoptera litura*]	up
TRINITY_DN22691	attacin [*Ostrinia furnacalis*]	up
TRINITY_DN30726	unknown secreted protein [*Papilio xuthus*]	up
TRINITY_DN8	beta-1,3-glucan-binding protein-like [*Spodoptera litura*]	up
TRINITY_DN188	PREDICTED: serine protease snake-like [*Amyelois transitella*]	up
TRINITY_DN1984	laminin subunit beta-1 [*Spodoptera litura*]	up
TRINITY_DN459	hemolin [*Ostrinia furnacalis*]	up
TRINITY_DN8177	hypothetical protein B5V51_3495 [*Heliothis virescens*]	up
TRINITY_DN8402	peptidoglycan recognition protein A [*Ostrinia nubilalis*]	up
TRINITY_DN18334	PREDICTED: serine-protein kinase ATM isoform X1 [*Amyelois transitella*]	up
TRINITY_DN14366	PREDICTED: inhibitor of nuclear factor kappa-B kinase subunit alpha isoform X1 [*Amyelois transitella*]	up
TRINITY_DN53	dVC-AP3-2, partial [*Manduca sexta*]	up
TRINITY_DN3412	hemolin [*Ostrinia furnacalis*]	up
TRINITY_DN1425	Cecropin OS = *Bombyx mori* OX = 7091 PE = 1 SV = 1	up
TRINITY_DN10069	attacin [*Ostrinia furnacalis*]	up
TRINITY_DN27900	matrix metalloproteinase-14-like [*Helicoverpa armigera*]	up
TRINITY_DN1542	glycogen phosphorylase [*Ostrinia furnacalis*]	down

