Bulletin of Entomological Research

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Molecular mechanism of *Serratia marcescens* Bizio infection in *Reticulitermes chinensis* Snyder based on full-length SMRT transcriptome sequencing

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Research Paper

Cite this article: Zhang L, Tang F (2024). Molecular mechanism of *Serratia marcescens* Bizio infection in *Reticulitermes chinensis* Snyder based on full-length SMRT transcriptome sequencing. *Bulletin of Entomological Research* **114**, 190–202. https://doi.org/10.1017/S000748532300072X

Received: 17 June 2023
Revised: 7 September 2023
Accepted: 24 December 2023
First published online: 8 February 2024

Keywords:
full-length transcriptome; pathogenic mechanism; *Reticulitermes chinensis* Snyder; *Serratia marcescens* Bizio; SMRT sequencing

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Abstract

Reticulitermes chinensis Snyder is an important pest in forestry and construction and is widely distributed in China. We found that *Serratia marcescens* Bizio strain SM1 has insecticidal activity to *R. chinensis*, but the pathogenic mechanism of SM1 to *R. chinensis* is not clear. Therefore, full-length transcriptome sequencing was performed on *R. chinensis* infected with SM1 and the control group. A total of 230 differentially expressed genes were identified by comparing SM1 infection group and the control group, among which 103 were downregulated and 127 were upregulated. We found downregulated genes in nine metabolic pathway categories, among which carbohydrate metabolism had the most downregulated genes, followed by energy metabolism and amino acid metabolism. We also found that some downregulated genes were related to pattern recognition receptors, cellular immunity, and humoral immunity, indicating that *R. chinensis* immunity was negatively affected by SM1 infection. In addition, some genes in signal transduction and genetic information processing pathways were downregulated. In this study, high-throughput full-length transcriptome analysis was used to analyse the pathogenic mechanism of SM1 to *R. chinensis*. The results of this study provide useful information for exploring the relationship between SM1 and *R. chinensis*, and provide theoretical support for the future application of SM1 and the prevention and treatment of *R. chinensis*.

Introduction

It is well known that termite is a social insect; some species of which are important worldwide pests. They have the characteristics of strong survival ability, long population life, hidden lifestyle, and wide range of harm, and they often cause serious harm to housing, water conservancy, transportation, communication, warehousing, agriculture, and forestry (Huang *et al.*, 2000; Zhang *et al.*, 2015). According to 2010 data, the global economic impact of termites is estimated at $40 billion (Rust and Su, 2012).

Reticulitermes chinensis Snyder is a widely distributed subterranean termite in China. As an important pest in forestry and construction, *R. chinensis* nest in soil and wood structures and feed on wood and its products (Huang *et al.*, 2000; Liu, 2003). Chemical control is an important means to control termites. Chemical control has quick and good effect, but long-term use of chemical insecticides has caused many adverse effects. So biological control has been paid more and more attention (Verma *et al.*, 2009; Lin, 2015). The use of pathogenic microorganism is an important pest control method, which has been studied with a variety of termites. As for *R. chinensis*, there are also some studies related to pathogenic microorganisms, such as Wang (2014) studied the lethal effect of *Metarhizium anisopliae* (Metschn.) Sorokin against *R. chinensis*, and the changes in genes and enzymes in *R. chinensis* infected with *M. anisopliae*. Tan (2022) studied the differences in lethality of four different fungal strains against *R. chinensis*.

Our research group isolated a *Serratia marcescens* Bizio strain SM1 (hereinafter referred to as SM1) from the disease insects and found that the strain has insecticidal activity to *R. chinensis* (Jiang *et al.*, 2023). As a biocontrol bacterium, *S. marcescens* can be used in the control of some pests, plant pathogenic fungi, and nematodes (Sezen *et al.*, 2001; Someya *et al.*, 2001; Hegazy *et al.*, 2019). In terms of insecticidal ability, *S. marcescens* has been shown to kill a wide variety of insects as an insect pathogen. Kwak *et al.* (2014) verified the pathogenicity and toxicity of *S. marcescens* by injecting and orally treating *Protaetia brevitarsis seulensis* (Kolbe) larvae with bacterial suspension of *S. marcescens*. Mohan *et al.* (2011) isolated the *S. marcescens* strain SRM from the flowers of summer squash and found that the strain was pathogenic against *Helicoverpa armigera* (Hübner). After infection

with *S. marcescens*, the egg production and eclosion rate of *Heliothis virescens* (Fabricius) adults decreased compared with the control group, while the larval mortality increased, and the female pupae became smaller and had a higher mortality (Inglis and Lawrence., 2001). In addition, *S. marcescens* strain TC-1 had been isolated and found to have larvicidal activity against *Anomala corpulenta* Motschulsky, *Plutella xylostella* (Linnaeus), *Spodoptera exigua* (Hübner), *H. armigera*, and *Bombyx mori* (Linnaeus) (Tao *et al*., 2022). *S. marcescens* is a biocontrol bacterium with great potential.

SM1 is a potential biocontrol bacterium against *R. chinensis*, and we have previously identified the immune defence mechanism of *R. chinensis* against SM1 by second-generation transcriptome sequencing technology (Luo *et al*., 2022), but the pathogenic mechanism of SM1 to *R. chinensis* has not been reported. Single-molecule real-time (SMRT) sequencing technology is a third-generation high-throughput sequencing technology, which is suitable for *de novo* genomic sequencing and high-quality assemblies of small genomes. SMRT sequencing technology can detect epigenetic modifications directly, and has the characteristics of being polymerase chain reaction (PCR)-free, having a high speed, and having long read lengths (Liu *et al*., 2015). Therefore, we intended to use SMRT sequencing technology to clarify the following questions: (1) whether SM1 has a destructive effect on the metabolism of *R. chinensis*; (2) whether SM1 can destroy the immunity of *R. chinensis*; (3) whether SM1 affects the signal transduction pathways of *R. chinensis*; (4) whether SM1 affects the pathways related to genetic information of *R. chinensis*. By clarifying the above questions, we clarified the pathogenic molecular mechanism of SM1 to *R. chinensis*.

Materials and methods

Insects and bacteria

R. chinensis populations were collected in Nanjing, Jiangsu province, China. Each *R. chinensis* colony was kept in separate plastic boxes (20 cm × 15 cm × 15 cm). In our laboratory, the colonies were set in 25 ± 1°C with 90 ± 5% relative humidity and dark conditions (0 h light:24 h dark). Healthy workers in the colonies were selected for the experiment.

We placed SM1 on solid bacterial basal medium and cultured it at 30°C for 12 h. After the generation of single colonies, we placed a single colony in 50 ml seed culture medium, and cultured it in a shaker at 30°C and 200 r min^{-1} for 12 h. Then, we added an appropriate amount of seed solution into 200 ml fermentation medium and cultured in a shaker at 30°C and 200 r min^{-1} for 36 h. Bioassays were performed using SM1 fermentation medium with a concentration of 1.52×10^{10} cells ml^{-1}.

Sample processing

We placed 20 healthy third-instar worker termites into each Petri dish with a diameter of 20 cm and starved them for 12 h. In the treatment group (SM_RC), 1 µl SM1 fermentation medium was dropped on the pronotum of *R. chinensis*, and 1 µl sterile fermentation medium was dropped on the same locations of *R. chinensis* in the control group (RC). After 20 h, ten live *R. chinensis* were deposited at −80°C respectively for subsequent experiment. Three replicates were set in the treatment group and the control group.

RNA sample preparation

Total RNA of the samples was extracted by the TRIzol method. The purity and concentration of RNA was determined by Nanodrop 2000 spectrophotometer, and agarose gel electrophoresis was used to detect the integrity of RNA.

