

Rui Guo, He Zang, Xiaoxue Fan, Dafu Chen

College of Bee Science and Biomedicine, Fujian Agriculture and Forestry University, Fuzhou 350002, China

Circular RNAs (circRNAs) are a class of non-coding RNAs (ncRNAs) that can participate in biological processes such as metabolism and development. However, little has been explored about the function of circRNAs in the development of *Apis cerana* larval guts. By using our previously gained deep sequencing data from the guts of *A. cerana* worker larvae at 4-, 5-, and 6-day-old (Ac4, Ac5, and Ac6 groups), the expression pattern and regulatory role of circRNAs during the development process was comprehensively investigated, with a focus on differentially expressed circRNAs (DEcircRNAs) relevant to immunity pathways and developmental signaling pathways, followed by validation of the binding relationships among a key competing endogenous RNA (ceRNA) axis. Here, 224 (158) DEcircRNAs were detected in the Ac4 vs. Ac5 (Ac5 vs. Ac6) comparison group. It's suggested that 172 (123) parental genes of DEcircRNAs were involved in 26 (20) GO terms and 138 (136) KEGG pathways like Hippo and Wnt signaling pathways. Additionally, ceRNA network analysis indicated that 21 (11) DEcircRNAs could target seven (three) DEmiRNAs, further targeting 324 (198) DEmRNAs. These DEmRNAs can be annotated to 33 (26) GO terms and 168 (200) KEGG pathways, including 12 (16) cellular and humoral immune pathways (endocytosis, lysosome, Jak-STAT, etc.) and 10 (nine) developmental signaling pathways (Hippo, mTOR, Hedgehog, etc.). Interestingly, DEcircRNAs in these two comparison groups could target the same ace-miR-6001-y, forming complex subnetworks. The results of PCR and Sanger sequencing confirmed the back-splicing sites within four randomly selected DEcircRNAs. RT-qPCR detection verified the reliability of the used transcriptome data. The results of dual-luciferase reporter assay verified the binding relationships between novel_circ_001627 and ace-miR-6001-y and between ace-miR-6001-y and apterous-like. Our data demonstrated that DEcircRNAs were likely to modulate the developmental process of the *A. cerana* worker larval guts via regulation of parental gene transcription and ceRNA network, and novel_circ_001627/ace-miR-6001-y/apterous-like was a potential regulatory axis in the larval gut development. Findings from this work offer a basis and a candidate ceRNA axis for illustrating the circRNA-modulated mechanisms underlying the *A. cerana* larval guts.

Fujian Agriculture and Forestry University

National & Local United Engineering Laboratory of Natural Biotoxin

Apitherapy Research Institute



Unravelling the modulatory manner and function of circRNAs in the Asian honeybee larval guts

Associate Prof. Dr. Rui Guo

Sep. 24th, 2025

Copenhagen, Denmark

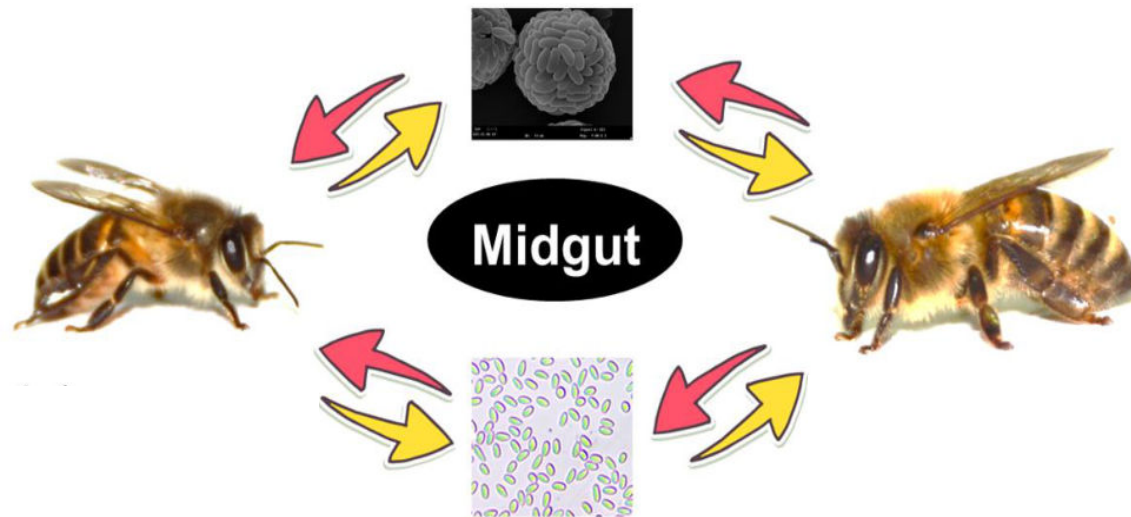
Self-introduction

- **Rui Guo**, PhD, Associate Prof., Doctoral supervisor
- College of Bee Science and Biomedicine, FAFU
- Deputy director of National & Local United Engineering Lab of Natural Biotoxin
- Deputy director of Apitherapy Research Institute of Fujian province
- Director of Experimental Centre of College of Bee Science and Biomedicine
- The Science of Nature, **Associate Editor**
- BMC Biology, Biology, IJMS, Animals, Insects, **Editor**
- Biomolecules, Frontiers in Bee Science, **Review Editor**
- Genes, Veterinary Sciences, Discover Developmental Biology, Frontiers in Physiology, Frontiers in Genetics, **Guest Editor**



Research direction

- Host—Pathogen/Parasite Interactions
- Non-coding RNAs (miRNA, piRNA, lncRNA, and circRNA)
- Omics (genomics, transcriptomics, proteomics, metabolomics, etc.)
- Effects of api-products on human diseases (tumors, inflammation, diabetes, etc.)



- 1. Background**
- 2. Research contents**
- 3. Conclusions**
- 4. Future direction**

Background

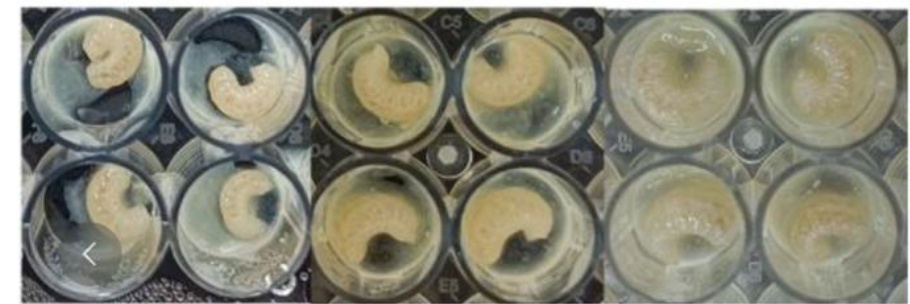
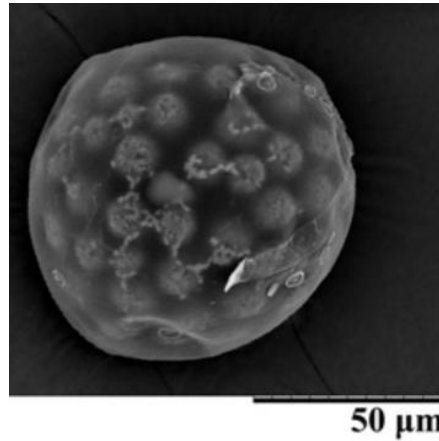
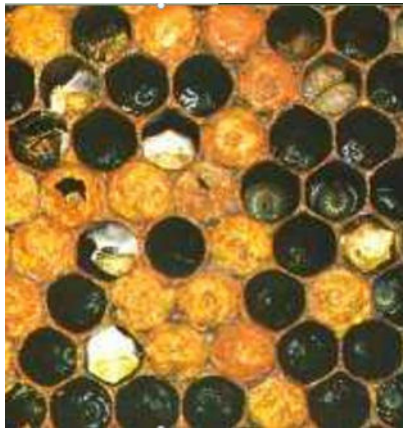
- Asian honeybee, *Apis cerana*

- Widely reared in Asian countries
- Environmental adaptation, tolerant to low temperature, high disease resistance, good at collecting scattered nectar source, etc.
- Resistant to chalkbrood disease at colony level (hygienic behavior)
- Innate immunity (cellular immune and humoral immune)



● Chalkbrood disease and *Ascosphaera apis*

- Sharply reduces the colony population and productivity
- Harms the colony health in combination with other factors



4-day-old

5-day-old

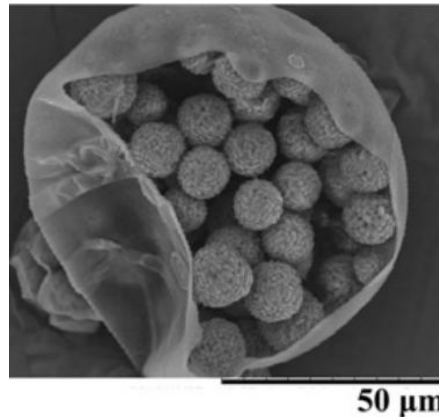
6-day-old



Chalkbrood



Pure culture



SEM

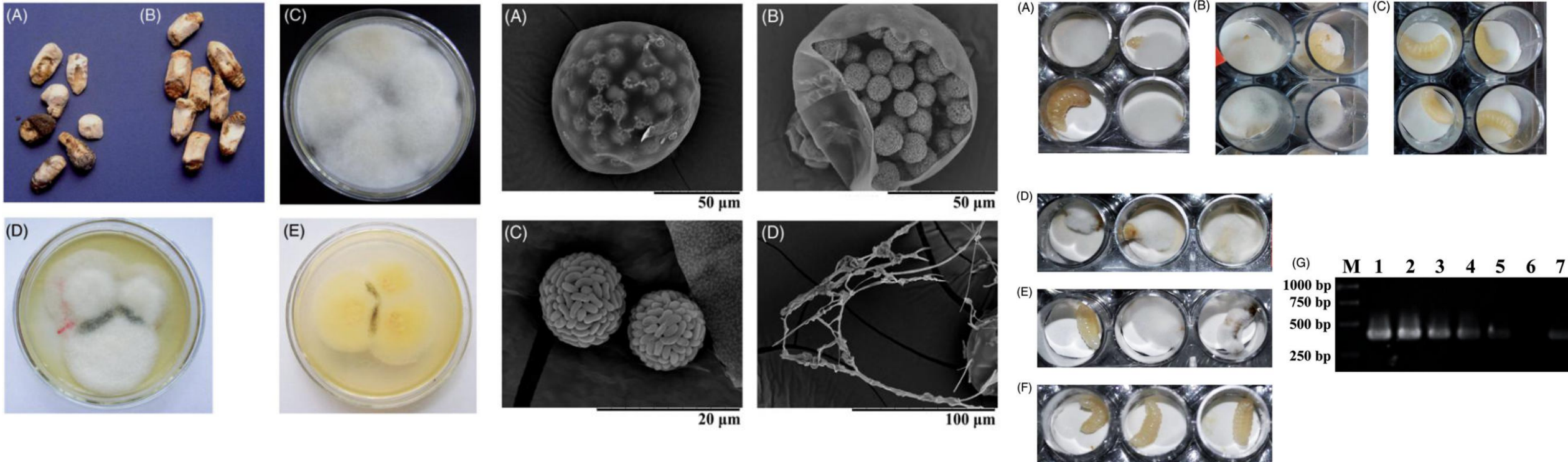


7-day-old
prepupa

8-day-old
prepupa

9-day-old
pupa

First validation of the *A. apis* infection of *A. cerana* worker and drone larvae

