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ORIGINAL PAPER

microRNAs shape social immunity: a potential target for biological control of the termite *Reticulitermes chinensis*

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Abstract
Eusocial insects can employ various behavioural and physiological disease defences to avoid, resist and tolerate pathogen infections in their closely related and packed colonies, termed social immunity. Recent studies have shown that several molecules serve insect social immunity, including chemical odours, insect venoms, immune-related proteins, etc. However, whether and how microRNAs (miRNAs), whose precursors are processed by *Dicer-1*, drive social immunity in insect colonies is still unknown. Here, we used a 'host–pathogen' system (host: *Reticulitermes chinensis*; pathogen: *Metarhizium anisopliae*) to explore the impact of miRNAs on social immunity in termite colonies. We found that RNAi-mediated silencing of *Dicer-1* led to decreased miRNA concentration, significantly inhibited carbohydrate and energy metabolism and affected other life processes, such as the immune response and oxidation–reduction reactions, in whole body of the termite. In behavioural defence, silencing *Dicer-1* significantly diminished defensive social behaviours such as locomotion, grooming, cannibalism and burial in termite groups when encountering fungal contamination. In physiological defence, *Dicer-1* silencing and miR-71-5 stimulation resulted in significantly decreased antifungal activities of termites. Furthermore, both *Dicer-1*-silenced and miR-71-5 stimulant-treated termite groups exhibited a high level of mortality during fungal contamination. Our findings demonstrated the important role of miRNAs in shaping social immunity in termite colonies, providing insights necessary to understand the potential mechanisms underlying behavioural and physiological disease defences in insects and hence laying the groundwork for miRNA-based pest control.

Keywords Eusocial insect · Entomopathogenic fungi · Disease defences · Dicer-1 · miR-71-5

Key message

- miRNAs mediated gene expressions related to social immune systems.
- miRNAs affected the behavioural disease defences in termite groups.
- miRNAs influenced the physiological disease defence in termite individuals.
- miRNA-based termiticides could be considered a novel accesses to biological pest control.

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Introduction

Termites are economically important pests, costing 30 billion dollars worldwide, imposing global damage to crops, trees, wooden structures, and cellulose materials. Chemical pesticides provide an effective control avenue for termite pests, but they also create serious problems, threatening human health and the environment. Biological control is a valuable alternative that can address these problems. Several entomopathogens, such as *Metarhizium* and *Beauveria,* have been successfully applied for biological pest control, which

is reasonable and effective (Verma et al. 2009; Kumar and Upadhyay 2021). However, the biological control of termites is unsatisfactory, mainly due to the unique disease defences in termite colonies.

Termites, being eusocial insects, have evolved collective behavioural and individual physiological defences to counteract the high risk of the spread of infectious pathogens among closely packed and closely related nestmates (termed 'social immunity') (Van Meyel et al. 2018; Liu et al. 2019a). It is well documented that termites deploy a wide repertoire of behavioural defences: avoidance is the first line of defence and helps to prevent termites from entering polluted areas (Yanagawa et al. 2015); alarm behaviour is exhibited with rapid longitudinal oscillatory movement to warn nestmates about the presence of pathogens (Rosengaus et al. 1999); grooming towards pathogen-contaminated nestmates can efficiently remove pathogens from nestmate cuticles (Liu et al. 2019b); burial and cannibalistic behaviours limit pathogen transmission from corpses to susceptible nestmates (Sun et al. 2016). Physiological responses such as immune responses and oxidation–reduction reactions also serve termite disease defence. Immune responses include humoral immunity-mediated antimicrobial peptides (AMPs) and cellular immunity-mediated phagocytosis and encapsulation, which are able to inhibit the replication and dissemination of pathogens in the insect body cavity (Hussain et al. 2013; Liu et al. 2015; Lopez-Uribe et al. 2016; Hong et al. 2018). Oxidation–reduction reactions mitigate the damage caused by toxins and reactive oxygen species (ROS) during pathogen infections, contributing to insect tolerance (Liu et al. 2015; Zhao et al. 2020; Zhou et al. 2021). In addition, previous studies on termite disease defences have shown that some gut symbionts were an important component of the host physiological defence (Rosengaus et al. 2014). Some AMPs could be employed as external disinfectants on the cuticle surface and in nest materials when in conjunction with behaviours such as grooming and nesting (Bulmer et al. 2009; Hamilton and Bulmer 2012). Therefore, weakening termite behavioural and physiological defences may be the key step to enhance the biocontrol effect of termites.