Library preparation and SMRT sequencing

Full-length cDNA of mRNA was synthesised by Clonetech SMARTer™ PCR cDNA Synthesis Kit. Primer with Oligo dT was used to pair the A–T bases with the structure of poly-A at the 3′ end of mRNA, and primer was added to the end of reverse-synthesised full-length cDNA. The full-length cDNA was amplified by PCR, purified by SMRTbell cleanup beads (PB) magnetic beads, and quantified by Qubit 3.0. The end of the full-length cDNA was repaired and attached to the SMRT dumbbell adapter, and the unattached fragments were digested by exonuclease. After purification by PB magnetic beads, the sequencing library was obtained. Accurate quantification was performed using Qubit 3.0 and library size was detected using Agilent 2100. The full-length transcriptome sequencing was performed by using a PacBio sequencer after the test results met the requirements.

Sequel data output and quality control

The original sequencing output data were preprocessed by using SMRTLink. The main parameters of SMRTLink were minimum subread length = 50, maximum subread length = 15,000, minimum number of passes = 3, minimum predicted accuracy = 0.99. The Iso-Seq analysis process was used to obtain full-length transcript sequences. The single-molecule polymerase reads were separated to obtain subreads, and circular consensus sequence (CCS) was formed by the self-correction of the subreads obtained from the same polymer reads. The 3′ poly-A sequences, primer sequences, and chimeric sequence of CCSs were detected to classify CCSs and find full-length non-concatemer (FLNC) sequences. The iterative clustering and error correction tool of SMRTLink software was used to cluster and eliminate redundancy of the FLNC sequences and then the arrow algorithm in SMRTLink was used to make further correction to obtain the polished transcripts. Finally, cd-hit software was used for clustering and redundancy removal (Li and Godzik, 2006).

Functional annotation of transcripts

Isoform sequences were aligned to gene ontology (GO), Swiss-Prot Protein Sequence Database (Swiss-Prot), Clusters of Orthologous Groups/Eukaryotic Orthologous Groups (COG/KOG), NCBI nonredundant protein sequences (NR), and Kyoto Encyclopedia of Genes and Genomes (KEGG) by diamond blastx to obtain protein IDs with high sequence similarity and the protein functional annotation information of the isoforms (Buchfink *et al*., 2015). The annotation status of all transcripts was statistically summarised, and the BLAST function of the NCBI website (https://www.ncbi.nlm.nih.gov/) was used to further verify the transcripts with contradictory or ambiguous annotations.

Digital gene expression library preparation and analysis

Using bowtie2 software, the clean reads of each sample were compared with the isoforms that had redundancy removed

https://doi.org/10.1017/S000748532300072X Published online by Cambridge University Press

Table 1. Statistics of sequencing data and transcript clustering data

Data type	Total bases (Gb)	Total number	Minimum length	Average length	Maximum length	N50	Average_accuracy	Average_passes
polymerase read	83.6	690,759	51	121,029	354,538	195,728	–	–
subread	80.0	43,588,684	51	1836	255,792	2605	–	–
CCS	1.482	537,340	59	2758	14,885	3485	0.99952	67
FLNC	0.901	357,749	50	2518	11,282	3265	–	–
polished transcript	0.078	27,570	57	2830	10,641	3612	–	–
non redundant isoform	0.073	26,097	57	2816	10,643	3596	–	–

(Langmead, 2010). RSEM was used to count the results of bowtie2 and perform fragments per kilobase per million bases (FPKM) conversion (Li and Dewey, 2011). When the differential expression analysis was performed by DESeq2 (Love *et al.*, 2014), the *P*-value threshold of multiple tests was determined by the false-discovery rate (FDR) method, and an FDR threshold of <0.05 and an absolute value of $|\log_2 \text{fold change (FC)}| > 1$ were set. Then, KEGG pathway enrichment analysis and GO enrichment analysis were performed.

Results

Overview of the full-length transcriptome database

Sequencing data output and transcript clustering analysis

The full-length transcriptome sequencing was performed by using a PacBio sequencer after the test results met the requirements, and 145 Gb of original data were generated and stored in the NCBI Sequence Read Archive with accession number SRR24891407. The number of polymerase reads was 690,759, and 80 Gbp of subreads remained after removing the adapter sequence of polymerase reads. A total of 537,340 sequences were CCSs, and a total of 357,749 FLNC were obtained by CCS classification. A total of 27,570 polished isoform sequences were assembled from FLNC, and a total of 26,097 isoforms were obtained by cd-hit-est software for sequence clustering and elimination of redundancy (table 1).

Functional annotation of transcripts

In this study, 3808 transcripts were not annotated, and 22,289 transcripts were annotated, and 5256 transcripts were annotated by all databases. A total of 22,148 transcripts were annotated in NR database, 7765 in GO database, 13,168 in KEGG database, 16,696 in KOG database, and 19,180 in Swiss-Prot database.

In the NR annotation, 44.6% of the *R. chinensis* sequences were aligned to *Cryptotermes secundus* (Hill), followed by *Zootermopsis nevadensis* Hagen (26.0%), *Coptotermes formosanus* (5.9%), *Blattella germanica* Linnaeus (1.6%), *Reticulitermes flavipes* (Kollar) (1.2%), and others (20.7%) (fig. 1).

In the GO annotation, the transcriptome was divided into three major functional processes including 52 terms. The most abundant terms in the biological process (BP) category were cellular process (3841), metabolic process (3048), and single-organism process (2508). In the cellular component (CC) category, cell (3564), cell part (3489)s and organelle (2689) had the most transcripts. The transcripts of binding (4082) and catalytic activity (3836) were far more numerous than other subcategories in the molecular function (MF) category (fig. 2).

A total of 13,168 transcripts were annotated to 287 KEGG pathways on 34 smaller branches on the five major branches. The terms with the largest number of transcripts in five major branches, including environmental information processing, organismal systems, metabolism, cellular processes, and genetic information processing, were signal transduction (2106), endocrine system (1427), carbohydrate metabolism (1031), transport and catabolism (1730), and folding, sorting, and degradation (1132), respectively (fig. 3).

A total of 16,696 transcripts were divided into 26 KOG groups. The three largest groups were general function prediction only (2563), posttranslational modification, protein turnover, chaperones (2027), and signal transduction mechanisms (1924) (fig. 4).

Differentially expressed genes in R. chinensis *in response to SM1 infection*

In order to explore the mechanism of SM1 infection, we used PacBio Sequel sequencing to identify differentially expressed genes (DEGs) in *R. chinensis*. The combined data from three biological replicates were used to compute FPKM values, and the

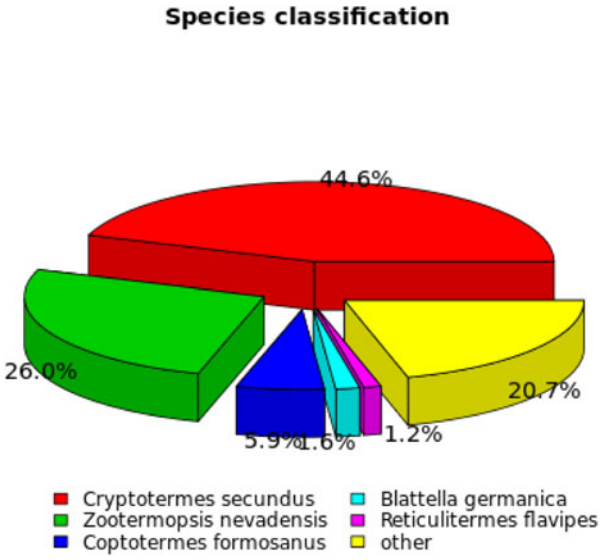

Figure 1. NR classification of all *R. chinensis* unigenes.