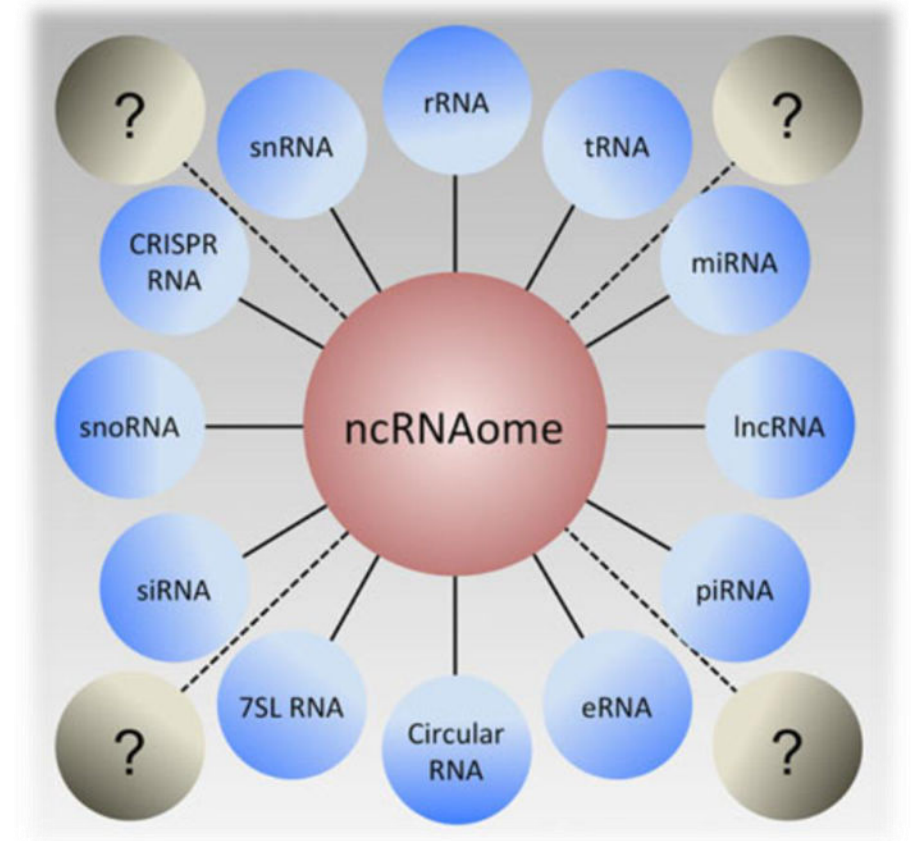
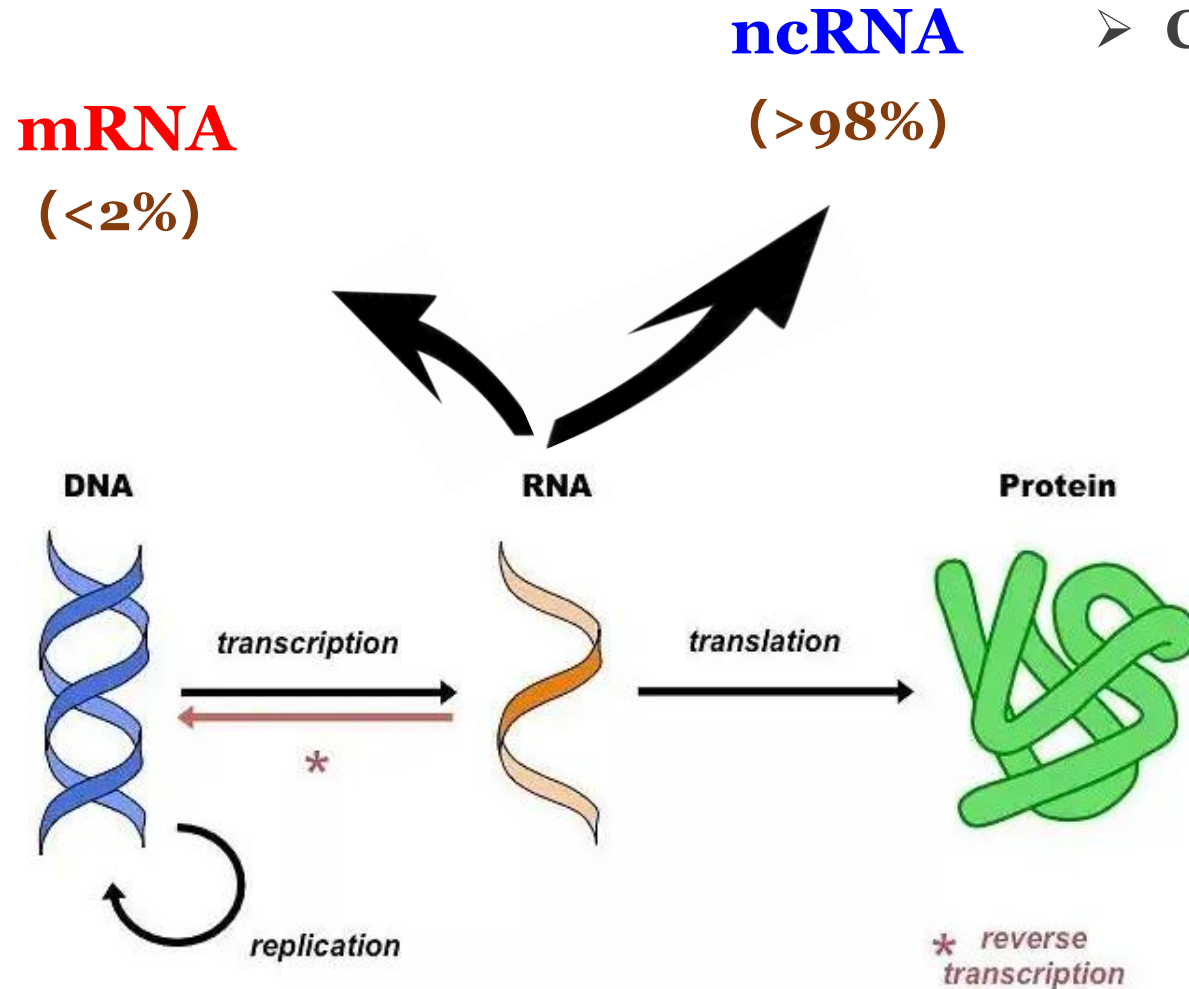


(J. Apicult. Res., 2018)

- **Non-coding RNAs, ncRNAs**

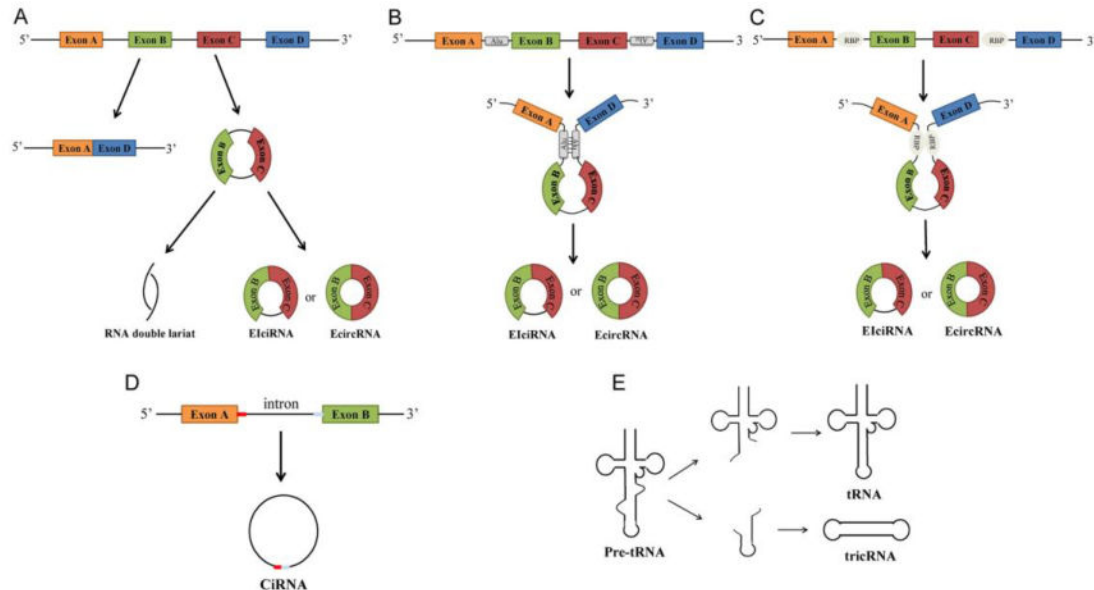
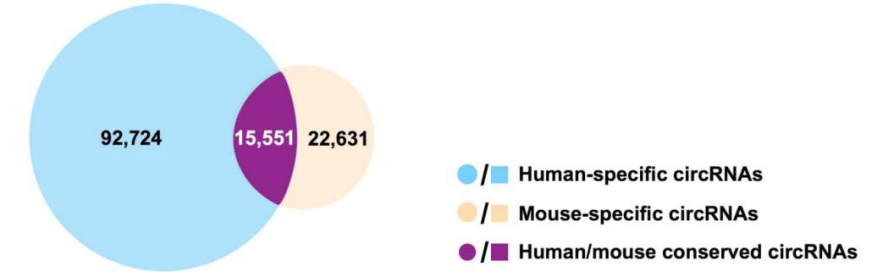
- Pivotal regulators for life activities

- Complex interactions via **ceRNA** networks

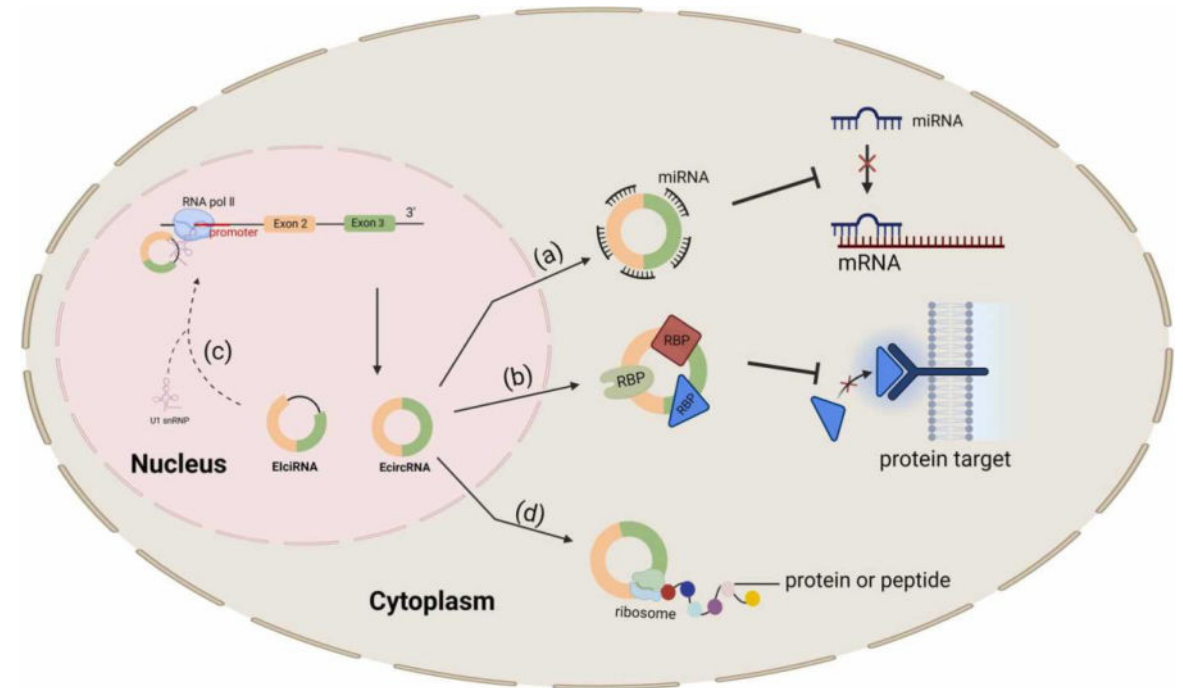


● Circular RNA, circRNA

- Covalently closed circular structure
- **Abundantly** expressed in diverse species
- High **stability**
- Ideal biomarkers

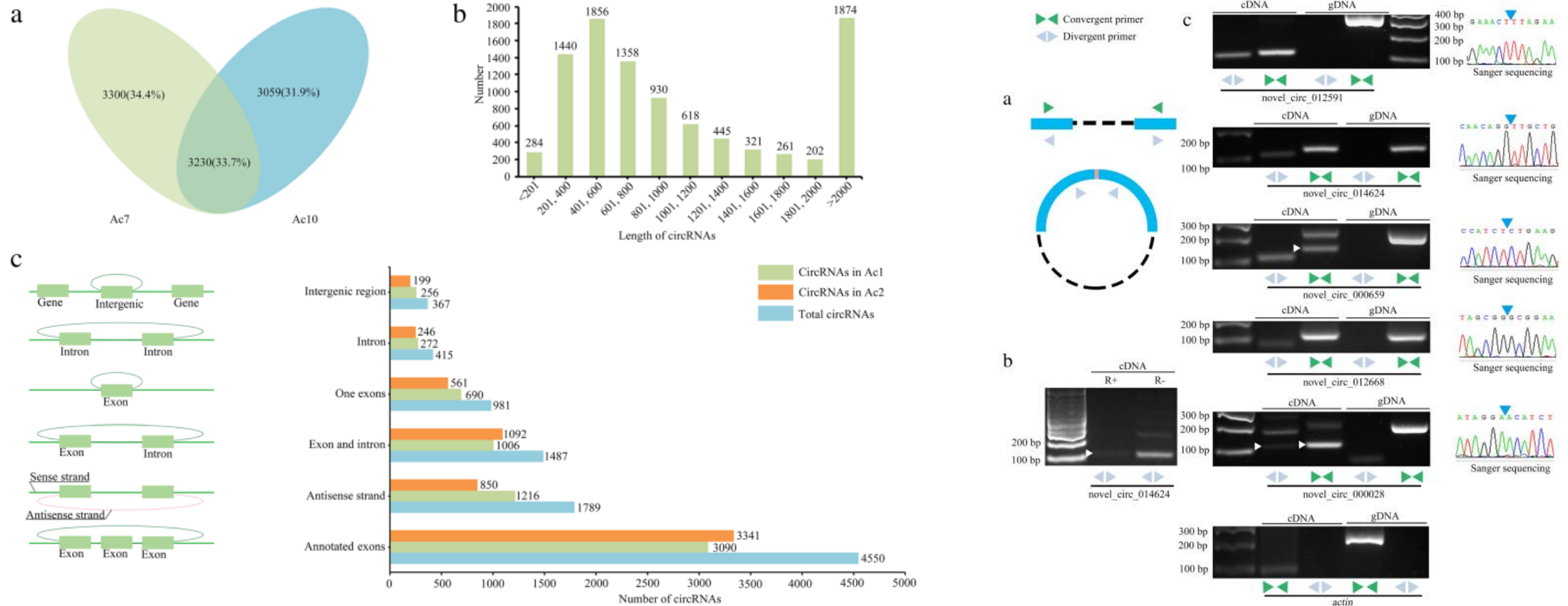


(World J. Surg. Oncol., 2022)