The molecular basis of social immunity has been reported in a growing number of studies. Several molecules, including chemical odours, external secretions, antibiotics, immune proteins, and chemoreceptors serving insect behavioural and physiological defences, have been identified by multiple omics techniques and gas/liquid chromatography–mass spectrometry (Seipke et al. 2011; Hussain et al. 2013; Terrapon et al. 2014; Liu et al. 2015; Sun et al. 2016; He et al. 2018). In termite groups, musty odour from pathogens induces enhanced grooming behaviour (Yanagawa et al. 2011). Death cues from termite corpses induce either cannibalism or burial behaviours (Sun et al. 2016). External secretions from the frontal and salivary glands and beneficial microorganisms-mediated antimicrobial activity help termites protect themselves, nestmates and even colonies (Bulmer et al. 2009; Rosengaus et al. 2000, 2014; Chouvenc et al. 2013). For the genetic and biochemical mechanisms driving insect social immunity, RNAi and gene editing technologies could be used. In termites, RNAi-mediated silencing of the *termicin*, *gram-negative binding protein 2* (*GNBP2*), *selenium-binding protein*, and *transglutaminase* (*TG*) genes has been demonstrated to significantly decrease the antifungal activity of termites and hence increase infection mortality (Hamilton and Bulmer 2012; Zhao et al. 2020; Zhou et al. 2021). *Isocitrate dehydrogenase* (*IDH*) could influence termite metabolism, and its dysregulation induces increased apoptotic lesions, leading to high levels of infections and mortalities (Liu et al. 2020). In addition, *GNBP2* and *TG* also serve the behavioural defence of termites, influencing cannibalistic and grooming behaviours, respectively (Zhao et al. 2020; Esparza-Mora et al. 2020). However, the molecular basis of social immunity has yet to be clearly understood, especially the molecular mechanism driving the complicated social behaviours in response to infectious pathogens. Furthermore, the genetic and biochemical mechanism of social immunity focuses on the coding RNA driving insect social immunity, but whether noncoding RNAs such as miRNAs take part in the regulation of insect social immunity is still unclear.

Dicer-1, an RNase III endonuclease, is essential for the final step of miRNA biosynthesis. miRNAs are endogenous, 18–25 nt, noncoding RNAs that negatively regulate gene expression at the posttranscriptional level by base pairing between the seed region in miRNAs and the matched region in target mRNAs (Gomez-Orte and Belles 2009; Wang et al. 2013; Yang et al. 2014; Lucas et al. 2015). As miRNA production depends on Dicer-1, the functions of miRNAs in modulating the biological processes of insects can be studied by functional losses of Dicer-1. By using *Dicer-1* mutants of fruit flies, miRNAs have been proven to function in embryogenesis and olfactory neuron morphogenesis (Lee et al. 2004; Berdnik et al. 2008). By RNAi-mediated silencing of *Dicer-1*, researchers have verified that miRNAs play a key role in regulating developmental processes in insect metamorphosis, such as those in cockroaches and locusts (Gomez-Orte et al. 2009; Wang et al. 2013). Moreover, miRNAs are also involved in modulating insect behaviours. miR-8 and miR-429 regulate virus-induced climbing behaviour in cotton bollworms by directly controlling *BrZ2* expression (Zhang et al. 2018). miR-133 regulates dopamine synthesis and hence inhibits behavioural aggregation in the locust (Yang et al. 2014). Based on such evidence for the function of miRNAs in insect physiology and behaviour, we aimed to determine the effect of miRNAs on termite physiology and behaviour involved in social immunity by using a 'host–pathogen' system (host: *Reticulitermes*

chinensis; pathogen: *Metarhizium anisopliae*). Our study mainly included three aspects of the research on social immunity: the effect of miRNAs on (1) the in vivo molecules driving social immunity; (2) the behavioural defensive response, such as locomotion in fungus-contaminated areas, grooming towards fungus-contaminated nestmates and cannibalism/burial towards fungus-contaminated corpses; and (3) the physiological defence response, such as antifungal activity. Furthermore, we tested the effect of miRNA dysregulation on the mortality of fungus-contaminated termites to evaluate the effect of miRNAs on biological control of the termites. Our study revealed the potential mechanism underlying behavioural and physiological disease defences and novel targets for pest control.