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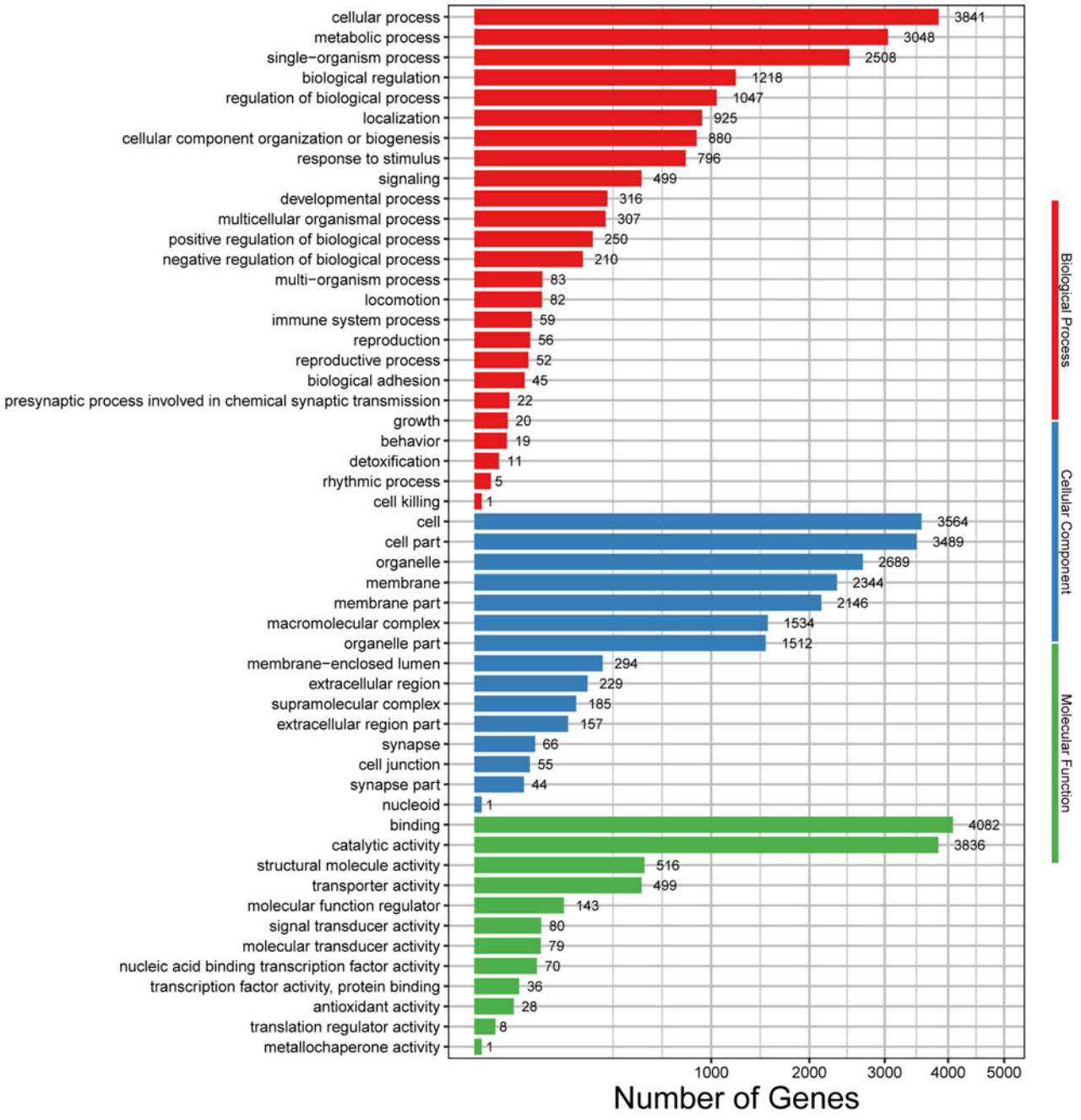

Figure 2. GO functional classifications of *R. chinensis* transcripts. MF, BP, and CC are represented in green, red, and blue, respectively. The *x*-axis and *y*-axis represent the number of transcripts and GO categories, respectively.

results were compared between the replicate RC and SM_RC groups. DEGs were significantly different when FDR < 0.05 and $|\log_2 FC| > 1$ (fig. 5A). A total of 230 DEGs were identified from 22,289 unigenes, including 103 downregulated DEGs and 127 upregulated DEGs (fig. 5B).

The functions of 61 DEGs in *R. chinensis* were divided into 30 groups in GO enrichment analysis. The most abundant terms in the BP category were cellular process (29), metabolic process (22), and single-organism process (19). In the CC category, cell (27), cell part (27), membrane (17), and organelle (17) had the most transcripts. The transcripts of binding (30) and catalytic activity (24) were far more numerous than other subcategories in the MF category (fig. 6).

Mapping DEGs to typical KEGG pathways identified biological pathways that responded to SM1 processing, 80 DEGs were assigned into 104 KEGG pathways (Supplementary table S1). *P*-values of pathways <0.05 indicated highly enriched pathways (table 2).

Transcriptomic response of R. chinensis *to SM1 infection*

SM1 affected the expression of metabolism-related genes of R. chinensis

Metabolism is a considerable component of life activities. By searching the KEGG database, we screened the genes related to metabolic pathways of *R. chinensis*, which could be divided into

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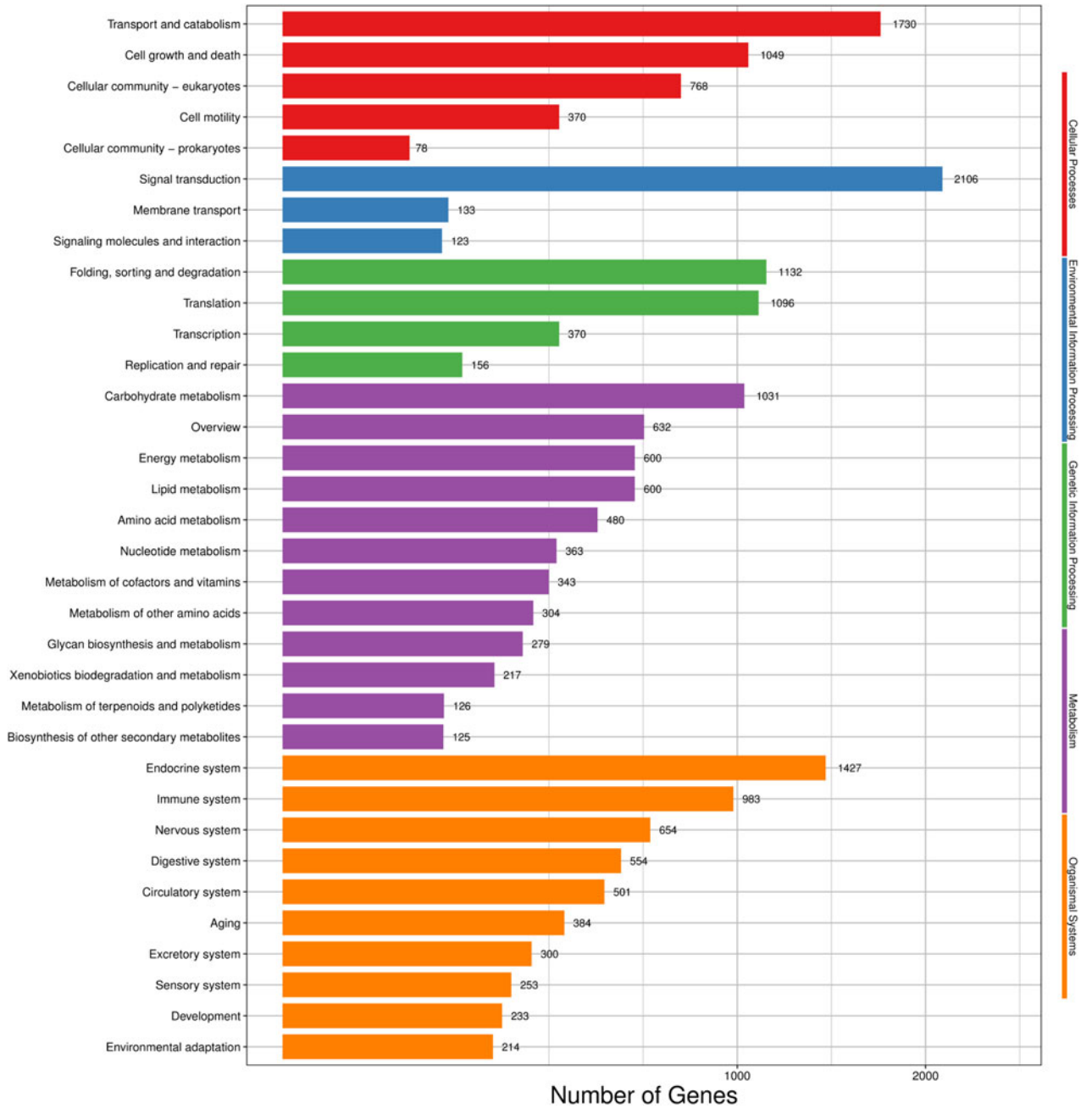