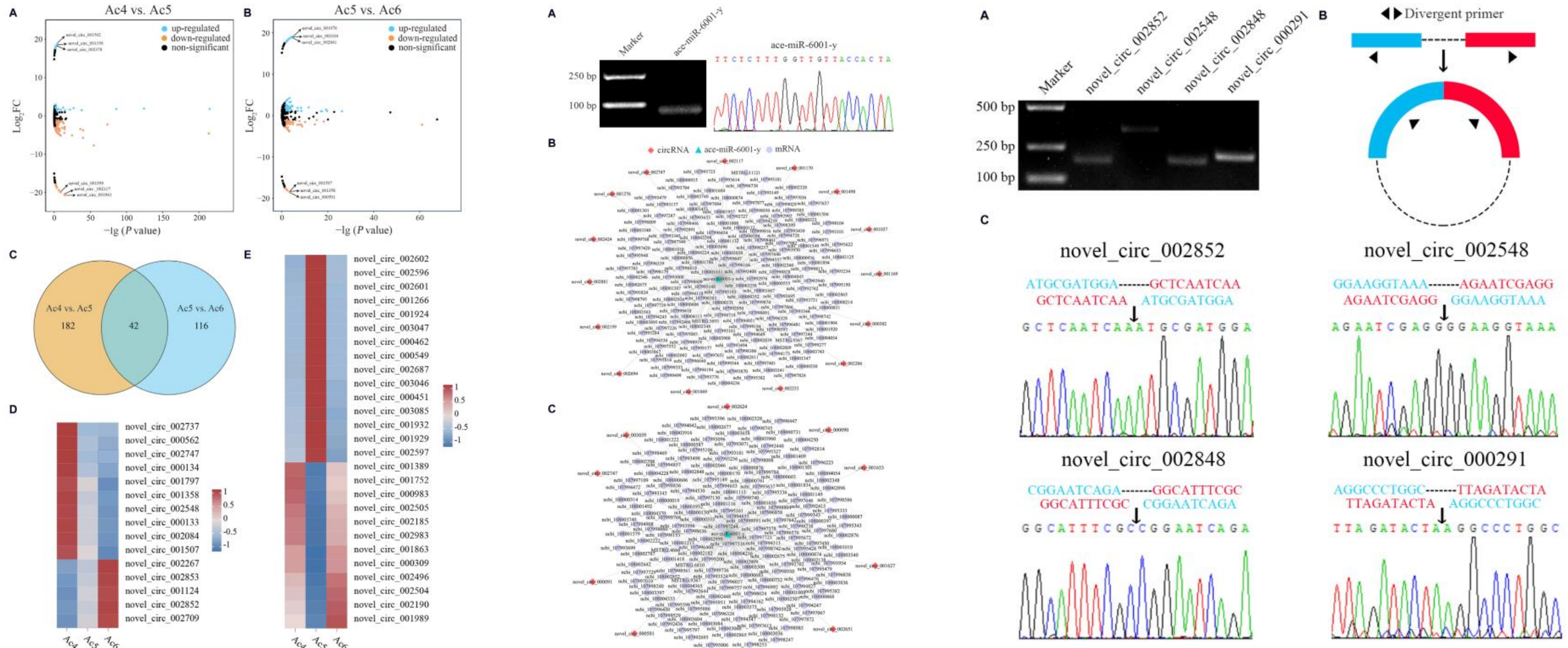
(Biomed. Pharmacother., 2023)

Systematic identification of circRNAs in the midgut of *A. cerana* worker



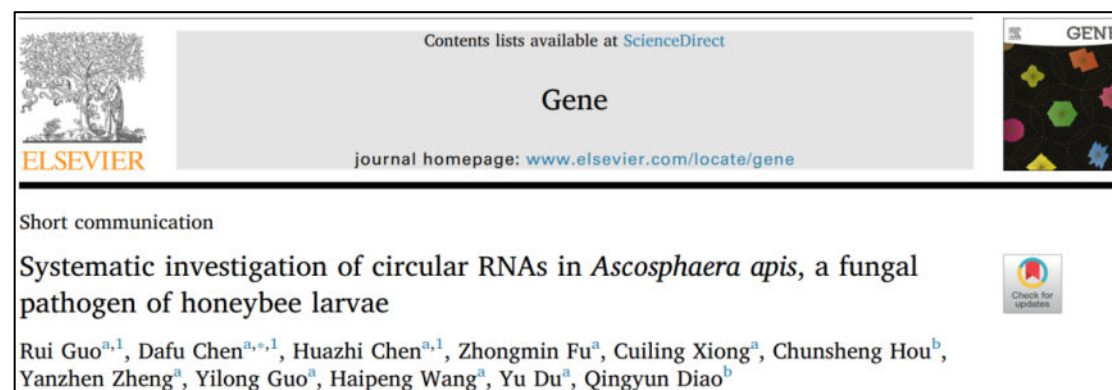
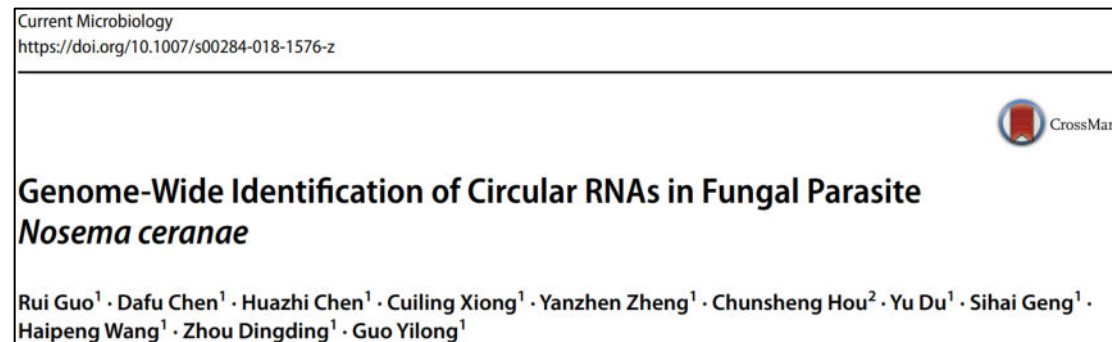
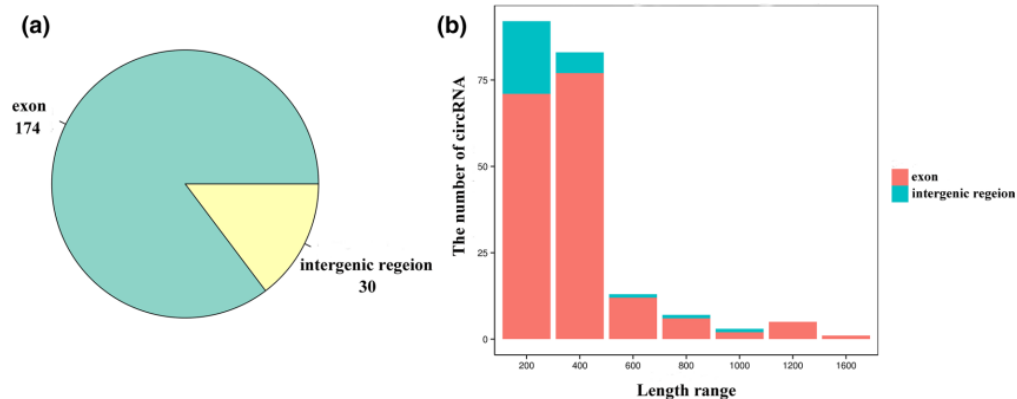
(Appl. Microbiol. Biot., 2020)

Analyzing the circRNA-regulated development of the *A. cerana* worker larval gut



(Front. Cell Dev. Biol., 2024)

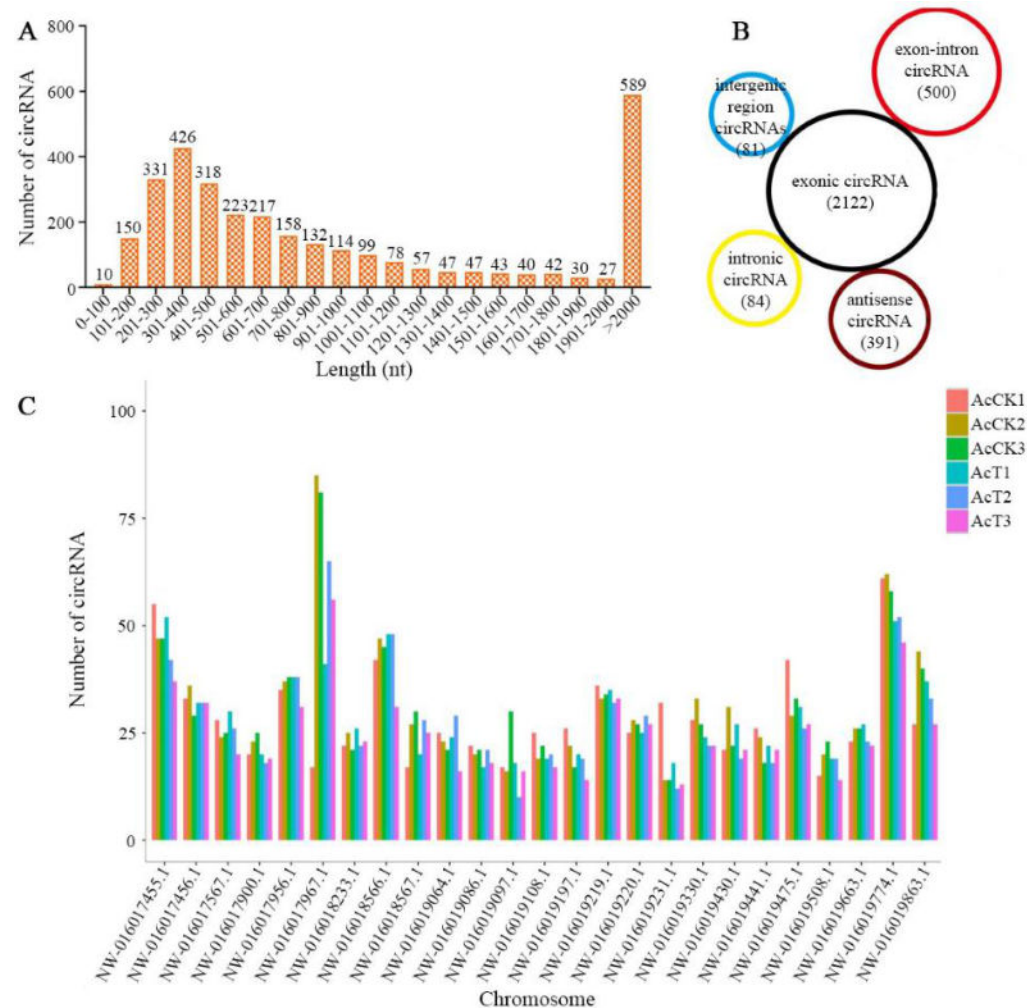
First discovery of circRNAs in *Ascospaera apis* and *Nosema ceranae*



(Curr. Microbiol., 2018; Gene, 2018)

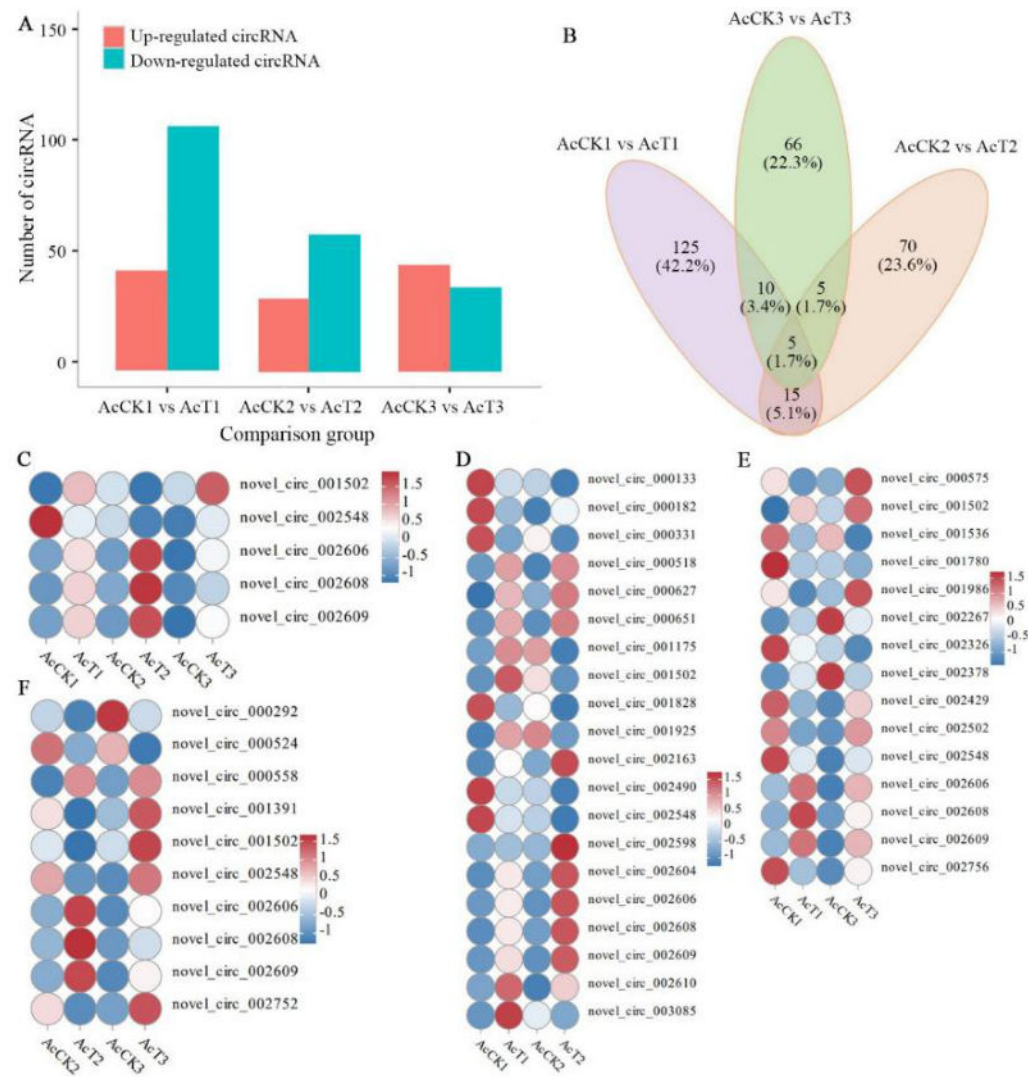
Research contents

1. Characterization and expression profile of circRNAs in the host guts



- **3178 circRNAs**
- **15~96007 nt**
- **301~400 nt, most abundant**
- **5 types**
- **NW_016019774.1 (AcCK1)**
NW_016017967.1 (AcCK2, AcCK3)
NW_016017455.1 (AcT1)
NW_016017967.1 (AcT2, AcT3)