Material and methods

Termites

All experimental termites in our study were workers of the termite *R. chinensis*. They were collected from Shizi Hill in Wuhan City, Hubei Province, China. Termites were reared in plastic containers (40 × 20 × 20 cm) containing small pine blocks in the laboratory at a temperature of 25 ± 1 °C, 80% relative humidity, and 24 h of darkness.

Entomopathogens, contaminated termites and corpses

The entomopathogenic fungus *M. anisopliae* (strain IBCCM321.93) was cultivated on potato dextrose agar (PDA) for 2 weeks at 25 ± 1 °C, 80% relative humidity, and 24 h of darkness. The fungal conidia were suspended using 1% Tween 80 and stored at a temperature of 4 °C for a maximum of 3–4 weeks. Before each experiment, the conidial suspension was tested for germination. We found that the conidial suspension used for contaminating termites had a germination rate of more than 90%.

To prepare fungus-contaminated termites, the abdomens of termites were contaminated by a 0.3 µL droplet of conidial suspension (10^8 conidia/mL) using a pipette (0.1–2.5 µL, Transferpette). Then, termites contaminated with the fungus were immediately refrigerated at 4 °C for one hour to inhibit their movement to precipitate fungal conidia on their cuticles (Liu et al. 2015).

To prepare fungus-contaminated corpses, termites were collected in a 1.5 mL centrifuge tube and then frozen in liquid nitrogen for 20 s to kill the termites. The corpses were immersed in the conidial suspension (10^8 conidia/mL) and then placed in a sterile petri dish with moist filter paper for 0 and 2 d of cultivation (Sun et al. 2016).

Synthesis of dsRNA and the miRNA simulant

Dicer-1 fragment (1697 bp; Supplementary material 1-Text S1) was amplified by specific primers (forward: 5′-CTG CGA CAG ATC ATT GCA CG-3′; reverse: 5′-CAC TGG CTG TTT TGG CAC TC-3′), and their PCR products were purified using the AxyPrepTM DNA Gel Extraction Kit (Axygen Scientific, USA), cloned into the pMD 18-T vector (TaKaRa, Japan) and then transformed using a DH5α chemically competent cell (Tsingke Biotechnology, China). A single colony from lysogeny broth (LB) medium with ampicillin was sent to Tsingke Biotechnology Co. Ltd. for sequencing. The sequence alignment showed that our fragment was *Dicer-1*. The T7 promoter sequence (5′-GGA TCC TAA TAC GAC TCA CTA TAG G-3′) was added to the 5′ end of the amplification primers of *Dicer-1* (519 bp; Forward: 5′-GTG ATG CTG GAG TTG GGT TT-3′; Reverse: 5′-AGA ATG AGT CGC CCA ATG TC-3′) and *GFP* (467 bp; Forward: 5′-CTT GAA GTT GAC CTT GAT GCC-3′; Reverse: 5′-TGG TCC CAA TTC TCG TGG AAC-3′) dsRNA templates (Supplementary material 1-Text S1). The PCR products of the dsRNA templates were extracted with hydroxybenzene/chloroform/isoamyl alcohol (25:24:1) (Solarbio, China). dsRNA was generated using a T7 RNAi Transcription Kit (Vazyme Biotech, China) and then purified by hydroxybenzene/chloroform/isoamyl alcohol (50:49:1) (Solarbio). Finally, dsRNA was assessed using agarose gel electrophoresis and a NanoDrop 2000 (Thermo Scientific, USA) and stored at − 80 °C.