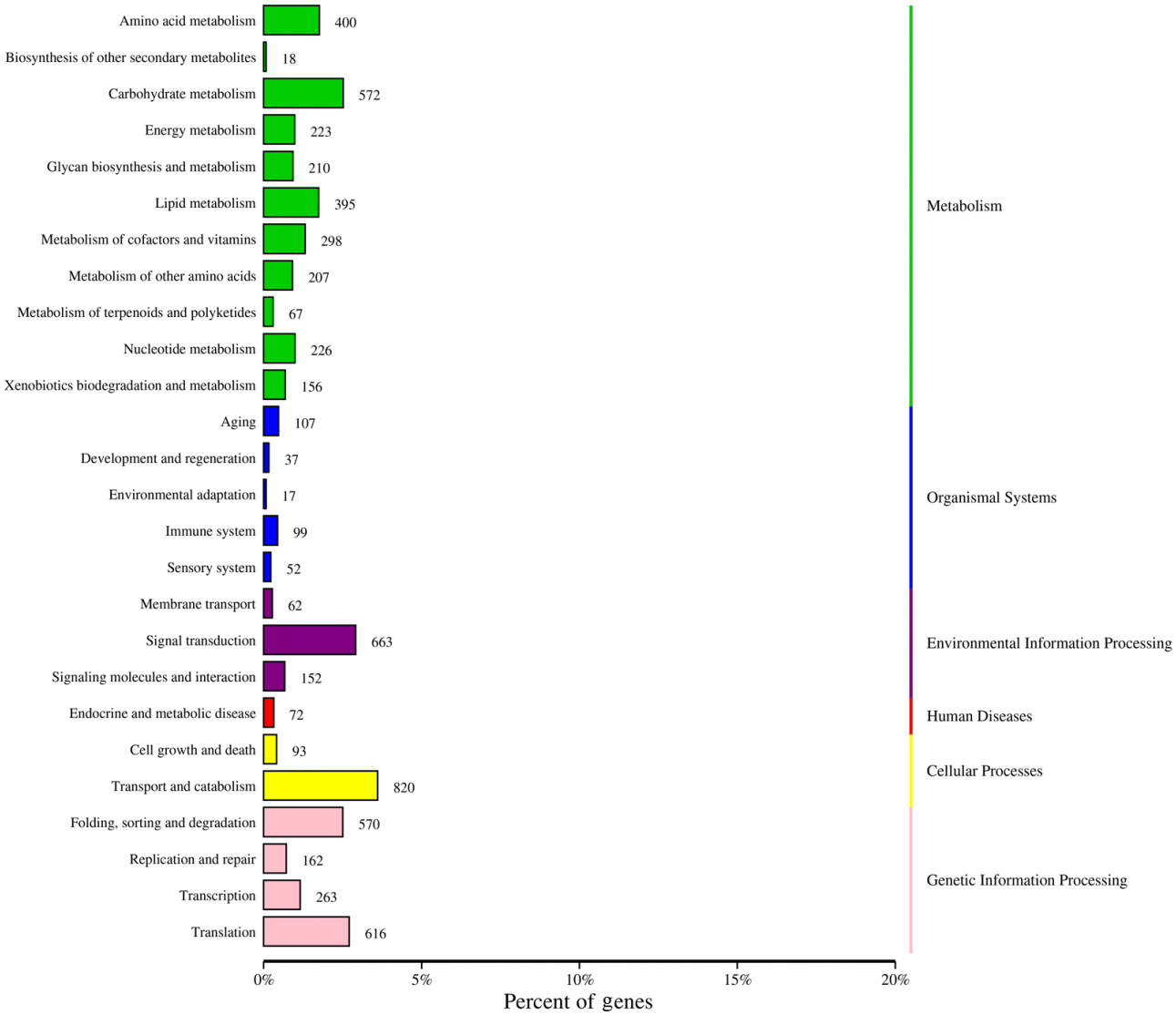

Fig. 3. Kyoto encyclopaedia of genes and genomes (KEGG) in *Ostrinia furnacalis*. Categories of metabolism, organismal systems, environmental information processing, human diseases, cellular processes and genetic information processing were analysed. The X-axis represents the percentage of genes and Y-axis the different functional categories.

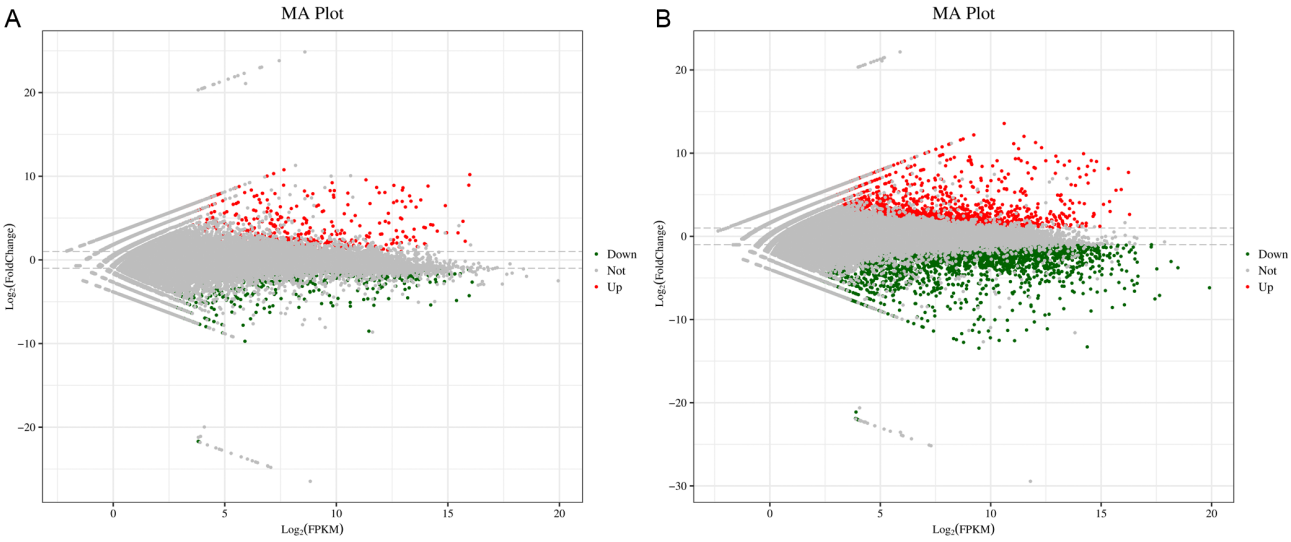

Fig. 4. The genes (DEGs) in *Ostrinia furnacalis* the expression of which differed after the injection with bacteria. A – Infection with *Escherichia coli*. B – Infection with *Staphylococcus aureus*. The X-axis represents the FPKM values of DEGs and Y-axis the fold change of DEGs. Red and green, respectively, represent upregulated and downregulated DEGs.