Fig. 1 Number, type, and chromosome distribution of *A. cerana* circRNAs



- **155 DEcircRNAs (4-day-old)**
- **95 DEcircRNAs (5-day-old)**
- **86 DEcircRNAs (6-day-old)**
- **4 up- and 1 down-regulated circRNAs shared**

Fig. 2 Differential expression pattern of circRNAs in the larval guts infected by *A. apis* infection

2. Investigation of parental genes of DEcircRNAs

- **26 terms and 144 pathways (4-day-old)**
- **20 terms and 114 pathways (5-day-old)**
- **17 terms and 61 pathways (6-day-old)**
- **MAPK signaling pathway**

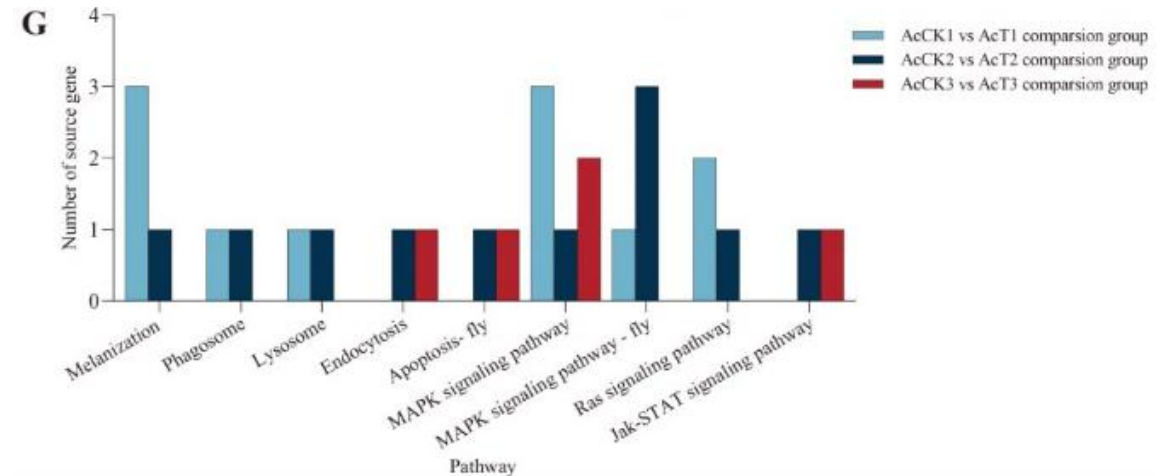
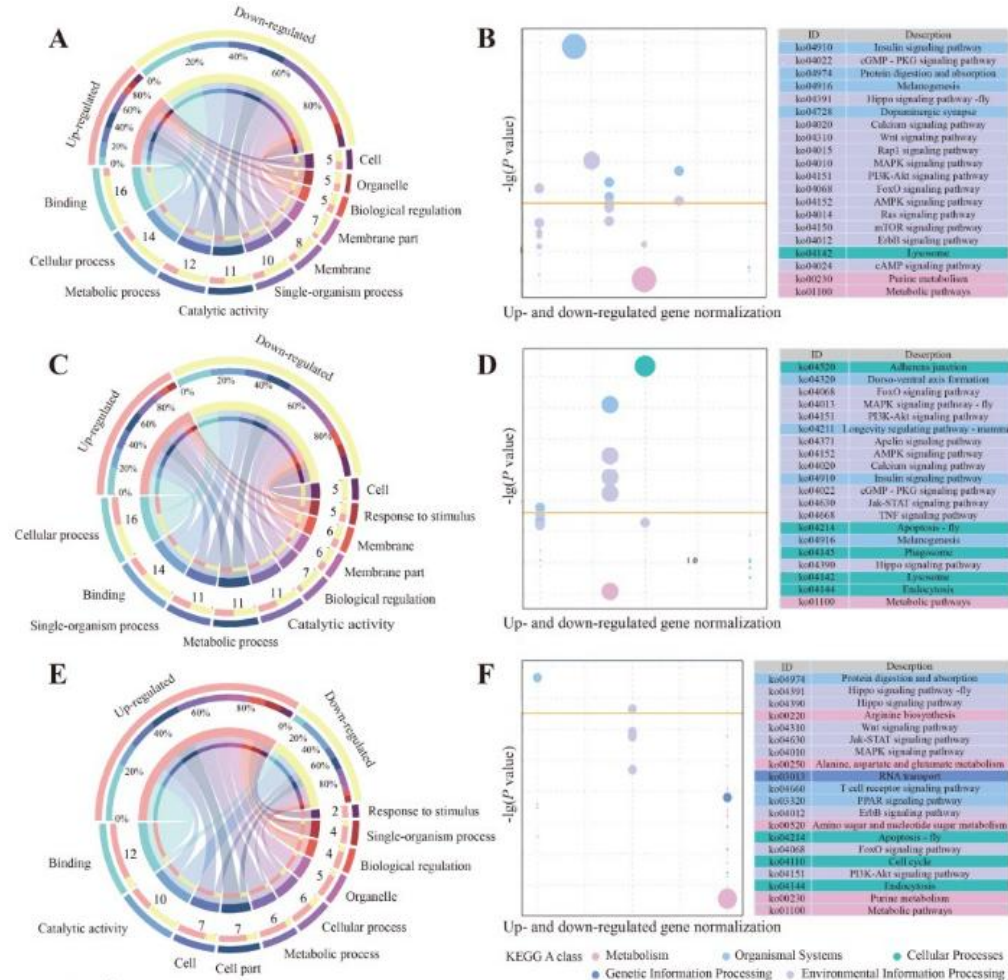


Fig. 3 Annotation of parental genes of DEcircRNAs in three comparison groups

- **41 DEcircRNAs/9 DEmiRNAs/760 DErnRNAs (4-day-old)**
- **31/26/4464 (5-day-old)**
- **59/54/5015 (6-day-old)**
- **One DEcircRNA target multiple DEmiRNAs (circ_002084, etc.)**
- **One DEmiRNA targeted by several DEcircRNAs (miR-1277-x, etc.)**