Figure 3. KEGG classification of *R. chinensis* transcripts. Genetic information processing, organismal systems, environmental information processing, cellular processes, and metabolism are represented in green, orange, blue, red, and purple, respectively. The *x*-axis and *y*-axis represent the number of transcripts and KEGG pathway categories, respectively.

nine categories: carbohydrate metabolism, xenobiotics biodegradation and metabolism, energy metabolism, nucleotide metabolism, lipid metabolism, metabolism of terpenoids and polyketides, amino acid metabolism, glycan biosynthesis and metabolism, and metabolism of cofactors and vitamins. In *R. chinensis* infected with SM1, 16 genes related to metabolic pathways were downregulated (fig. 7A) (Supplementary table S2). The pathway related to carbohydrate metabolism had the largest number of downregulated genes, with six downregulated genes, followed by amino acid metabolism and energy metabolism, with four downregulated genes respectively. Downregulated genes related to carbohydrate metabolism include UTP-glucose-1-phosphate uridylyltransferase (*UGP*), inositol-3-phosphate synthase (*ISYNA*), 2-oxoglutarate dehydrogenase (*OGDH*), phosphoglycerate mutase (*PGAM*), pyruvate kinase (*PK*), and β-glucuronidase (*GUSB*). UGP plays central roles in carbohydrate interconversion and glycogen synthesis. ISYNA is involved in the formation of inositol compounds. OGDH is involved in the citrate cycle in carbohydrate metabolism. PGAM is a protease that plays an important role in glycolysis and gluconeogenesis. PK is an enzyme that regulates glycolysis. The activity of animal GUSBs often exhibit changes in various physiological and pathological processes. In addition to carbohydrate metabolism, GUSB was not only related to xenobiotics biodegradation and metabolism,

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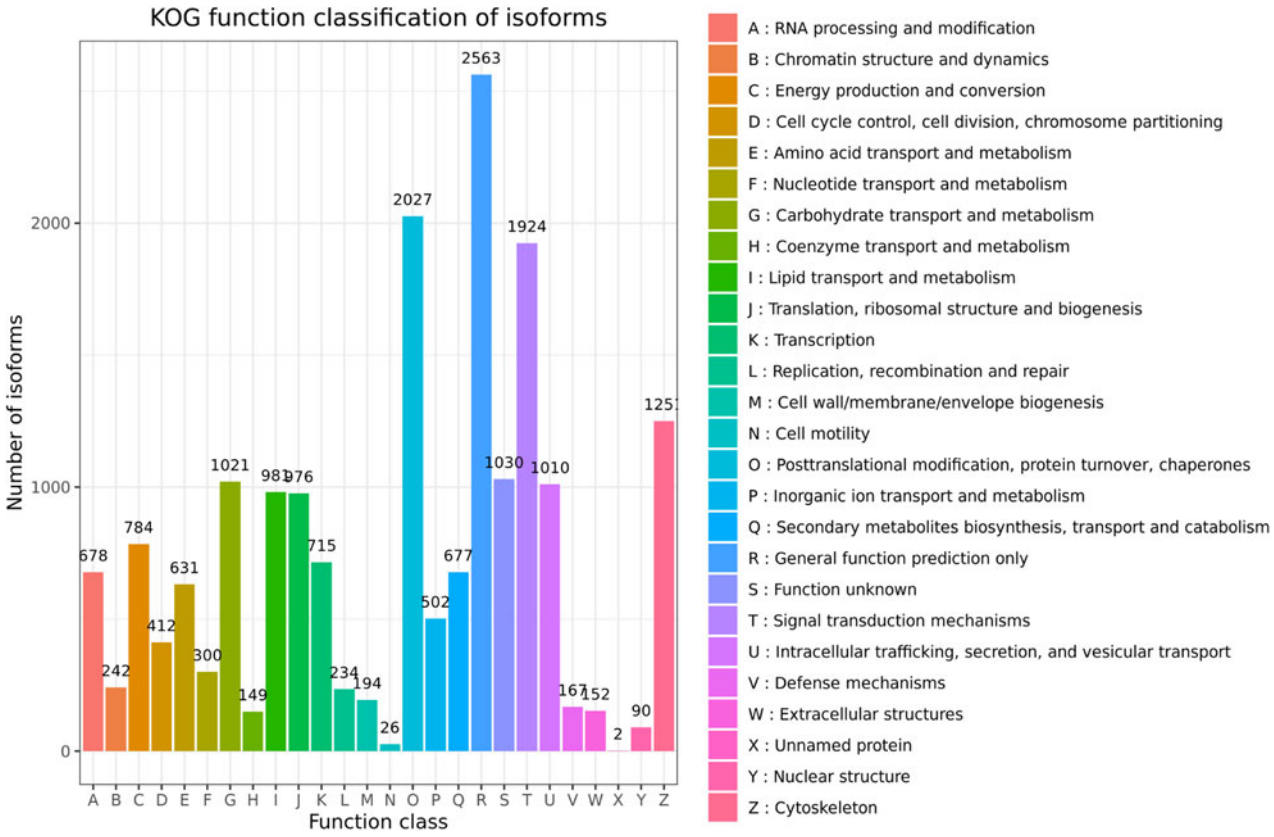

Figure 4. Eukaryotic orthologous groups of protein (KOG) annotation of *R. chinensis* transcripts. The *x*-axis and *y*-axis represent KOG categories and the number of transcripts, respectively.

glycan biosynthesis and metabolism, and metabolism of cofactors and vitamins, but also located in immune-related pathway. In addition to *OGDH* and *PGAM*, downregulated genes in amino acid metabolism included arginine kinase (*AK*) and ornithine decarboxylase (*ODC*). AK is a phosphotransferase in invertebrates, and ODC is important for polyamine biosynthesis. The four downregulated genes related to amino acid metabolism were located in arginine-, glycine-, proline-, serine-, lysine-, tryptophan-, and threonine-related metabolic pathways. In addition to *PGAM*, one NADH dehydrogenase (ubiquinone), one beta subcomplex subunit 8 (*NDUFB8*) gene, and 2V-type proton ATPase (*ATPeV1B* and *ATPeV0C*) genes were downregulated in the energy metabolism pathway. NDUFB8 is associated with oxidative phosphorylation. As a class of transporters, ATPeV1B and ATPeV0C located in the energy metabolism pathway were also present in immune-related pathways. In addition, we found that in the metabolism of terpenoids and polyketides, the expression of juvenile hormone epoxide hydrolase (*JHEH*), which is essential for juvenile hormone metabolism, was downregulated. All in all, SM1 downregulated some metabolism-related genes in *R. chinensis* and negatively regulated metabolic pathways.