Table 4. The immune-related genes in *Ostrinia furnacalis* that differed in expression after infection with *Staphylococcus aureus*.

Gene ID	Identification	Regulation
TRINITY_DN3414	proline-rich protein [*Galleria mellonella*]	up
TRINITY_DN459	hemolin [*Ostrinia furnacalis*]	up
TRINITY_DN791	attacin [*Ostrinia furnacalis*]	up
TRINITY_DN11788	cecropin A [*Hyphantria cunea*]	up
TRINITY_DN3414	proline-rich protein [*Galleria mellonella*]	up
TRINITY_DN6436	peptidoglycan-recognition protein LB-like [*Bicyclus anynana*]	up
TRINITY_DN2950	antimicrobial peptide cecropin 6 [*Manduca sexta*]	up
TRINITY_DN508	gloverin-like [*Pieris rapae*]	up
TRINITY_DN2313	cecropin-like [*Pieris rapae*]	up
TRINITY_DN4799	antimicrobial peptide cecropin 6 [*Manduca sexta*]	up
TRINITY_DN410	gloverin-like [*Pieris rapae*]	up
TRINITY_DN10601	peptidoglycan recognition protein [*Ostrinia furnacalis*]	up
TRINITY_DN6827	attacin [*Ostrinia furnacalis*]	up
TRINITY_DN2877	beta-1,3-glucan-binding protein-like [*Spodoptera litura*]	up
TRINITY_DN4279	PREDICTED: beta-1,3-glucan-binding protein [*Amyelois transitella*]	up
TRINITY_DN868	peptidoglycan recognition protein B [*Ostrinia nubilalis*]	up
TRINITY_DN4458	beta1-3 glucan recognition protein [*Ostrinia furnacalis*]	up
TRINITY_DN30726	unknown secreted protein [*Papilio xuthus*]	up
TRINITY_DN15537	PREDICTED: gloverin-like [*Amyelois transitella*]	up
TRINITY_DN2	PREDICTED: uncharacterized protein LOC106720577 [*Papilio machaon*]	up
TRINITY_DN6405	MKRN2 opposite strand protein [*Spodoptera litura*]	up
TRINITY_DN4872	peptidoglycan recognition protein D [*Ostrinia nubilalis*]	up
TRINITY_DN8402	peptidoglycan recognition protein A [*Ostrinia nubilalis*]	up
TRINITY_DN1249	beta-1,3-glucan-binding protein-like [*Helicoverpa armigera*]	up
TRINITY_DN1762	serine protease inhibitor 010 [*Chilo suppressalis*]	up
TRINITY_DN247	peptidoglycan recognition protein A [*Ostrinia nubilalis*]	up
TRINITY_DN1531	PREDICTED: NF-kappa-B inhibitor cactus [*Amyelois transitella*]	up
TRINITY_DN8	beta-1,3-glucan-binding protein-like [*Spodoptera litura*]	up
TRINITY_DN1593	modular serine protease-like [*Spodoptera litura*]	up
TRINITY_DN11125	modular serine protease-like [*Spodoptera litura*]	up
TRINITY_DN102	PREDICTED: suppressor of tumorigenicity 14 protein homolog [*Papilio xuthus*]	up
TRINITY_DN459	hemolin [*Ostrinia furnacalis*]	up
TRINITY_DN1741	serine protease snake-like [*Pieris rapae*]	up
TRINITY_DN3592	serine protease inhibitor 003 [*Chilo suppressalis*]	up
TRINITY_DN1417	putative phosphatidate phosphatase isoform X2 [*Bicyclus anynana*]	up
TRINITY_DN1020	leucine-rich repeat-containing protein 15-like [*Helicoverpa armigera*]	up
TRINITY_DN22691	attacin [*Ostrinia furnacalis*]	up
TRINITY_DN2198	cecropin 2 [*Helicoverpa armigera*]	up
TRINITY_DN188	PREDICTED: serine protease snake-like [*Amyelois transitella*]	up
TRINITY_DN8177	hypothetical protein B5V51_3495 [*Heliothis virescens*]	up
TRINITY_DN1564	PREDICTED: serine/threonine-protein kinase pelle [*Amyelois transitella*]	up
TRINITY_DN3412	hemolin [*Ostrinia furnacalis*]	up
TRINITY_DN311	PREDICTED: lysosome membrane protein 2 [*Papilio polytes*]	up
TRINITY_DN1984	laminin subunit beta-1 [*Spodoptera litura*]	up