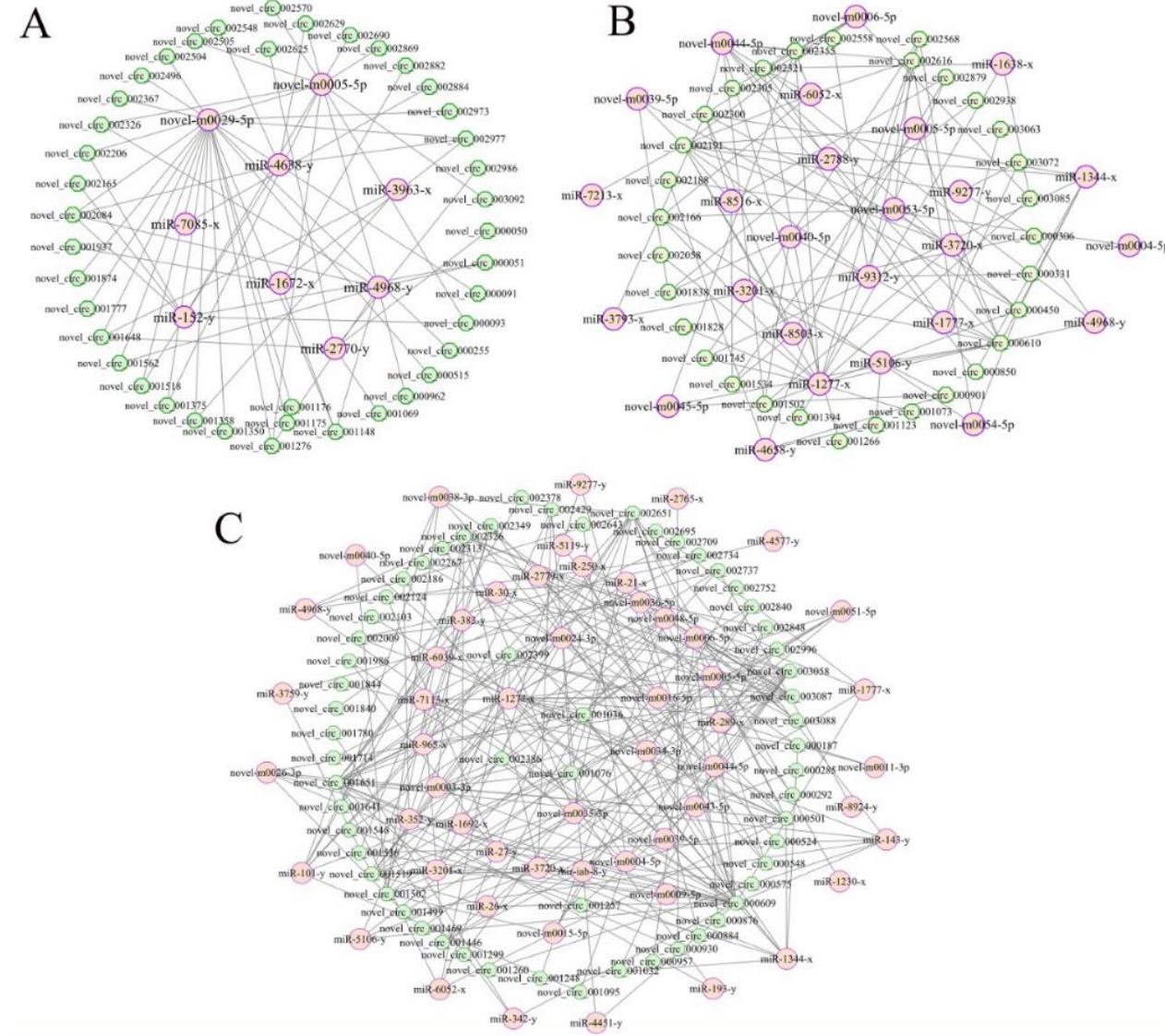


Fig. 4 Regulatory networks between DEcircRNAs and DEmiRNAs

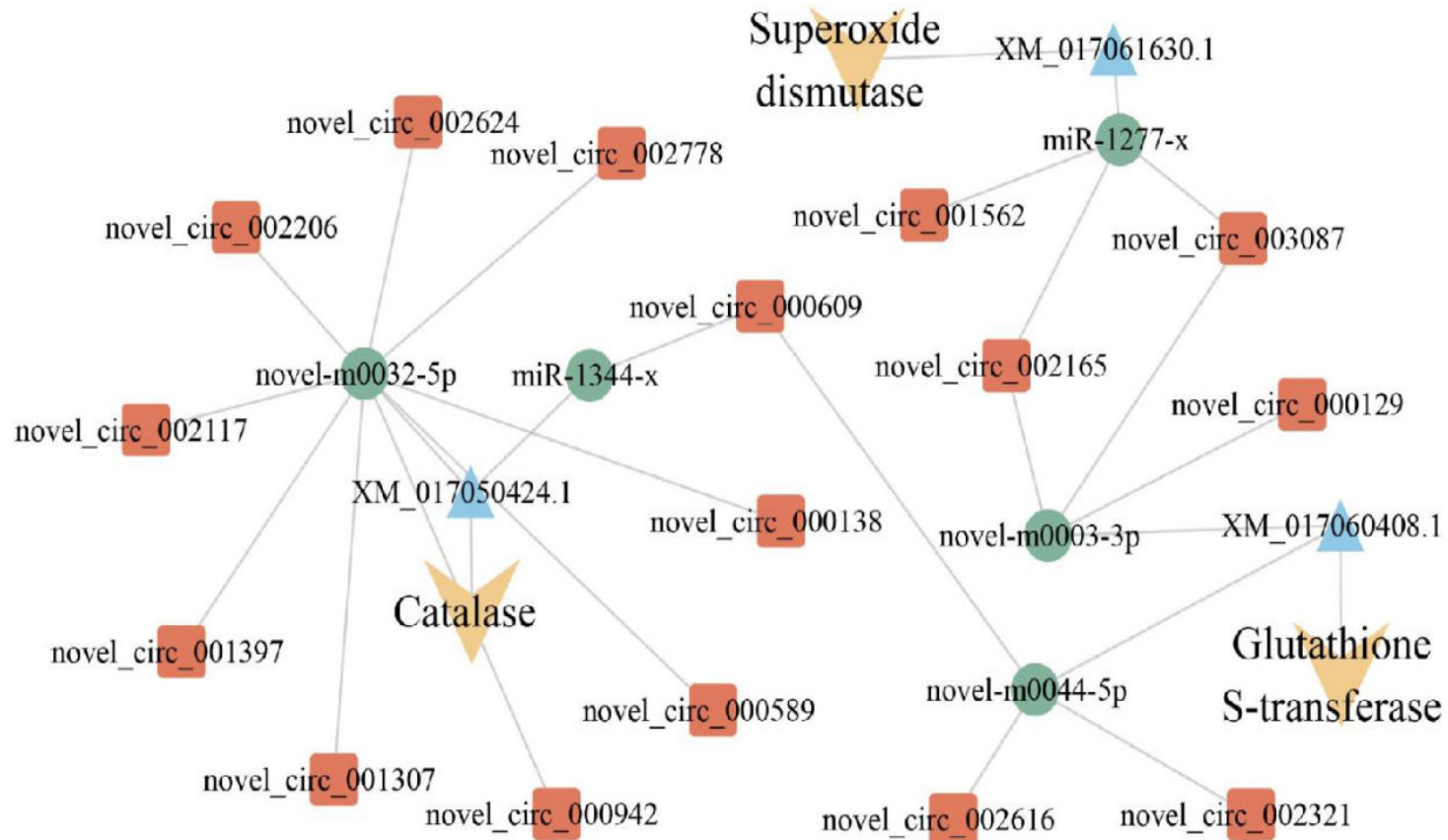
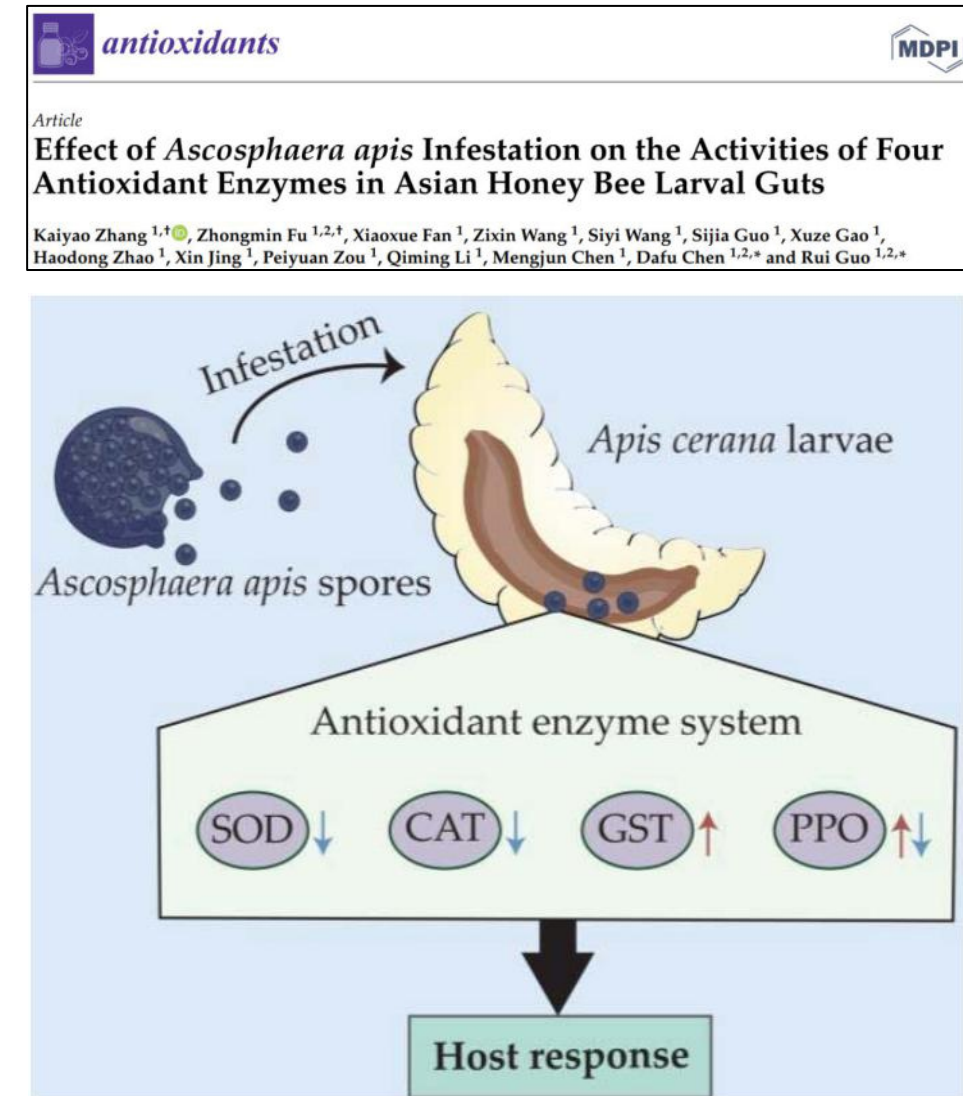


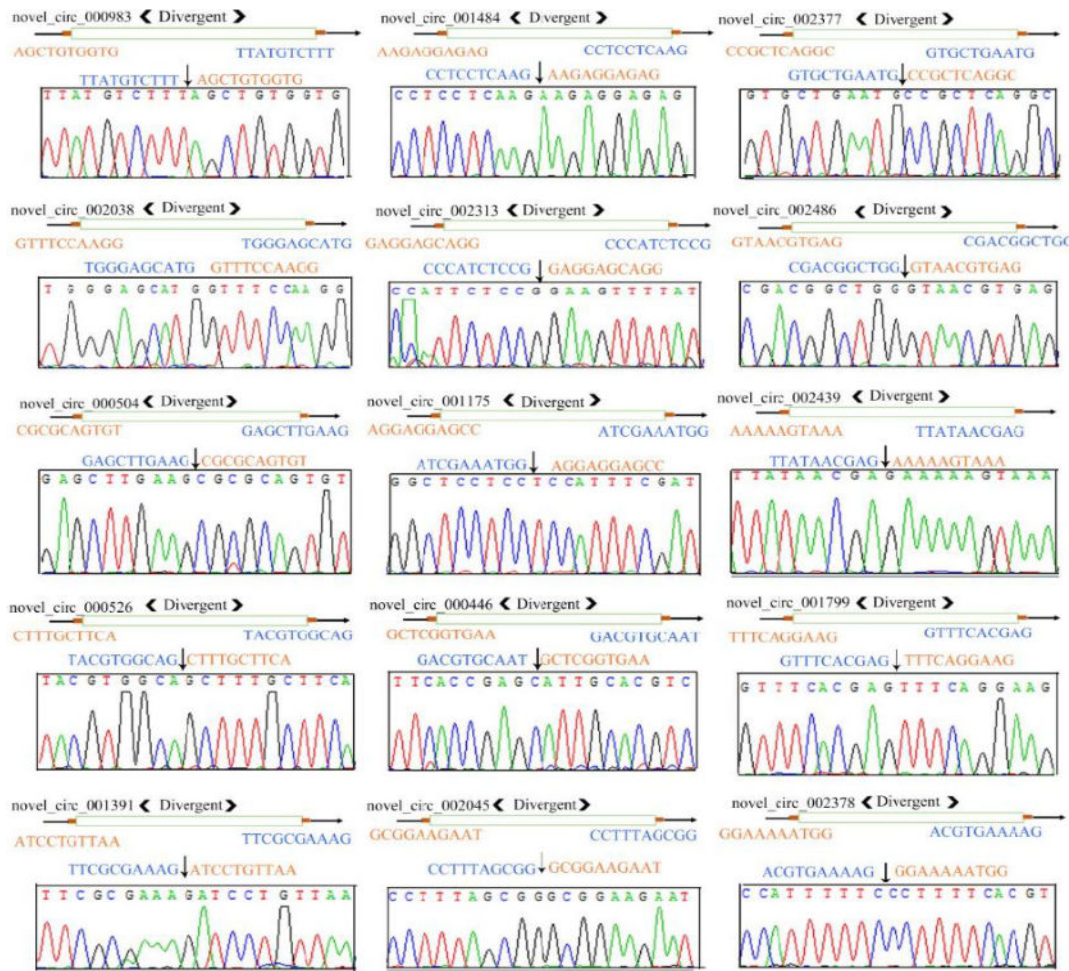
Fig. 5 CeRNA regulatory networks of antioxidant enzyme-associated DEcircRNAs



(Antioxidants, 2023)

3. Molecular validation of DEcircRNAs in the host guts

● BS sites were authentic



● Omics data were reliable

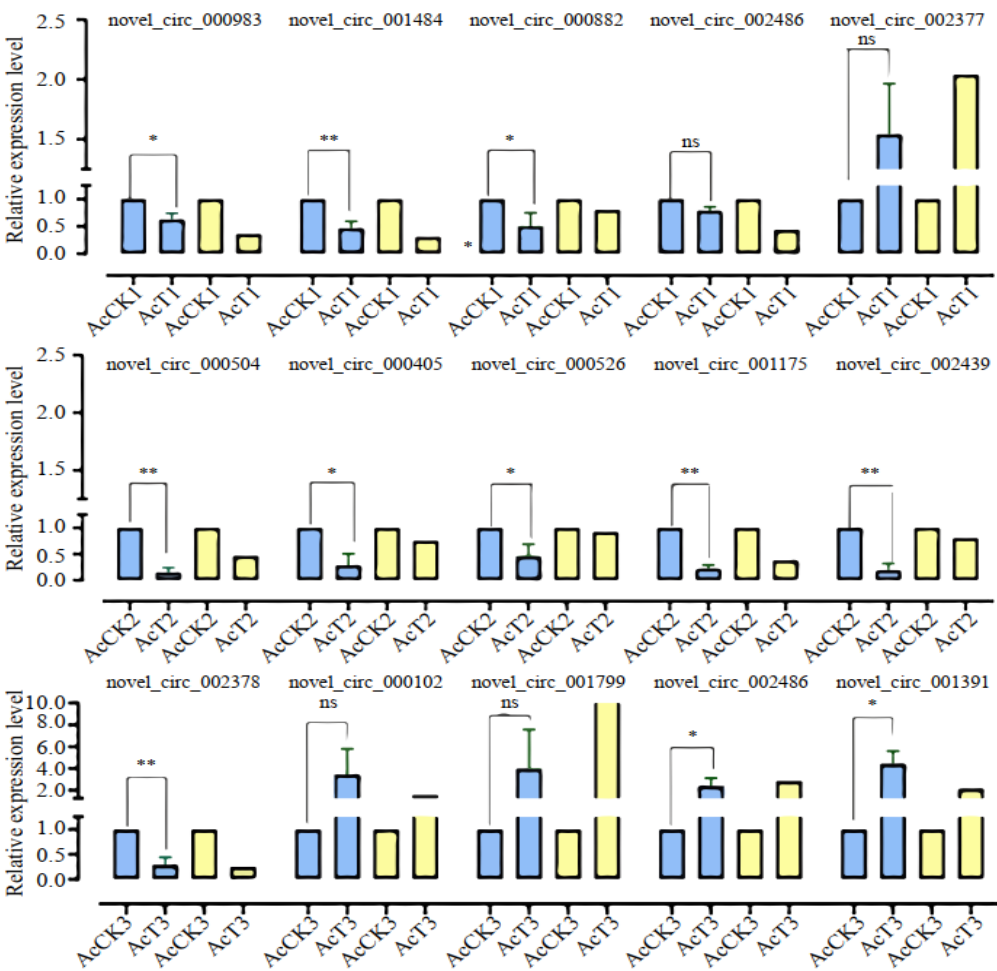


Fig. 6 Verification of DEcircRNAs by PCR and RT-qPCR

4. Molecular verification and expression pattern of ac2651 in the host guts

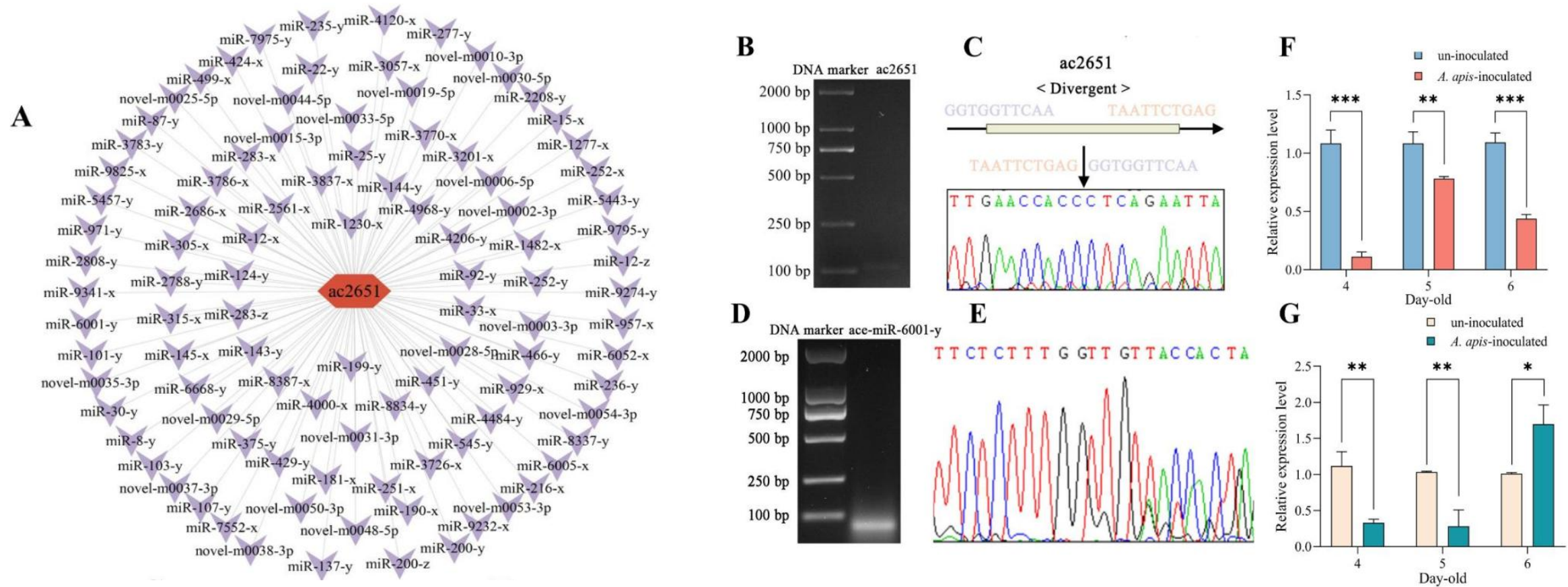
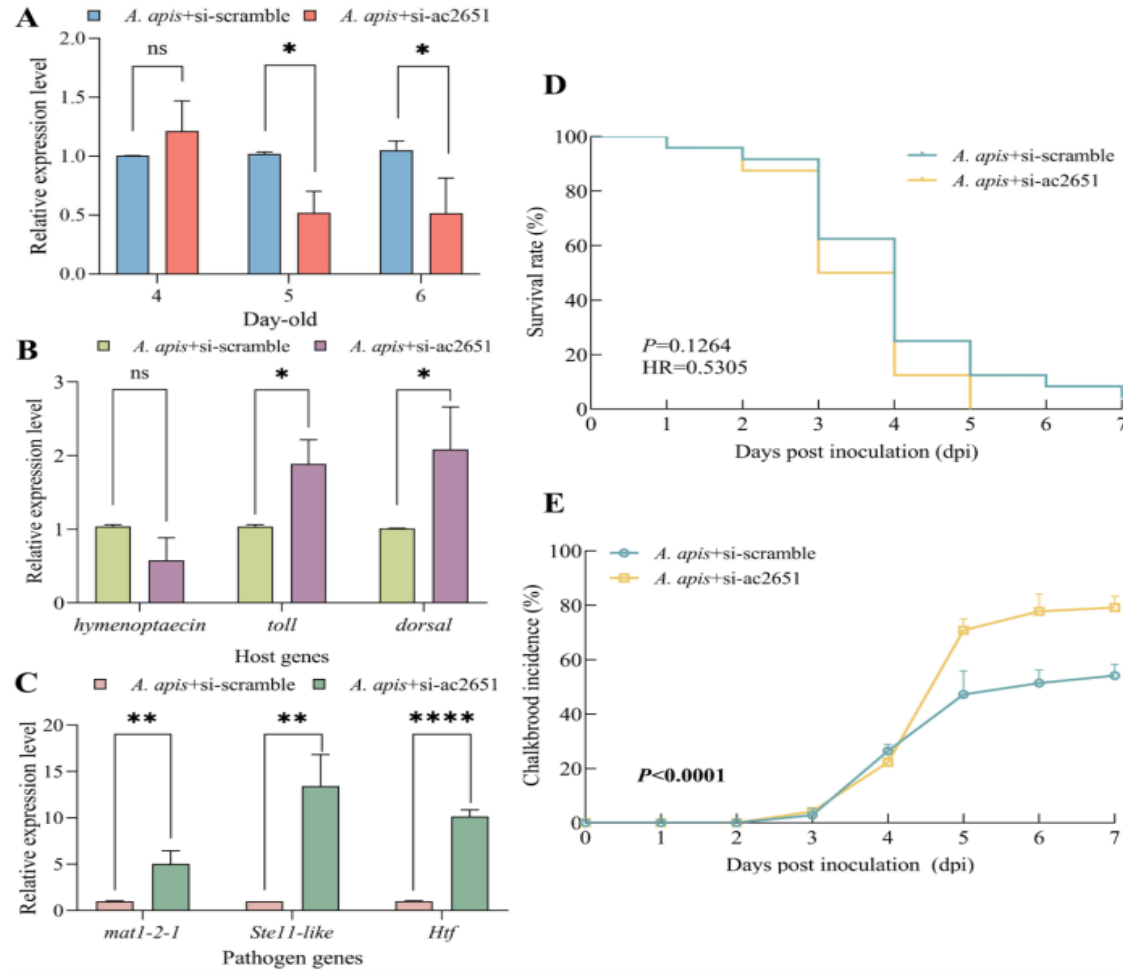