SM1 negatively affects the immune response in R. chinensis

By screening the annotations of genes, we found that some immune-related genes of *R. chinensis* were downregulated after infection with SM1 (fig. 7B) (Supplementary table S2). We found that three pattern recognition receptor (PRR) genes were downregulated, and they were lipopolysaccharide binding protein (*LBP*), galectin-3 (*Gal-3*), and apolipophorin-III (*apoLp*-III). Regarding cellular immunity-related genes, we found downregulated genes in endocytosis, lysosome, and phagosome pathways. *ATPeV0C* and *ATPeV1B* both had one downregulated gene in the phagosome pathway. In the lysosome pathway, one gene each of cathepsin F (*CTSF*), lysosomal-associated transmembrane protein (*LAPTM*), insulin-like growth factor 2 receptor (*IGF2R*), *ATPeV0C*, and *GUSB* was downregulated. Cathepsin is associated with various physiological processes. LAPTMs regulate lysosomal function. IGF2R is a multifunctional binding protein, and *IGF2R* gene was not only related to lysosome, but also located in the endocytosis pathway in our data. In addition, downregulated genes located in the endocytosis pathway also included one ADP-ribosylation factor (*ARF*) gene. ARF is involved in regulating membrane trafficking pathways. Insect hexamerin is primarily considered a storage protein, but this protein is also involved in other biological functions. In humoral immunity, two hexamerin (*Hex-1* and *Hex-2*) genes were downregulated. In addition, our results showed that transferrin (*Tf1* and *Tf2*) had two downregulated genes. Besides transporting iron, this protein has other physiological functions.

SM1 downregulated genes associated with multiple signal transduction pathways in R. chinensis

We found the presence of DEGs in nine signal transduction pathways, and six signal transduction pathways were found to have downregulated genes after *R. chinensis* was infected by SM1. The six signal transduction pathways were phospholipase D (PLD) signalling pathway, mammalian target of rapamycin (mTOR) signalling pathway, mitogen-activated protein kinase

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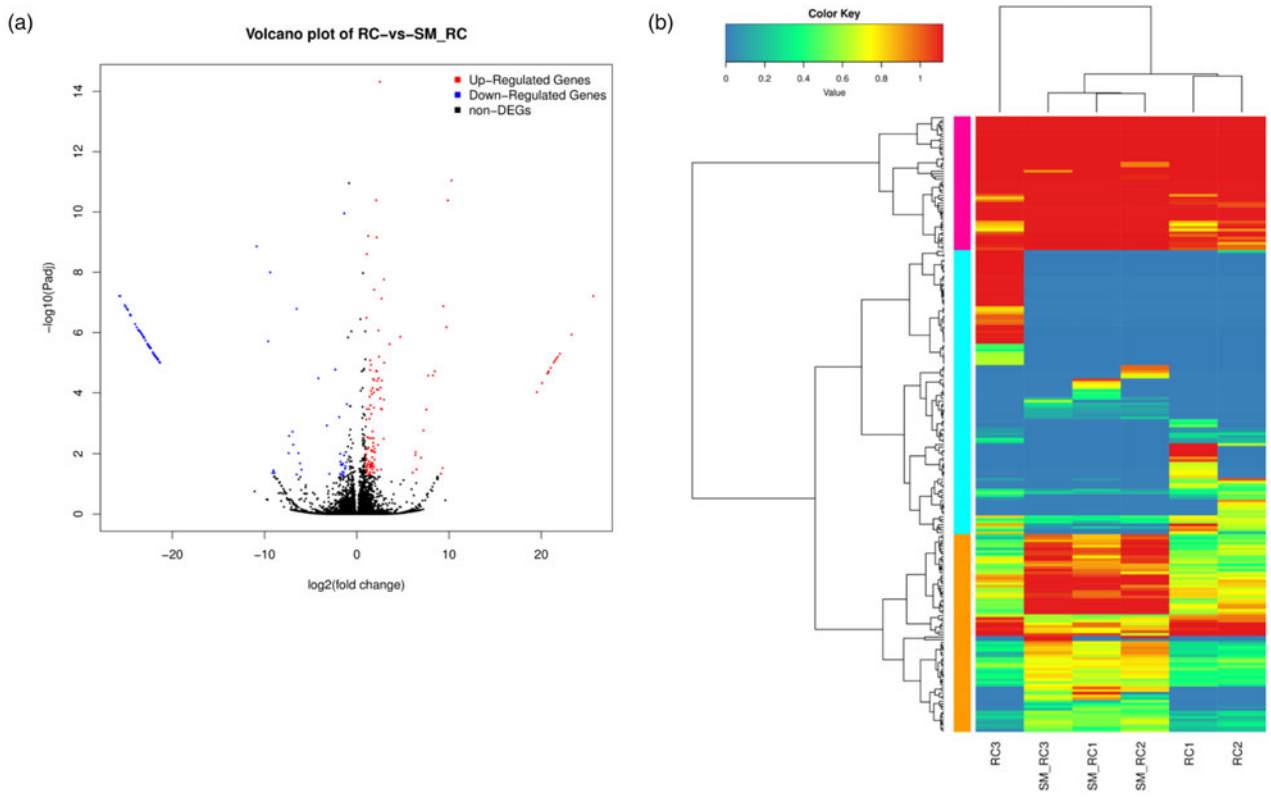

Figure 5. Overview of DEGs. (A) Comparison of DEGs between the RC library and SM_RC library. Red, blue, and black spots represent significantly upregulated genes, significantly downregulated genes, and genes with no significant difference in expression, respectively. (B) Heatmaps illustrating differences in normalised log signal intensity for the identified *R. chinensis* genes. *R. chinensis* treatment groups were labelled as SM_RC1, SM_RC2, and SM_RC3, and control groups were labelled as RC1, RC2, and RC3. Red and blue indicates genes expressed at high levels and genes expressed at low levels, respectively. The colours from red to blue indicate gradual decrease in expression.

(MAPK) signalling pathway, cyclic guanosine monophosphate-protein kinase G (cGMP-PKG) signalling pathway, cyclic adenosine monophosphate (cAMP) signalling pathway, and calcium signalling pathway. A total of six downregulated genes were found in these six signal transduction pathways (fig. 7C) (Supplementary table S2). Both cAMP signalling pathway and cGMP-PKG signalling pathway had two downregulated genes, and one gene was downregulated in other pathways, respectively.

SM1 downregulated genes in pathways related to genetic information in R. chinensis

In this study, we found DEGs in eight pathways related to genetic information processing in *R. chinensis* infected with SM1, among which six pathways had downregulated genes. The six pathways were basal transcription factors, RNA degradation, RNA transport, ribosome biogenesis in eukaryotes, ribosome and protein processing in endoplasmic reticulum, and there are six downregulated genes in these pathways (fig. 7D) (Supplementary table S2). RNA degradation pathway had the highest number of downregulated genes, with two downregulated genes, while other pathways had only one downregulated gene each.