miR-71-5 simulant was a small double-strand RNA designed according to the miR-71-5 sequence (21 nt, Supplementary material 1-Text S1). The template of the miR-71-5 simulant consisted of two double-stranded DNAs containing the T7 promoter: one was generated by oligonucleotide A1 (5′-GAT CAC TAA TAC GAC TCA CTA TAG GGT GAA AGA CAT GGG TAA TGA GAA A-3′) and oligonucleotide A2 (5′-TTT CTC ATT ACC CAT GTC TTT CAC CCT ATA GTG AGT CGT ATT AGT GAT C-3′) using touchdown PCR; the other was generated by oligonucleotide B1 (5′-GAT CAC TAA TAC GAC TCA CTA TAG GGT CTC ATT ACC CAT GTC TTT CAA A-3′) and oligonucleotide B2 (5′-TTT GAA AGA CAT GGG TAA TGA GAC CCT ATA GTG AGT CGT ATT AGT GAT C-3′) using touchdown PCR. A control simulant was designed according to the *GFP* sequence (19 nt, Supplementary material 1-Text S1). The template of the control simulant was also acquired by two double-stranded DNAs containing the T7 promoter: one was generated by oligonucleotide C1 (5′-GAT CAC TAA TAC GAC TCA CTA TAG GGG CAA GCT GAC CCT GAA GTT AA-3′) and oligonucleotide C2 (5′-TTA ACT TCA GGG TCA GCT TGC CCC TAT AGT GAG TCG TAT TAG TGA TC-3′) using touchdown PCR; the other was generated by oligonucleotide D1 (5′-GAT CAC TAA TAC GAC TCA CTA TAG GGA ACT TCA GGG TCA

GCT TGC AA-3′) and oligonucleotide D2 (5′-TTG CAA GCT GAC CCT GAA GTT CCC TAT AGT GAG TCG TAT TAG TGA TC-3′) using touchdown PCR. The miR-71-5 and control simulants were generated using a T7 RNAi Transcription Kit and then extracted by hydroxybenzene/chloroform/isoamyl alcohol (50:49:1). The extract was assessed using agarose gel electrophoresis and a NanoDrop 2000 and stored at − 80 °C.

dsRNA feeding

Termites were prestarved for 12 h before being fed (Zhou et al. 2008; Hamilton and Bulmer 2012). A total of 18 termites were placed in a cell petri dish ($D = 35$ mm) with a piece of filter paper ($D = 18$ mm) that was moistened by 60 µL of RNase-free water containing 80 µg *dsDicer-1*, 80 µg *dsGFP*, 60 µg miRNA simulant, or 60 µg control simulant. For *Dicer-1* dsRNA feeding bioassays, termites were allowed to ingest the moistened paper for 24 h before the experiments. *GFP*-treated termites were regarded as controls. For miRNA simulant feeding bioassays, termites were allowed to ingest the moistened paper for 48 h before the experiments. Control simulant-treated termites were regarded as controls.

RT-qPCR

Five or six replicates from three colonies were used for gene expression using RT-qPCR. Three termites per replicate per treatment were pooled for total RNA extraction using the Direct-zol™ RNA MiniPrep Kit (Zymo Research, USA). The purity and concentration of the extracted RNA were determined using a NanoDrop 2000. RNA was converted to cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TakaRa, Japan). RT-qPCR was performed with a QuantStudio™ 3 Real-Time PCR System (Thermo Scientific) using ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech). The specific primers are listed in Supplementary material 2-Table S1**.**

miRNA concentration

Six replicates from three colonies were used to determine the concentration of miRNAs using a MiPure Cell/Tissue miRNA Kit (Vazyme Biotech, China). Three termites per replicate per treatment were pooled for miRNAs. The purity and concentration of the extracted miRNAs were determined using a NanoDrop 2000.