Fig. 7 Regulatory network, molecular validation, and expression profile of *ac2651* in the guts of *A. cerana* worker larvae inoculated with *A. apis*

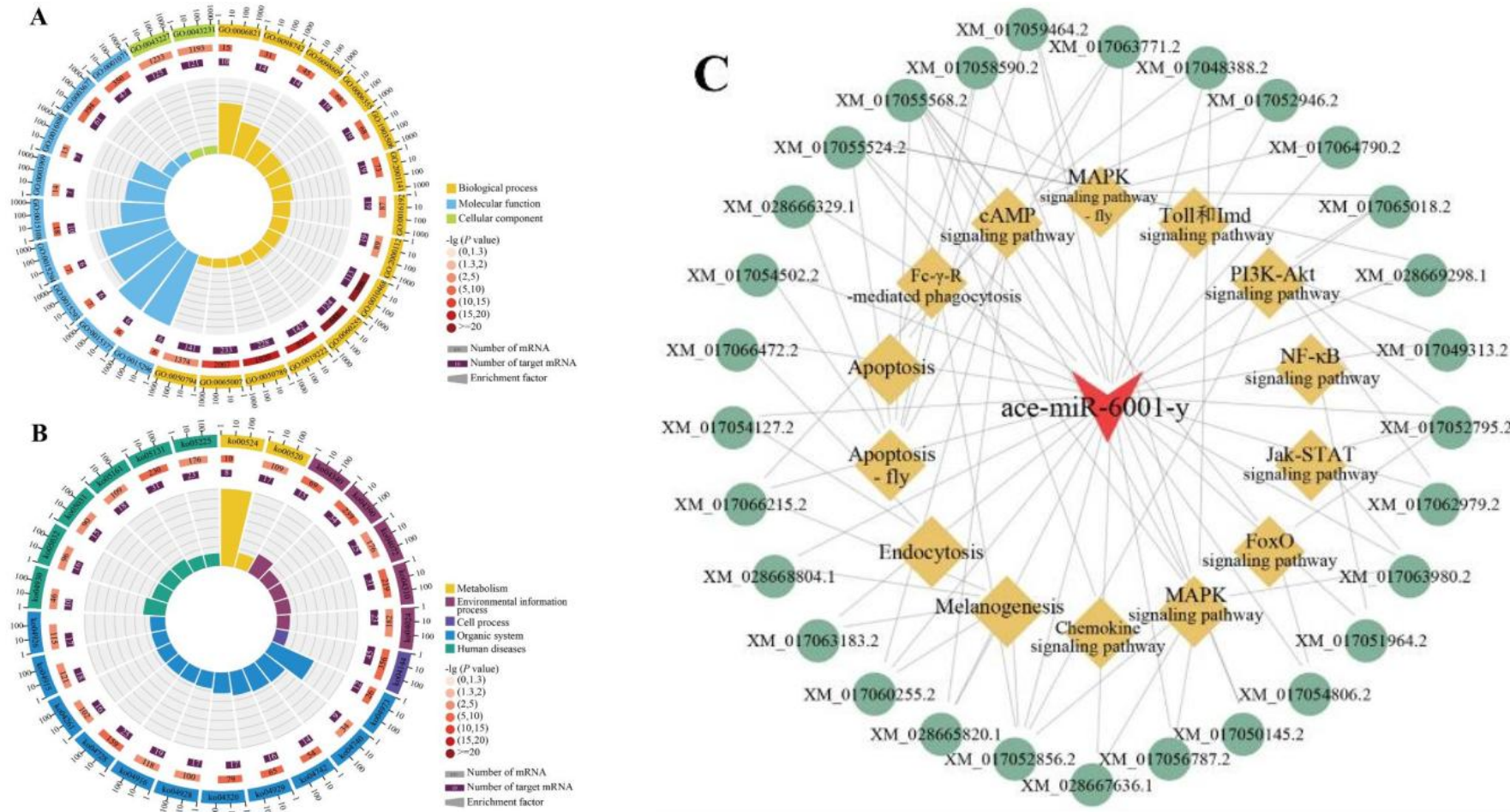
5. Silencing *ac2651* affected host immune response, survival, and chalkbrood incidence



- Effective silencing of *ac2651*
53.60% (5-day-old)
48.30% (6-day-old)
- Activation of host immune genes (*toll* and *dorsal*)
- Upregulation of fungal genes (*mat1-2-1*, *Ste11-like*, and *Htf*)
- Decreased host survival rate
- Significantly elevated host chalkbrood incidence

Fig. 8 Effect of *ac2651* silencing on the *A. cerana* immune gene expression, survival, and chalkbrood incidence as well as and the expression of proliferation-associated genes in the *A. apis*

5. Regulatory network and functional dissection of ace-miR-6001-y



- **37 terms**
- **37 pathways**
- **5 cellular immune pathways**
- **9 humoral immune pathways**

Fig. 9 Functional term and pathway annotations of mRNAs targeted by ace-miR-6001-y

- Effective overexpression and knockdown
- **Negatively regulates** host **dorsal** gene and fungal **Stell-like** gene

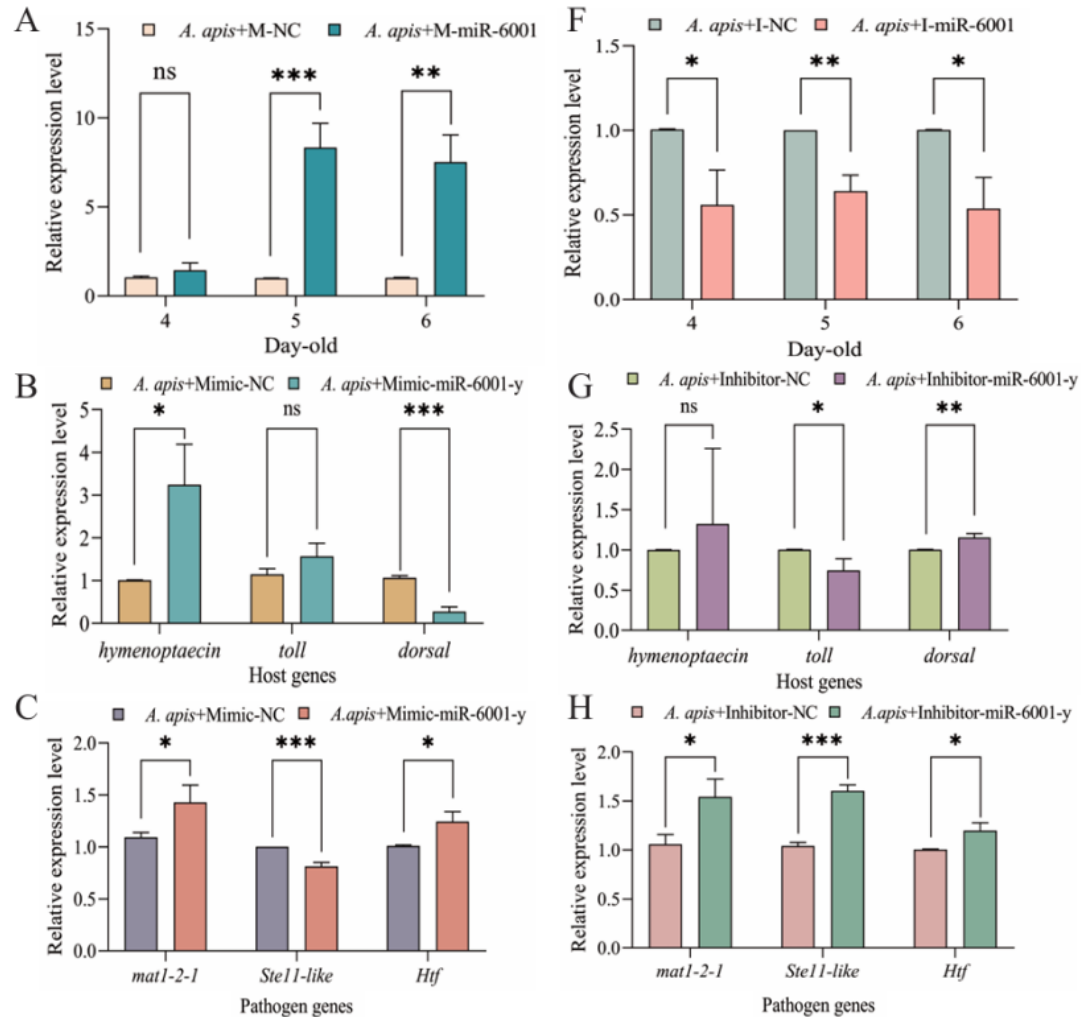


Fig. 10 Functional term and pathway annotations of mRNAs targeted by ace-miR-6001-y