Discussion

In our study, SM1 infection had different effects on nine categories metabolic pathways of *R. chinensis*, among which carbohydrate metabolism had the most downregulated genes, followed by amino acid metabolism and energy metabolism. A similar situation also appeared in *H. armigera* infected with nucleopolyhedrovirus (NPV), most of the downregulated genes were enriched in the gene cohorts of carbohydrate, amino acid and energy metabolism, and the downregulated proteins participated in carbohydrate, amino acid, and energy metabolism pathways (Xing *et al.*, 2017). In studies of arthropods such as *Eriocheir sinensis* (H. Milne Edwards), *S. exigua*, and *B. mori*, some genes related to carbohydrate metabolism were downregulated after infection with pathogenic microorganisms, which suggested that pathways related to carbohydrate metabolism were negatively affected to some extent (Huang *et al.*, 2009; Ding *et al.*, 2018; Li *et al.*, 2018). With regards to energy metabolism, in *Epiphyas postvittana* (Walker) and *Musca domestica* Linnaeus, some genes involved in energy metabolism were downregulated after infection by pathogenic microorganisms, indicating that their energy metabolism was disrupted (Gatehouse *et al.*, 2007; Tang *et al.*, 2014). In the study of *Ceracris kiangsu* Tsai, when energy metabolism was disrupted and related supply and demand was unbalanced, the cell activities were affected, and eventually the body died (Zhao *et al.*, 2004). In organisms, amino acids not only participate in the synthesis of nucleic acids, proteins and enzymes, but also play roles in immune regulation. Different amino acids have different functions in organisms (Li *et al.*, 2007). The changes of genes related to amino acid metabolism will undoubtedly affect the normal function of the organism. In the study of *Scylla paramamosain* Estampador, Cheng *et al.* (2022) found that the levels of some amino acids were changed by mud crab reovirus infection and some genes in amino acid

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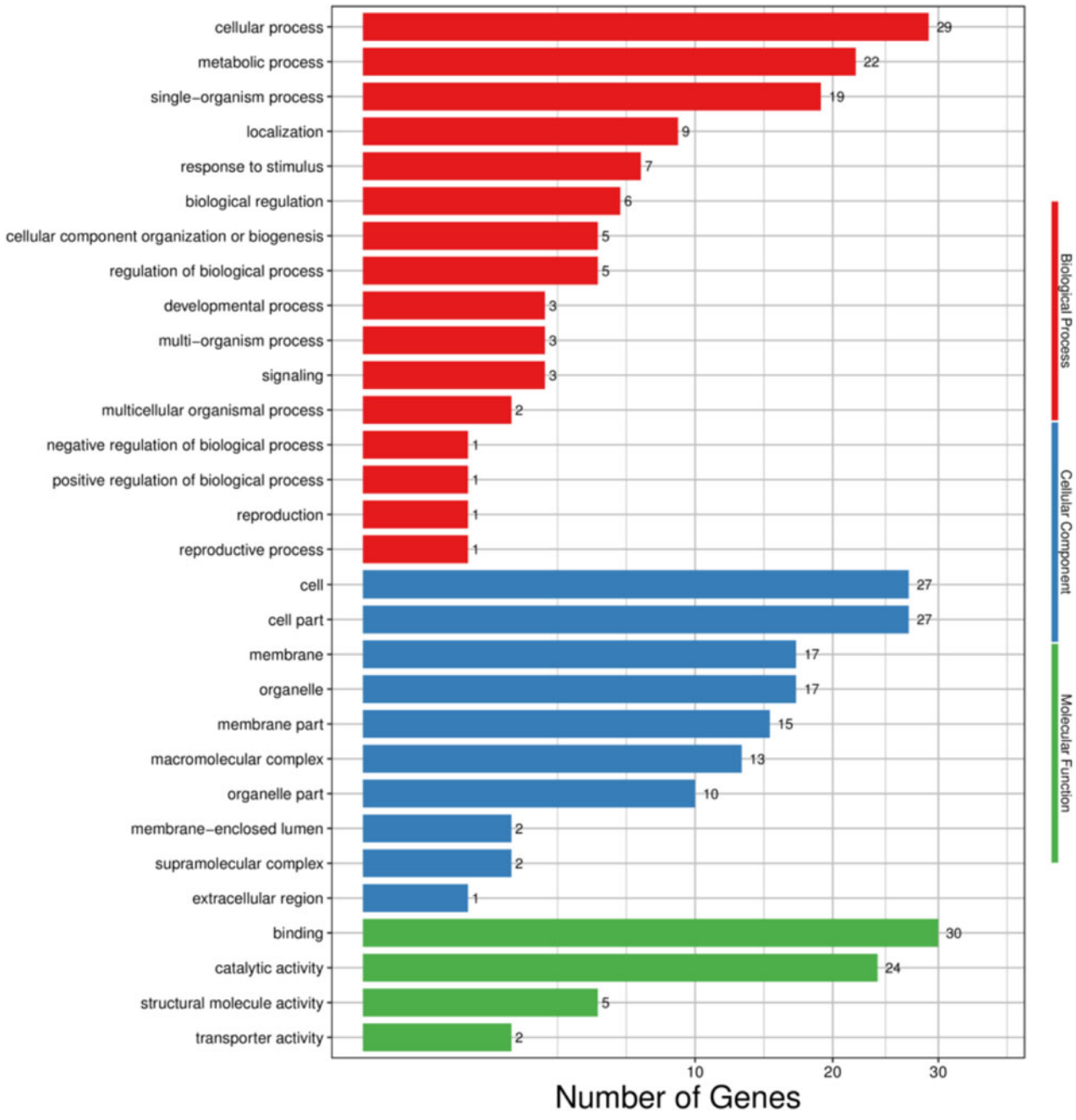

Figure 6. GO functional classification of DEGs in *R. chinensis*. MF, BP, and CC are represented in green, red, and blue, respectively. The *x*-axis and *y*-axis represent the number of transcripts and GO categories, respectively.

metabolism pathways were affected, which could have an impact on the regulation of oxidative stress and metabolic processes. In this study, we found that *JHEH* genes were downregulated. After *JHEH* gene expression was inhibited by RNAi technology, the survival rate of *Apolygus lucorum* (Meyer-Dur) nymphs decreased significantly, and some nymphs showed difficulty in moulting (Tusun *et al.*, 2017). After *JHEH* of *Mythimna separata* (Walker) larva was treated with RNAi technology, the emergence rate of *M. separata* was reduced (Li *et al.*, 2021). In conclusion, SM1 infection affected the expression of genes related to metabolism in *R. chinensis*, and negatively regulated its metabolism.

PRRs are a class of receptors that recognise and bind to pathogen-associated molecular patterns (PAMPs) that are specific to the pathogen but not present in the host when insects are infected with pathogenic microorganisms, thus activating relevant immune pathways in insects (Akira *et al.*, 2006; Kumar *et al.*, 2011; Brubaker *et al.*, 2015; Stokes *et al.*, 2015). Lipopolysaccharide is a special structure of Gram-negative bacteria, which can be recognised by LBP as PAMP. LBP has been reported in some insects, including *B. mori*, *Galleria mellonella* (Linnaeus), and *Periplaneta americana* (Linnaeus) (Jomori *et al.*, 1990; Dunphy and Halwani, 1997; Koizumi *et al.*, 1997). Studies on *B. mori* had found that LBP can remove bacteria by promoting the formation of haemocyte nodules (Koizumi *et al.*, 1999). Chen *et al.* (2023) found that LBP regulates the immune response of *C. formosanus* to Gram-negative bacteria by affecting

https://doi.org/10.1017/S000748532300072X Published online by Cambridge University Press

Table 2. Highly enriched KEGG pathways of DEGs in the *R. chinensis* transcriptome