TRINITY_DN7381	PREDICTED: insulin receptor substrate 1 [*Papilio xuthus*]	up
TRINITY_DN53	dVC-AP3-2, partial [*Manduca sexta*]	up
TRINITY_DN8594	death-associated inhibitor of apoptosis 2-like [*Bicyclus anynana*]	up
TRINITY_DN26	phospholipid phosphatase 2-like isoform X1 [*Helicoverpa armigera*]	up
TRINITY_DN17704	beta1-3 glucan recognition protein [*Ostrinia furnacalis*]	up
TRINITY_DN16257	beta-1,3-glucan-binding protein-like [*Spodoptera litura*]	up
TRINITY_DN8365	PREDICTED: slit homolog 3 protein-like [*Amyelois transitella*]	up
TRINITY_DN1425	Cecropin OS = *Bombyx mori* OX = 7091 PE = 1 SV = 1	up
TRINITY_DN1340	peptidoglycan recognition protein B [*Ostrinia nubilalis*]	up
TRINITY_DN5749	peptidoglycan-recognition protein LC-like isoform X1 [*Spodoptera litura*]	up
TRINITY_DN6380	tyrosine-protein kinase PR2 [*Bombyx mori*]	up
TRINITY_DN8594	PREDICTED: baculoviral IAP repeat-containing protein 7-like [*Papilio machaon*]	up
TRINITY_DN14366	PREDICTED: inhibitor of nuclear factor kappa-B kinase subunit alpha isoform X1 [*Amyelois transitella*]	up
TRINITY_DN27900	matrix metalloproteinase-14-like [*Helicoverpa armigera*]	up
TRINITY_DN9248	PREDICTED: suppressor of tumorigenicity 14 protein homolog [*Papilio xuthus*]	up
TRINITY_DN10069	attacin [*Ostrinia furnacalis*]	up
TRINITY_DN1149	peptidoglycan recognition protein [*Ostrinia furnacalis*]	up
TRINITY_DN20937	PREDICTED: catalase-like [*Papilio machaon*]	up
TRINITY_DN43990	antimicrobial peptide cecropin 6 [*Manduca sexta*]	up
TRINITY_DN16172	cadherin-99C isoform X4 [*Spodoptera litura*]	down
TRINITY_DN12685	Coatomer subunit beta [*Papilio machaon*]	down
TRINITY_DN25280	putative serine protease K12H4.7 [*Helicoverpa armigera*]	down
TRINITY_DN14931	hypothetical protein B7P43_G02381 [*Cryptotermes secundus*]	down
TRINITY_DN19880	coatomer subunit beta [*Helicoverpa armigera*]	down
TRINITY_DN48250	PREDICTED: gamma-interferon-inducible lysosomal thiol reductase-like [*Papilio xuthus*]	down
TRINITY_DN18576	Proline-rich antimicrobial peptide 1 OS = *Galleria mellonella* OX = 7137 PE = 1 SV = 1	down
TRINITY_DN9033	PREDICTED: exocyst complex component 2 [*Papilio xuthus*]	down
TRINITY_DN27809	proline-rich protein [*Galleria mellonella*]	down
TRINITY_DN2665	leucine-rich repeat-containing G-protein coupled receptor 5-like [*Helicoverpa armigera*]	down
TRINITY_DN3831	hypothetical protein B5V51_11622 [*Heliothis virescens*]	down
TRINITY_DN1604	Mitochondrial import inner membrane translocase subunit Tim10 [*Papilio xuthus*]	down
TRINITY_DN3079	PREDICTED: sulfotransferase family cytosolic 1B member 1 [*Amyelois transitella*]	down
TRINITY_DN6923	PREDICTED: protein croquemort-like isoform X1 [*Amyelois transitella*]	down
TRINITY_DN4557	peptidoglycan recognition protein A [*Ostrinia nubilalis*]	down
TRINITY_DN148	hypothetical protein B5V51_13101 [*Heliothis virescens*]	down
TRINITY_DN6228	serine proteinase diverged [*Ostrinia nubilalis*]	down
TRINITY_DN2356	putative serine protease K12H4.7 [*Pieris rapae*]	down
TRINITY_DN1542	glycogen phosphorylase [*Ostrinia furnacalis*]	down
TRINITY_DN3467	beta-1,3-glucanase [*Ostrinia nubilalis*]	down
TRINITY_DN2824	catalase [*Chilo suppressalis*]	down