De novo sequencing and transcriptome analysis flow

Fifteen termites from three colonies per treatment were pooled for total RNA extraction using TRIzol Reagent (Invitrogen Life Technologies, USA). The concentration, quality and integrity of the total RNA were determined using a NanoDrop spectrophotometer (Thermo Scientific). Sequencing libraries were generated using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, USA) and sequenced on a HiSeq platform (Illumina) by Shanghai Personal Biotechnology Cp. Ltd. (China). Sequencing data were filtered by Cutadapt (v1.15) software. For the transcriptome sequencing project without a reference genome, Trinity (v2.5.1) software was used to montage clean reads for the transcripts for later analysis. NR (NCBI non-redundant protein sequences), GO (Gene Ontology), KEGG (Kyoto Encyclopedia of Genes and Genome), EggNOG (Evolutionary genealogy of genes: Non-supervised Orthologous Groups), and Swiss-Prot databases were used for gene functional annotation. Furthermore, DESeq (1.30.0) was used to analyse the differentially expressed genes under the condition of $|\log 2^{\mathrm{FoldChange(FC)}}| > 1$ and $p < 0.05$. The genes were then mapped to GO terms, and the terms with significant enrichment were calculated by hypergeometric distribution under the condition of $p < 0.05$ to reveal the possible functions of the candidate genes. Additionally, the genes were mapped to KEGG pathways to determine their possible functions.

Locomotion

Nine replicates from three colonies were analysed to compare the movement distance of *Dicer-1*-silenced vs. *GFP*-treated termites. After one day of oral feeding of *dsDicer-1* or *dsGFP*, one termite per replicate per treatment was placed in a new cell petri dish ($D = 90$ mm) with a piece of filter paper moistened by the conidial suspension (10^8 conidia/mL). Then, we used a black marker to trace the movement of the termite on a transparent lid for 10 s. The movement distance was measured using grid paper (1×1 mm).

Grooming

Nine replicates from three colonies were analysed to compare the number of grooming behaviours between *Dicer-1*-silenced and *GFP*-treated termites. After one day of oral feeding of *dsDicer-1* or *dsGFP*, three termites per replicate per treatment were placed in a new cell petri dish ($D = 35$ mm) with a piece of moistened filter paper ($D = 35$ mm) and then contaminated with fungal conidia (10^8 conidia/mL). Behaviours were video-recorded for 15 min using an HD digital camera (SONY, Japan). The videos were scanned every 10 s to observe whether grooming behaviours (termite mouths towards nestmate bodies) occurred between fungus-contaminated termites.

Cannibalism

Nine replicates from three colonies were analysed for the effect of *Dicer-1* vs. *GFP* on the cannibalistic behaviour towards fungus-contaminated corpses. Six termites per replicate per treatment were placed in a cell petri dish ($D = 35$ mm) with a piece of moist filter paper ($D = 18$ mm) containing *dsDicer-1* or *dsGFP*. They were allowed one day of oral feeding of dsRNA, and then, one fungus-contaminated corpse incubated for 0 d was placed into the cell petri dish where the six treated termites were reared. After one day of rearing together, the fungus-contaminated corpse was either completely eaten (high test score = 2), partially eaten (low test score = 1), or not eaten (no test score = 0) (Supplementary material 1-Fig. S1) by the six termites.

Burial

Nine replicates from two colonies were analysed for the effect of *Dicer-1* vs. *GFP* on the burial behaviour towards fungus-contaminated corpses. Six termites per replicate per treatment were placed in a cell petri dish ($D = 35$ mm) with a piece of moist filter paper ($D = 18$ mm) containing *dsDicer-1* or *dsGFP*. They were allowed one day of oral feeding of dsRNA. Then, one fungus-contaminated corpse incubated for 2 d and soil were added to the petri dish in which the six treated termites were reared. After one day of rearing together, the fungus-contaminated corpse was either completely buried (high test score = 2), partially buried (low test score = 1), or not buried (no test score = 0) (Supplementary material 1-Fig. S2) by the six termites.