No.	KEGG pathway	DEGs with pathway annotation (gene number)	All genes with pathway annotation (background number)	*P*-Value	Pathway ID
1	Endocytosis	8	394	0.030073798	ko04144
2	Antigen processing and presentation	7	262	0.010990207	ko04612
3	Oestrogen signalling pathway	6	216	0.015236596	ko04915
4	Carbohydrate digestion and absorption	5	116	0.004451214	ko04973
5	Pentose and glucuronate interconversions	4	111	0.019656025	ko00040

the immune deficiency (IMD) pathway. They also found that *C. formosanus* treated with both Gram-negative bacteria and the dsRNA of *LBP* had a higher mortality rate than *C. formosanus* treated with only Gram-negative bacteria or dsRNA. Gal has been found to have an affinity for galactoside, and has functions of inducing apoptosis, immune regulation, and parasitic immune evasion in different organisms (Advedissian *et al.*, 2017; Lu *et al.*, 2017; Xue *et al.*, 2017). Among the arthropods, the Gal-4 of *B. mori* showed obvious binding to fungi, Gram-positive bacteria and Gram-negative bacteria, and Gal of *Apis cerana* Fabricius had binding reactions with Chinese sacbrood virus and chronic bee paralysis virus. After the *Gal* gene was silenced, the number of bacteria in the haemolymph of *Marsupenaeus japonicus* (Bate) increased, and the survival rate of *Fenneropenaeus merguiensis* (de Man) against pathogenic microorganisms decreased (Shi *et al.*, 2014; Wang *et al.*, 2014; Yue *et al.*, 2018; Praparatana *et al.*, 2022). Studies have shown that apoLp-III is a multifunctional protein involved in immune response and lipid transport. The study on *Antheraea pernyi* (Guerin-Meneville) found that apoLp-III shows important role for binding of PAMPs, activation of prophenoloxidase, and production of antimicrobial peptides (AMPs) (Wen *et al.*, 2016). Injection of purified BmApoLp-III into *B. mori* larvae infected with *Beauveria bassiana* (Bals.-Criv.) Vuill. delayed the onset and death of the larvae. In contrast, silencing *BmApoLp-III* gene with RNAi led to early onset and death of *B. mori* larvae (Wu *et al.*, 2021). These three PRRs all play important roles in insect immunity, but in *R. chinensis* infected with SM1, these three PRRs were downregulated, which to some extent reflects the negative effects of SM1 on *R. chinensis* immunity.

Unlike vertebrates, insect immune systems do not have acquired immunity and rely on non-specific innate immunity to fight pathogens, including cellular immunity and humoral immunity (Lemaitre and Hoffmann, 2007). Cellular immunity is mediated by haemolymphatic cells of insects and consists mainly of nodulation, phagocytosis, and encapsulation (Kojour *et al.*, 2020). Endocytosis is an important way for organisms to remove pathogens (Ferreira and Boucrot, 2018; Kaksonen and Roux, 2018). IGF2R, also called cation-independent mannose-6-phosphate receptor, is a multifunctional receptor that performs a variety of tasks essential for normal cell function (Ghosh *et al.*, 2003). In our results, one *IGF2R* and one *ARF* were downregulated in the endocytosis pathway of *R. chinensis* infected with SM1. Ma *et al.* (2010) found that *ARF1* gene may be important in innate immunity of *M. japonicus*. V-ATPase is a class of transporters that are important for cellular processes and plasma membrane proton transport (Wagner *et al.*, 2004; Beyenbach and Wieczorek, 2006; Forgac, 2007; Hinton *et al.*, 2009). In addition to endocytosis, V-ATPase plays roles in many aspects, including amino acid transport, intracellular pH homoeostasis, intracellular waste disposal, neurotransmitter uptake, and protein degradation (Forgac, 1989; Harvey, 1992; Zhao *et al.*, 2015; McGuire *et al.*, 2017). After RNA interference on V-ATPase genes of arthropods such as *Tribolium castaneum* (Herbst), *Acyrthosiphon pisum* (Harris), *Cimex lectularius* Linnaeus, and *Neoseiulus californicus* (McGregor), their physiological activities and survival were severely affected (Basnet and Kamble, 2018; Cao *et al.*, 2018; Ghazy and Suzuki, 2022). *E. sinensis* increased mortality and decreased moulting rate after RNA interference with the *ATPeV1B* gene (Hou *et al.*, 2020). In *R. chinensis* infected with SM1, *ATPeV0C* and *ATPeV1B* each had one downregulated gene in the phagosome pathway. Degradation and recycling of cellular waste is an important function of lysosome. Substances reach lysosomes through endocytosis, phagocytosis, or autophagy, and then are degraded by lysosomal hydrolases (Saftig and Klumperman, 2009; Ballabio, 2016). In addition to *ATPeV0C* and *IGF2R*, *LAPTM*, *GUSB*, and *CTSF* were also downregulated in the lysosome pathway of *R. chinensis* infected with SM1. In other arthropod studies, these three genes had also been linked to immunity (Lanz *et al.*, 1993; Liu *et al.*, 2018; Yang *et al.*, 2021). Humoral immunity consists mainly of melanisation and production of AMPs (Zänker, 2010). Phenoloxidase (PO) is a key enzyme for melanism, and the study of *Neoaliturus haematoceps* (Mulsant and Rey) found that Hex was necessary for PO to obtain optimal activity (Cotter *et al.*, 2008; Eliautout *et al.*, 2016). In this study, two *Hex* genes were downregulated after *R. chinensis* infection with SM1. To sum up, SM1 infection had negative effects on both cellular and humoral immunity of *R. chinensis*.

Tf is a glycoprotein with multiple functions. It plays an important role in immunity, iron transport, and oxidative stress prevention in insects (Geiser and Winzerling, 2012). Studies have found that *Tf*, as an immune-related gene, was upregulated by pathogenic microorganism infection in a variety of insects (Yoshiga *et al.*, 1999; Valles and Pereira, 2005; Yun *et al.*, 2009; Brummett *et al.*, 2017). But in some cases, *Tf* was downregulated after insects were infected with pathogenic microorganisms, such as *Aedes aegypti* (Linnaeus) infected with Sindbis virus and *Spodoptera littoralis* Boisduval infected with NPV (Kim and Muturi, 2013; Hamama *et al.*, 2016). These may be because *Tf* will be induced as an immune-related gene to respond to the infection of pathogenic microorganisms on the one hand, and the infection of pathogenic microorganisms will cause damage to insects on the other. This may depend on the time, dose,