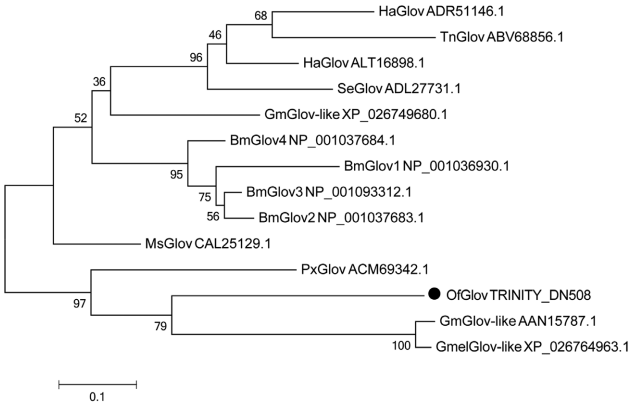

Fig. 5. Phylogenetic analysis of gloverin amino acid sequences. The amino acid sequences from *Bombyx mori* (Bm), *Helicoverpa armigera* (Ha), *Spodoptera exigua* (Se), *Trichoplusia ni* (Tn), *Manduca sexta* (Ms), *Plutella xylostellaand* (Px), *Galleria mellonella* (Gm) and *Ostrinia furnacalis* (Of) gloverin were used to build this tree. The value of each branch was tested using 1000 bootstrap trials.