● *ace-miR-6001-y* directly targeted both *ac2651* and *Ac14-3-3ζ*

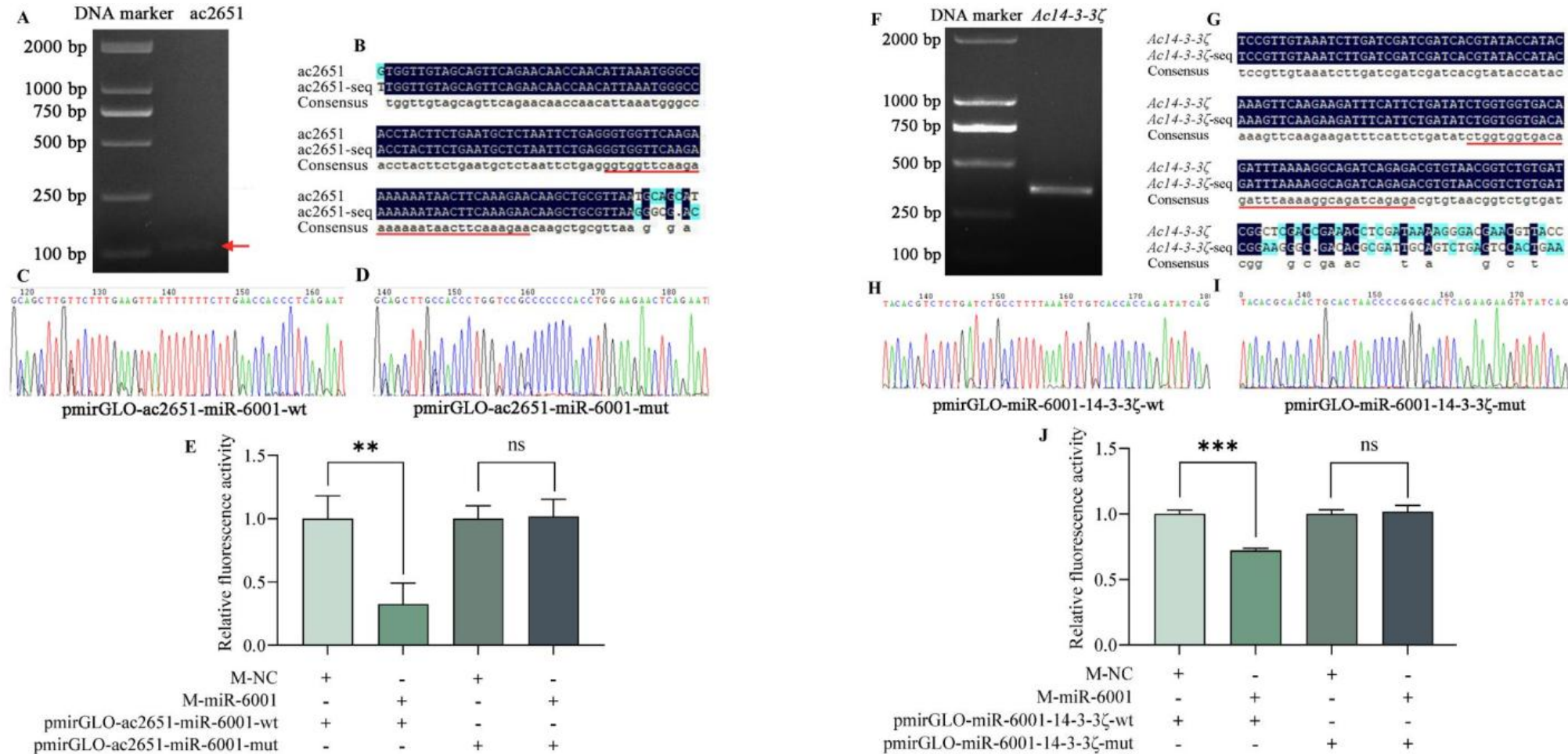
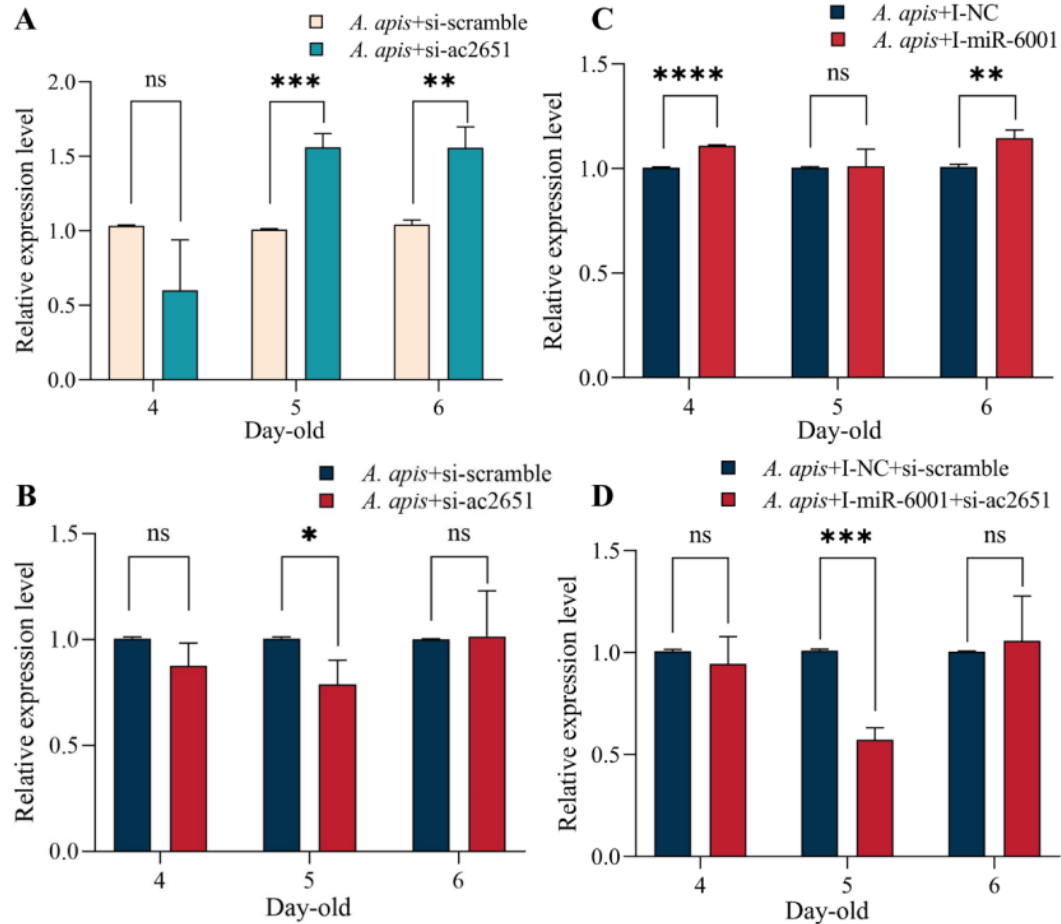


Fig. 11 Dual-luciferase reporter assay for the targeting relationships between *ac2651* with *ace-miR-6001-y* and between *ace-miR-6001-y* with *Ac14-3-3ζ*

6. *ac2651*, *ace-miR-6001-y*, and *Ac14-3-3ζ* formed a ceRNA network



● *ac2651* silencing

- *ace-miR-6001-y* up-regulated ↑
- *Ac14-3-3ζ* down-regulated ↓

● *ace-miR-6001-y* knockdown

- *Ac14-3-3ζ* up-regulated ↓

● *ac2651* silencing and *ace-miR-6001-y* knockdown

- *Ac14-3-3ζ* significantly down-regulated ↓

Fig. 12 RT-qPCR detection of relative expression levels of *ac2651*, *ace-miR-6001-y*, and *Ac14-3-3ζ*

7. Simultaneous silencing of ac2651 and knockdown of ace-miR-6001-y significantly enhanced the host immune response

- Host **immune genes** significantly increased ↑
- Fungal **mat1-2-1** and **Ste11-like** significantly increased ↑
- Fungal **Htf** significantly reduced ↓
- Host **chalkbrood incidence** significantly increased ↑

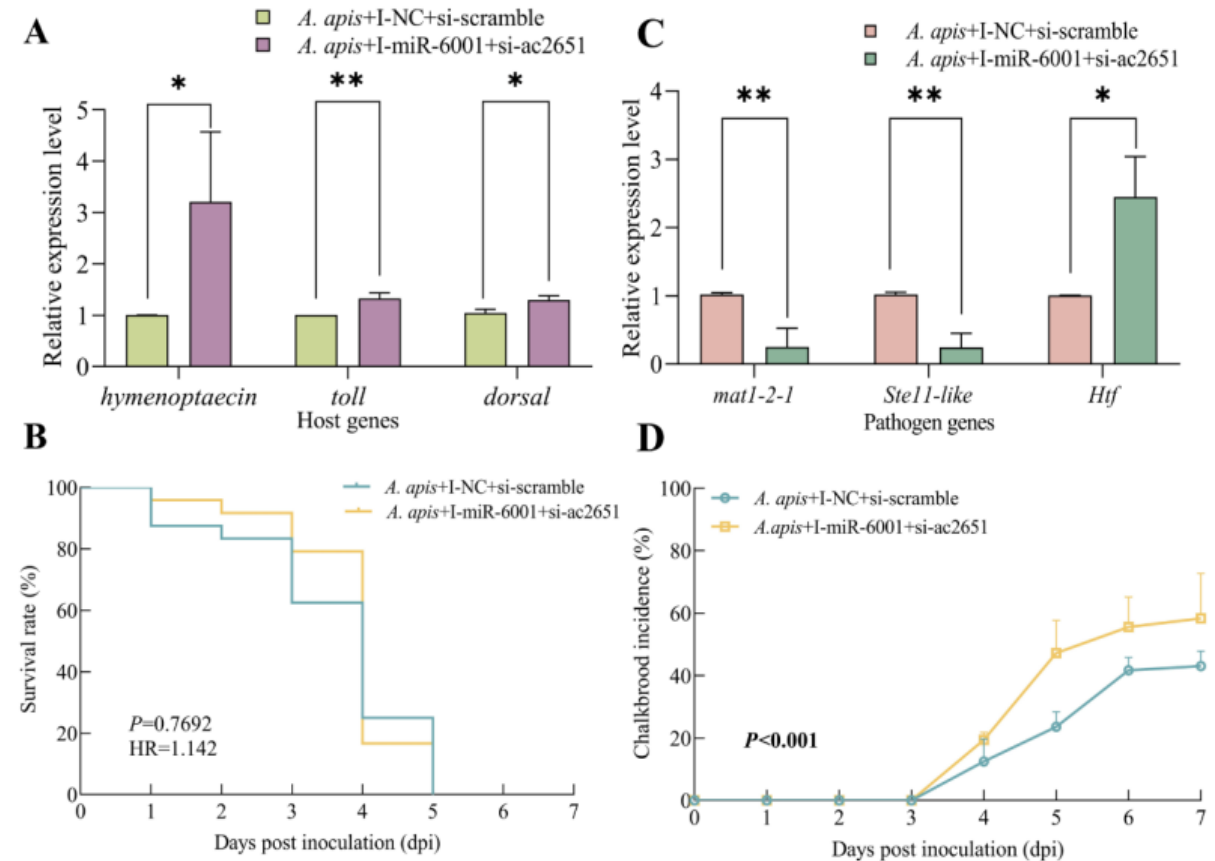


Fig. 13 Effect of simultaneous silencing of ac2651 and knockdown of ace-miR-6001-y on host immune response, survival, and chalkbrood disease

Conclusions

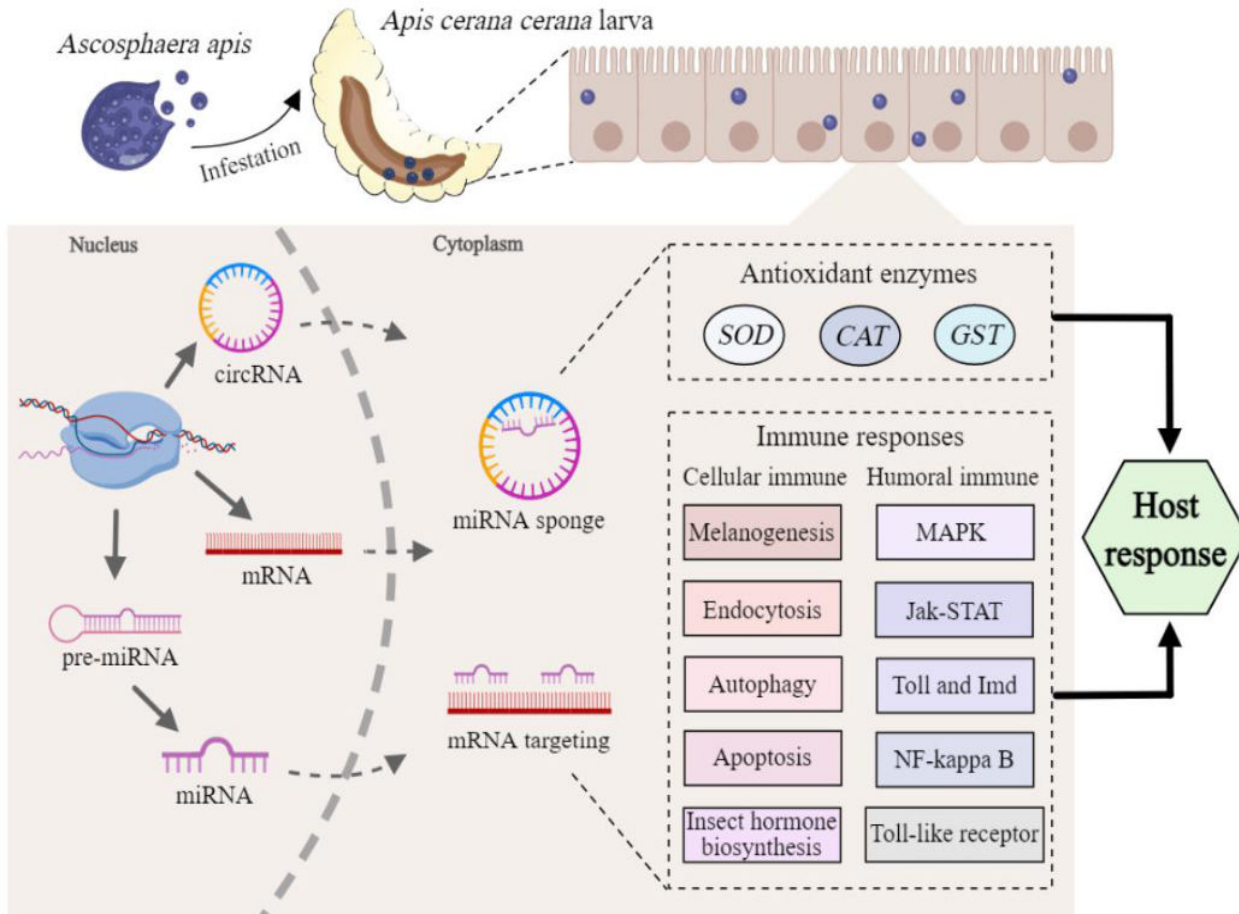
1. 3178 circRNAs were identified in the midgut tissues of *A. cerana* worker larvae
2. The *A. apis* invasion altered the expression profile of circRNAs in larval guts
3. DEcircRNAs may modulate host immune response by versatile manners
 - Regulation of parental gene transcription
 - Absorption of target miRNAs via ceRNA networks
 - Translation into proteins as templates

4. **ac2651** targets **ace-miR-6001-y** as a “**molecular sponge**”, directly controlling the **Ac14-3-3ζ** expression