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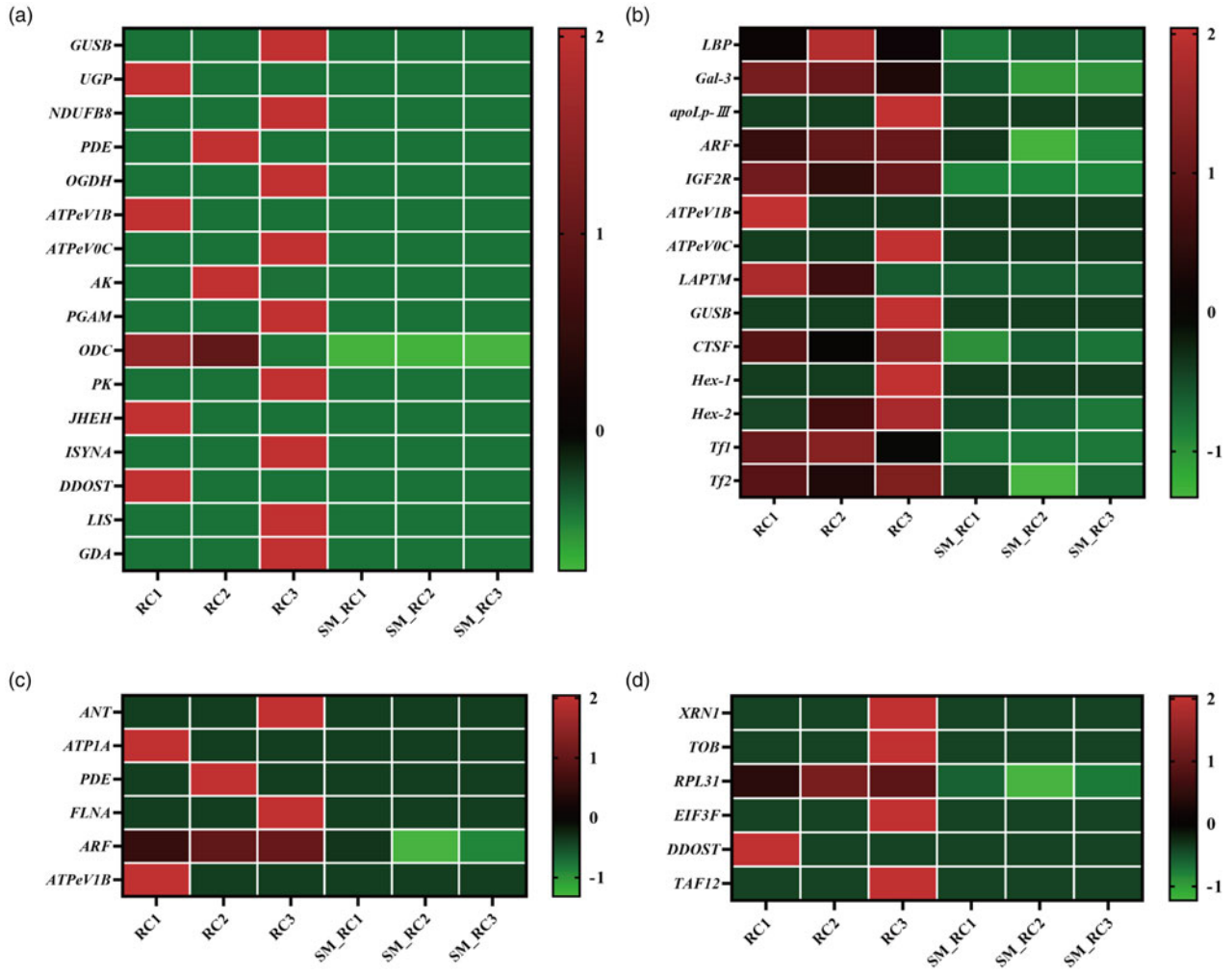

Figure 7. Heatmap analysis of downregulated genes in *R. chinensis* infected with SM1. RC1, RC2, and RC3 are the control groups. SM_RC1, SM_RC2, and SM_RC3 are the treatment groups. (A) Metabolism, (B) immune, (C) signal transduction, and (D) genetic information.

and microorganism species of infection. When *Tf* expression is inhibited, insect immunity will be affected. For example, after *Tf* inhibition, *P. xylostella* showed increased susceptibility to pathogenic microorganisms, and the production of blood nodules was inhibited (Kim and Kim, 2010; Xu *et al.*, 2020). After *R. chinensis* infection with SM1, *Tf* expression was downregulated. This also represents the negative effect of SM1 infection on *R. chinensis* immunity.

In terms of signal transduction pathways, we found downregulated genes in six pathways, each of which is important for the normal physiological activities of insects. Calcium signalling pathway is related to cold sensitivity and diapause regulation in insects (Teets *et al.*, 2013; Zhao *et al.*, 2017; Dong *et al.*, 2019). Studies on a variety of insects have found that the cAMP signalling pathway is involved in important physiological activities such as memory, immunity, heat stress, moulting, and cell death in insects (Venkatesh *et al.*, 2001; Kimura *et al.*, 2004; Armstrong *et al.*, 2006; Matsumoto *et al.*, 2009; Sajjadian and Kim, 2020). Regarding the cGMP-PKG signalling pathway, researchers have found that PKG signalling plays a role in neuronal plasticity of motor, sensory, and cognitive functions, including response to pheromones, appetitive learning, stress response, aggression, and phototaxis (Lucas and Ben-Shahar, 2021). MAPK regulates important cellular processes such as external stress, immune defence, apoptosis, cell proliferation, and intracellular metabolism, and the MAPK signalling pathway is involved in reproduction, drug resistance, immunity, moulting, and other aspects of insects (Covi *et al.*, 2012; Yang *et al.*, 2020; Zhang *et al.*, 2021; Huang *et al.*, 2022). As for the PLD signalling pathway, PLD is an important enzyme in the production of lipid second messenger phosphatidic acid, involved in a variety of basic cellular processes, and modulated a variety of cellular responses (Weernink *et al.*, 2007). The mTOR signalling pathway is also a multifunctional pathway involved in autophagy, moulting, hypoxia adaptation, and other aspects of insects (Covi *et al.*, 2012; Zhang *et al.*, 2017; Li *et al.*, 2022). The downregulation of genes in these signal transduction pathways represents the interference of SM1 infection on *R. chinensis* signal transduction pathways, and further affects various physiological activities of *R. chinensis*.

Ribosomes are basic macromolecular machines that play an important role in translation mechanism, converting encoded information in mRNA into protein (Thomson *et al.*, 2013). RPL31 is a component of the large ribosome subunit and may be involved in the normal function of the chaperone complex (Peisker *et al.*, 2008). In *R. chinensis* infected with SM1, *RPL31* gene expression level in the ribosome pathway was

https://doi.org/10.1017/S000748532300072X Published online by Cambridge University Press

downregulated. Ribosome biogenesis is the basic process that provides cells with the molecular factories that produce cellular proteins (Kressler *et al*., 2010). In our results, there is a downregulated gene, 5′–3′ exoribonuclease 1 (*XRN1*), in the ribosome biogenesis in the eukaryote pathway of *R. chinensis* infected with SM1, which is also located in the RNA degradation pathway. RNA degradation systems in organisms have demonstrated powerful efficacy in removing defective or no longer required RNA and RNA–protein complexes (Houseley and Tollervey, 2009). In this study, RNA degradation pathway had two downregulated genes. The gene downregulated in the RNA transport pathway is eukaryotic translation initiation factor 3 subunit F (*EIF3F*). Eukaryotic translation initiation factor 3 (EIF3) is a large multisubunit protein that plays a central role in translation initiation, and EIF3F may play a regulatory role in EIF3 (Mayeur, 2001). One gene of transcription initiation factor TFIID subunit 12 (*TAF12*) was downregulated in the basal transcription factors pathway of *R. chinensis* infected with SM1. TAF12 is one of the subunits of TFIID, and the general transcription factor TFIID is very important for the initiation of mRNA gene transcription (Sanders *et al*., 2002). The endoplasmic reticulum is a subcellular organelle. The unfolded protein enters the endoplasmic reticulum and uses the protein chaperones and catalysts of protein folding to form the final suitable conformation (Malhotra and Kaufman, 2007). In our results, one gene in the protein processing in the endoplasmic reticulum pathway was downregulated. All these pathways are related to genetic information processing, and genetic information of organisms is associated with various life activities. In this study, SM1 infection downregulated some of the genes in these pathways, which may indicate the influence of SM1 infection on the genetic information processing of *R. chinensis* and on other life activities directly or indirectly.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S000748532300072X.

Acknowledgements. This research was supported by The Natural Science Foundation of the Jiangsu Higher Education Institutions of China [grant number 20KJA220003], Postgraduate Research & Practice Innovation Program of Jiangsu Province [grant number KYCX23_1223], and the Priority Academic Program Development Fund of Jiangsu Higher Education Institutions. No additional external funding was received for this study.

Competing interests. None.

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https://doi.org/10.1017/S000748532300072X Published online by Cambridge University Press