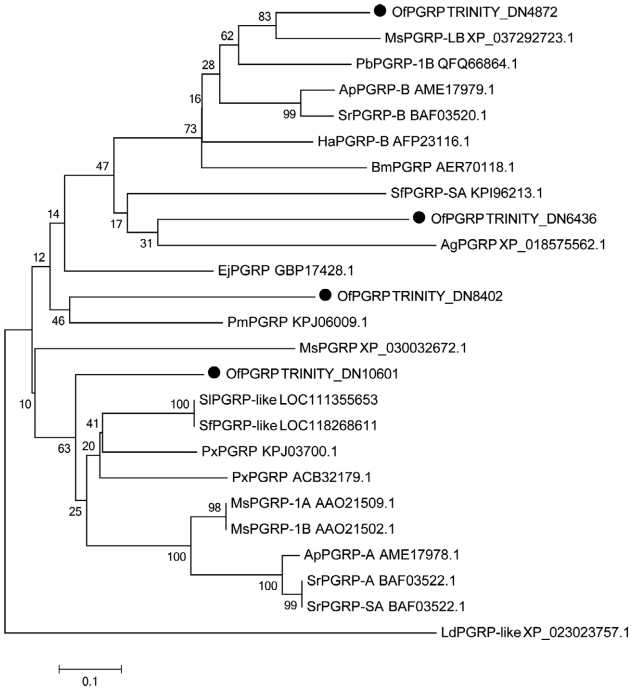

Fig. 6. Phylogenetic analysis of peptidoglycan recognition protein (PGRP). The amino acid sequences from *Leptinotarsa decemlineata* (Ld), *Bombyx mori* (Bm), *Plutella xylostellaand* (Px), *Helicoverpa armigera* (Ha), *Eumeta japonica* (Ej), *Manduca sexta* (Ms), *Papilio machaon* (Pm), *Samia ricini* (Sr), *Antheraea pernyi* (Ap), *Protaetia brevitarsis* (Pb), *Anoplophora glabripennis* (Ag), *Spodoptera litura* (Sl), *Spodoptera frugiperda* (Sf) and *Ostrinia furnacalis* (Of) PGRP were used to build this tree. The values of each branch were tested using 1000 bootstrap trials.

(572 genes), ageing (107 genes), signal transduction of environmental information (633 genes), transport and catabolism (820 genes) and translation of genetic information (616 genes). In addition, 99 genes were associated with immune system-related pathways that play an important role in initiating the immune response to a bacterial invasion (Fig. 3).

DEG analysis

The DESeq2 analysis showed that the expression of 748 genes differed after PBS injection compared with those in the CK group, including 304 up-regulated genes and 442 down-regulated genes.

E. coli injection led to a different expression of 1084 genes, 610 of which showed up-regulated expression (Fig. 4A). Of the up-regulated DEGs a total of 36 immune-related genes were screened. Among them, PRPs (8 genes), cecropins (5 genes), attacins/glycine-rich proteins (5 genes), gloverins (3 genes), haemolins (3 genes), serine proteases (3 genes) and proline-rich proteins (2 genes) were significantly up-regulated, and only glycogen phosphorylase was significantly down-regulated (Table 3).

S. aureus injection resulted in 4,835 DEGs, which is greater than the number resulting from *E. coli* injection. Among these DEGs, 2,132 genes were up-regulated and 2,703 were down-regulated (Fig. 4B). Of the DEGs in the SA group a total of 84 immune-related genes were screened, including PRPs (16 up-regulated, 2 down-regulated), cecropins (7 up-regulated), attachin /glycine-rich proteins (3 up-regulated), gloverins (3 up-regulated), haemolins (3 genes), serine proteases (7 up-regulated and 3 down-regulated) and proline-rich proteins (2 up-regulated and 2 down-regulated) (Table 4).

Phylogenetic analysis

According to the DEG analysis, the PGRP and gloverin genes were significantly the most active after gram-positive and gram-negative bacteria induction and were therefore selected for phylogenetic analysis. The phylogenetic tree was constructed by comparing the putative *O. furnacalis* gloverin amino acid sequence obtained in this study with the reported sequences of gloverin in Lepidoptera, which are in the same cluster. The obtained sequence was confirmed to belong to the gloverin gene family of *O. furnacalis* and was most closely related to that of *Galleria mellonella* (Fig. 5). Similarly, the PGRP phylogenetic tree was constructed based on the four putative *O. furnacalis* PGRP sequences screened and the reported amino acid sequences of Lepidoptera. The four genes clustered various species of Lepidoptera, showing a high degree of diversity, which indicates that these four genes may have different biological functions (Fig. 6).

DISCUSSION

Understanding the immune mechanism of *O. furnacalis* could enhance the efficacy of microbial agents used in its biological control. In the present study, gram-positive and gram-negative bacteria were selected as representative invasive pathogens and used to induce an immune response in *O. furnacalis* larvae followed by transcriptome sequencing. DEG analysis showed that 1,084 genes were differentially expressed following *E.coli* induction and 4,835 genes following *S. aureus* induction; 36 and 84 of the DEGs were related to immunity, respectively. After the functional classification, the putative PGRP and gloverin genes, which were significantly up-regulated after induc-

tion by both gram-positive and gram-negative bacteria, were selected for phylogenetic analysis. DEGs at different infection stages were screened and functionally analysed. These results provide a basis for further study on the molecular mechanism underlying the immune response of *O. furnacalis*.