5. **ac2651** **indirectly modulates** the expression of host immune genes (***hymenoptaecin***, ***toll***, and ***dorsal***) as well as fungal proliferation-associated genes (***mat1-2-1***, ***Ste11-like***, and ***Htf***)

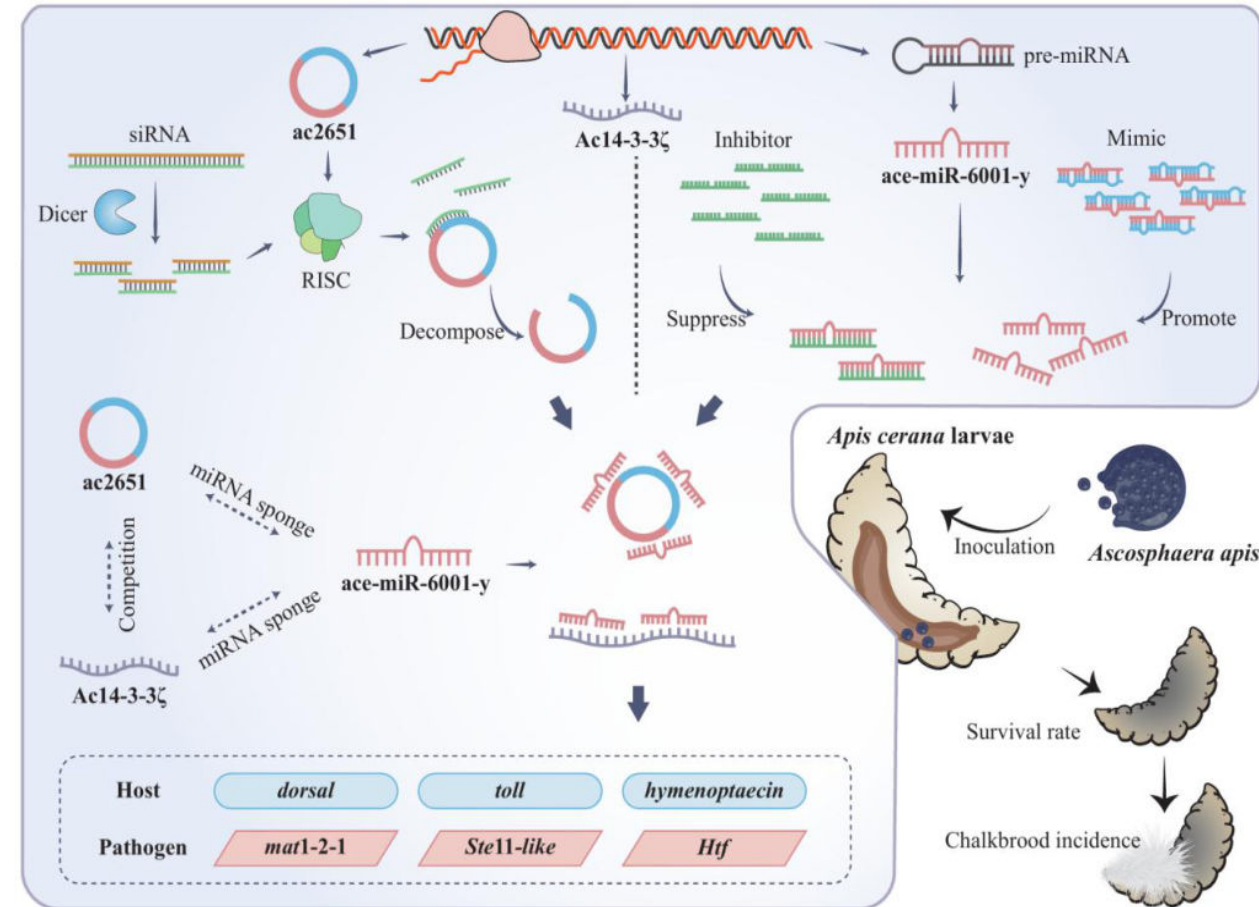
6. **ac2651/ace-miR-6001-y/Ac14-3-3ζ axis** is a vital **ceRNA network** engaged in the regulation of host immune defense against fungal invasion

Omics level



(Appl. Microbiol. Biot., 2024)

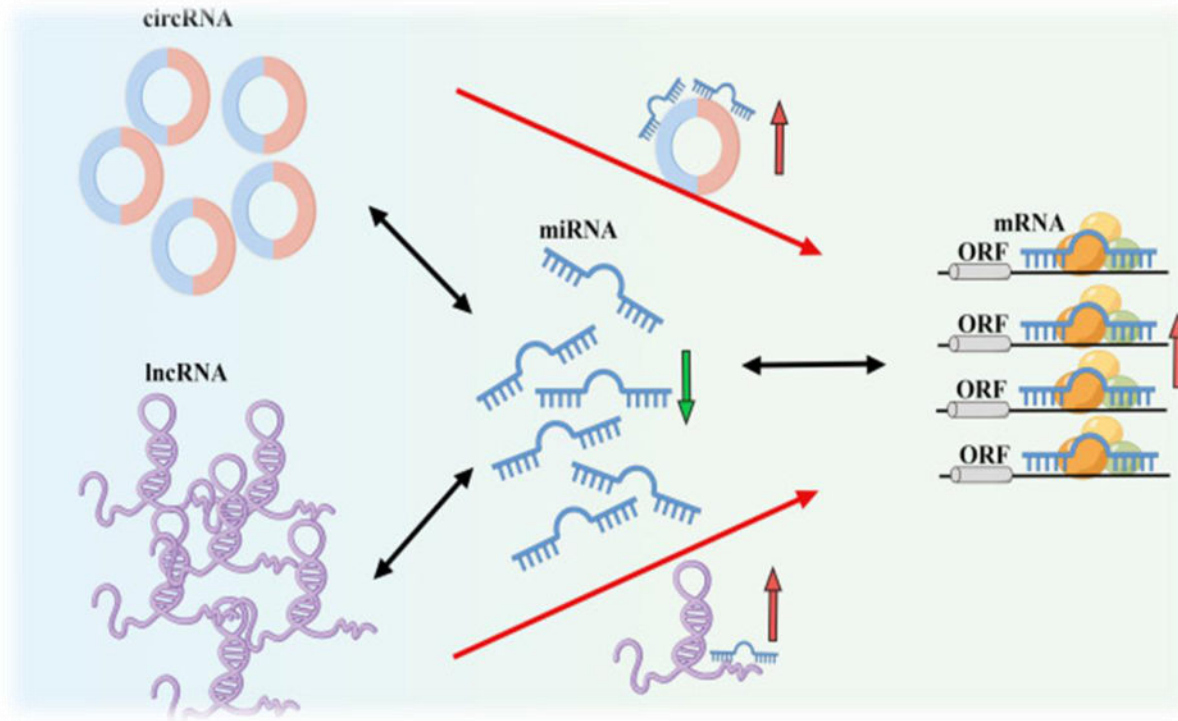
Molecular level



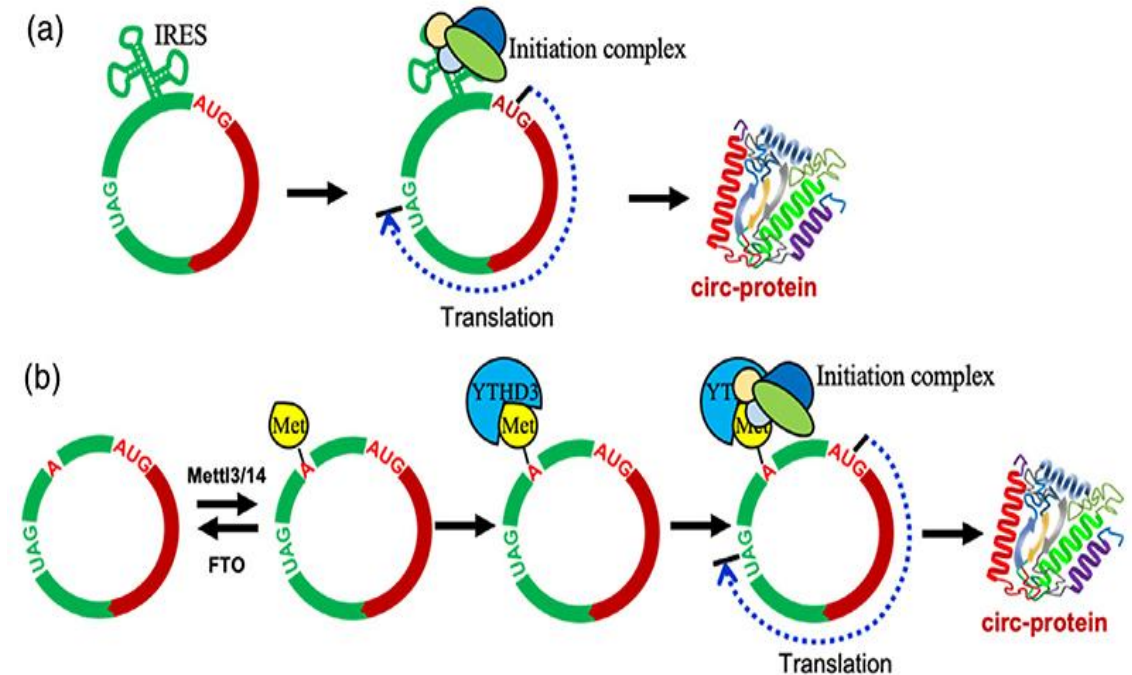
Submitted to the Communications Biology
(Under Review)

Future direction

1. **Whether** and **how** DEcircRNAs regulate host immune response to *A. apis* invasion via **translation** into proteins ?

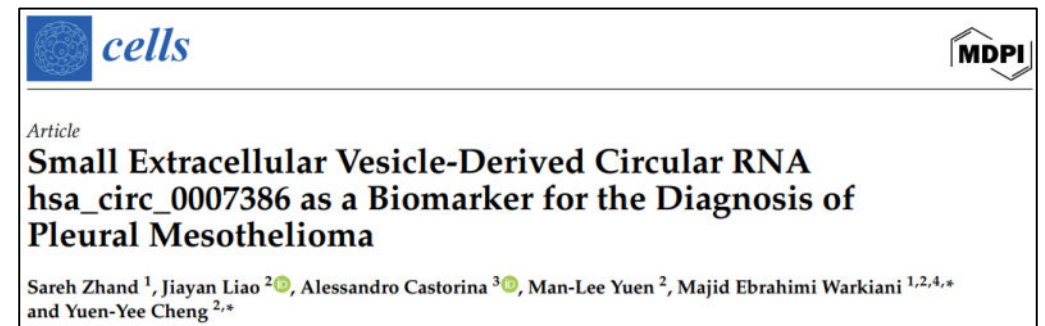
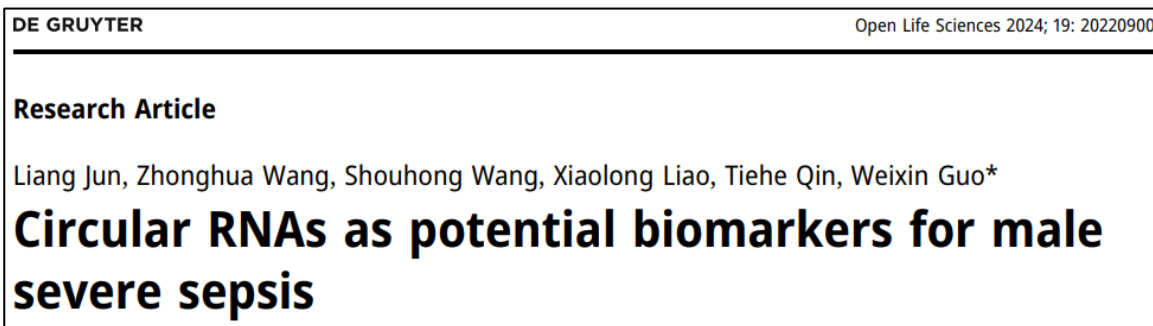
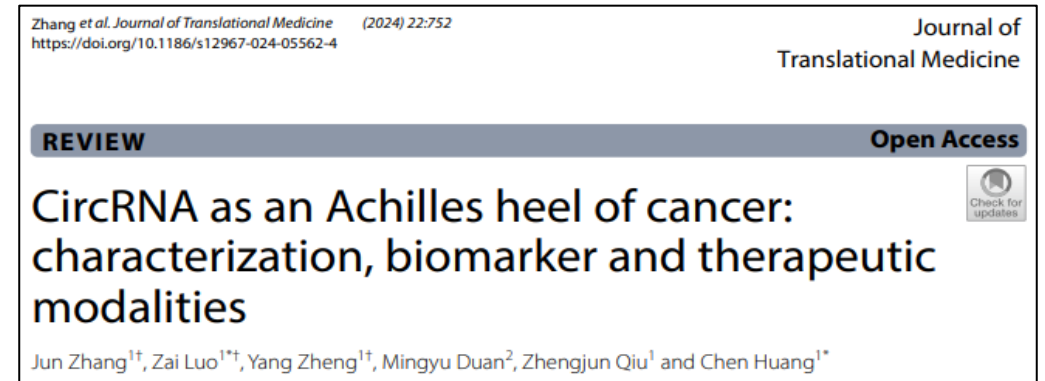


(Peer J., 2023)



(Wiley Interdiscip. Rev RNA., 2022)

2. High-throughput development of novel biomarkers for diagnosis and new targets for control of chalkbrood disease





Acknowledgement

Host—Pathogen Interaction Mechanism and Precision Medicine



Prof. Dafu Chen

Prof. Guojun Xu

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Associate Prof. Zhongmin Fu

Associate Prof. Qingwei Tan

Dr. Jianfeng Qiu

Dr. Xiaoxue Fan

Dr. He Zang

Dr. Kaiyao Zhang

Dr. Chenyan Wang

A total of 33 postgraduates