Wang et al. (2009) report lysozyme antimicrobial activity against both gram-positive and gram-negative bacteria in *O. furnacalis* and Zhang et al. (2013) that the expression levels of lysozyme and attacin significantly increase after microbial induction in *O. furnacalis*, which indicate their important roles in the antimicrobial activity of insects. In this study, the expression levels of lysozyme and attacin-encoding genes were also significantly up-regulated after larvae were injected with bacteria. However, we also found induced expression of a large number of AMP-encoding genes and PRPs, including PGRP, proline-rich protein, beta-1,3-glucan-binding protein, cecropin, gloverin and serine protease inhibitor. After injection with *E. coli* and *S. aureus*, the expression of 36 and 84 immune-related genes differed and were, respectively, screened out. Therefore, injection of gram-positive bacteria induced an increase in the expression of a greater number of immune response-related genes than the injection of gram-negative bacteria.

AMP production is regulated at the transcriptional level by two classical pathways, the Toll and IMD pathways, which are associated with NF-κB signalling and are activated by different classes of microorganisms. The Toll pathway responds primarily to gram-positive bacteria and fungi and thus plays a major role in the defence against these organisms. By contrast, the IMD pathway is activated by both gram-negative and gram-positive bacteria with diaminopimelate (DAP) type peptidoglycan and mutations affecting this pathway result in high susceptibility to gram-negative bacteria (Georgel et al., 2001; Lemaitre et al., 1996).

Gloverin is an inducible antibacterial insect protein that was first isolated from the pupae of the giant silk moth *Hyalophora gloveri* (Axén et al., 1997). Previous studies have shown that the antimicrobial activity of gloveins against gram-negative bacteria involves an interaction with lipopolysaccharide in the bacterial envelope (Axén et al., 1997). The gloverins in *B. mori* against *E. coli* are mediated by NF-κB- immune responses (Kawaoka et al., 2008). In addition, gloverins are transferred from the cytoplasm by the downstream signalling of Toll receptors in response to gram-positive bacteria. For example, the expression of gloverin in *Spodoptera exigua* is reported to be significantly up-regulated following induction by a gram-positive bacterium (Hwang & Kim, 2011).

PGRP is a highly conserved protein that plays a key role in recognizing invading pathogens, activating the innate immune response and killing invading pathogens (Xiong et al., 2015). For example, PGRP-LB, -SC1a, -SC1b and SC2 in *D. melanogaster* cleave a meso-DAP-type peptidoglycan from gram-negative bacteria and negatively regulate the IMD pathway (Bischoff et al., 2006; Zaidman-Rémy et al., 2006; Royet et al., 2011). In addition, recognition of peptidoglycan by some PGRPs can activate the phenol oxidase pathway, promote wound healing, elicit a melanin response, and promote antimicrobial activity (Yoshida et al., 1996; Mellroth & Steiner, 2006; Chen et al., 2016). For example, PGRP-SA and PGRP-SD in *D. melanogaster* bind to Lys-type peptidoglycan of gram-positive bacteria and further trigger the Toll pathway activating the prophenoloxidase cascade (Bischoff et al., 2004). Several studies have shown that PGRP can also activate the transcription and synthesis of AMPs. Shen et al. (2021) found that short-type PGRP1 is involved in the immune response of *O. furnacalis* induced by both bacteria and fungi. In addition, the expression levels of AMP-encoding genes in *O. furnacalis* larvae were significantly increased by the injection of recombinant PGRP1.

To better understand the functions of immune-related genes in *O. furnacalis* after microbial induction, future research needs to focus on the function of the identified immune genes using gene cloning and RNA interference technology, which will clarify the specific roles of these genes in innate immunity and explore their associated immune signal transduction pathways. This information is expected to provide new target genes for the development of more efficient biological agents and achieve a green and sustainable control of *O. furnacalis*.

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