Antifungal activity

Six replicates from three colonies were analysed for the impact of *Dicer-1* and miR-71-5 on the ability of termites to inhibit fungal growth. Five termites per replicate per treatment were pooled and crushed using liquid nitrogen and then dissolved in 100 µL of 0.9% saline. The homogenate was centrifuged at 6000×*g* for five minutes at 4 °C to yield 80 µL of supernatant. Then, the supernatant was centrifuged again to yield 50 µL of supernatant. For antifungal activity, a 96-well microplate was used to measure the absorbance of samples, growth controls and standard blanks to calculate the reduction of the fungal growth: (1) 50 µL of potato dextrose (PD), 2 µL of the fungal conidia (10^8 conidia/mL), and 5 µL of the supernatant per well were mixed for the sample; (2) 50 µL of potato dextrose (PD), 2 µL of the fungal conidia (10^8 conidia/mL), and 5 µL of 0.9% saline per well were mixed for the fungal growth control; (3) 50 µL of potato dextrose (PD) and 7 µL of 0.9% saline per well were mixed for the standard blank. After 24 h of cultivation in a constant temperature shaker (150 rpm at 25 ± 1 °C), measurement was performed in a microplate spectrophotometer (600 nm; Thermo Scientific).

Survival

To determine the effect of oral feeding of *dsDicer-1* on termite survival, termites from three colonies were deployed to determine the survival of *Dicer-1*-silenced (42 termites in total, 14 termites per colony) and *GFP*-treated (42 termites in total, 14 termites per colony) termites during fungal contamination. For controls, 56 termites from two colonies were deployed to determine the survival of *Dicer-1*-silenced (28 termites in total, 14 termites per colony) and *GFP*-treated termites (28 termites in total, 14 termites per colony). When performing the experiments, seven termites were reared in a cell petri dish ($D = 35$ mm) with a piece of filter paper ($D = 18$ mm) moistened with RNase-free water containing *dsDicer-1* or *dsGFP*, while termites were contaminated with fungus and then reared for ten days for daily observation of their survival. To determine the effect of oral feeding of miR-71-5 simulant on termite survival, termites from three colonies were deployed to determine the survival of miR-71-5 (42 termites in total, 14 termites per colony) and control (42 termites in total, 14 termites per colony) simulant-treated termites during fungal contamination. Additionally, 56 termites from two colonies were deployed to determine the survival of miR-71-5 (28 termites in total, 14 termites per colony) and control (28 termites in total, 14 termites per colony) simulant-treated termites. When performing the experiments, seven termites were reared in a cell petri dish ($D = 35$ mm) with a piece of filter paper ($D = 18$ mm) moistened with RNase-free water containing miR-71-5 simulant or control simulant. After two days of oral feeding, termites were contaminated with fungus and then reared for ten days for daily observation of their survival. Dead termites were removed in time.

Statistical analysis

All data analyses were conducted in IBM SPSS, version 19. The Shapiro–Wilk test was used to detect whether datasets were normally distributed. Given that the gene expression, cannibalism, and burial data were abnormally distributed, the Wilcoxon test was used. Alternatively, data from the miRNA concentration, locomotion, grooming, and antifungal activity were normally distributed, and a paired t test was used. Survival was analysed using the Kaplan–Meier method for the lifespans under the different treatment conditions. The significance level was $p < 0.05$ in this study.

Results

mRNA expression profile of *Dicer-1*-silenced termites

To determine the effect of *Dicer-1* on the gene expression profile of termites, mRNA sequencing of *Dicer-1*-silenced termites was performed. After 1 day of oral feeding of *dsDicer-1*, *Dicer-1* expression (Fig. 1A; $n = 6$, $p < 0.05$; Wilcoxon test) and hence miRNA concentration (Fig. 1B; $df = 5$, $p < 0.05$; paired *t* test) were significantly decreased in termites. A total of 1262 mRNAs were significantly altered in the whole bodies of *Dicer-1*-silenced termites (upregulated: 562 mRNAs; downregulated: 700 mRNAs; Fig. 1C and Supplementary material 2-Table S2). The differentially expressed genes (DEGs) were mapped to the salivary secretion, glycolysis, citrate cycle, and 29 other KEGG pathways (Fig. 1D–F and Supplementary material 2-Table S3). In addition, the DEGs were clustered into the ATP generation from ADP, structural constituent of cuticle, defense response, glutathione transferase activity, oxidation–reduction process, and 171 other GO terms (Fig. 2 and Supplementary material 2-Table S4).

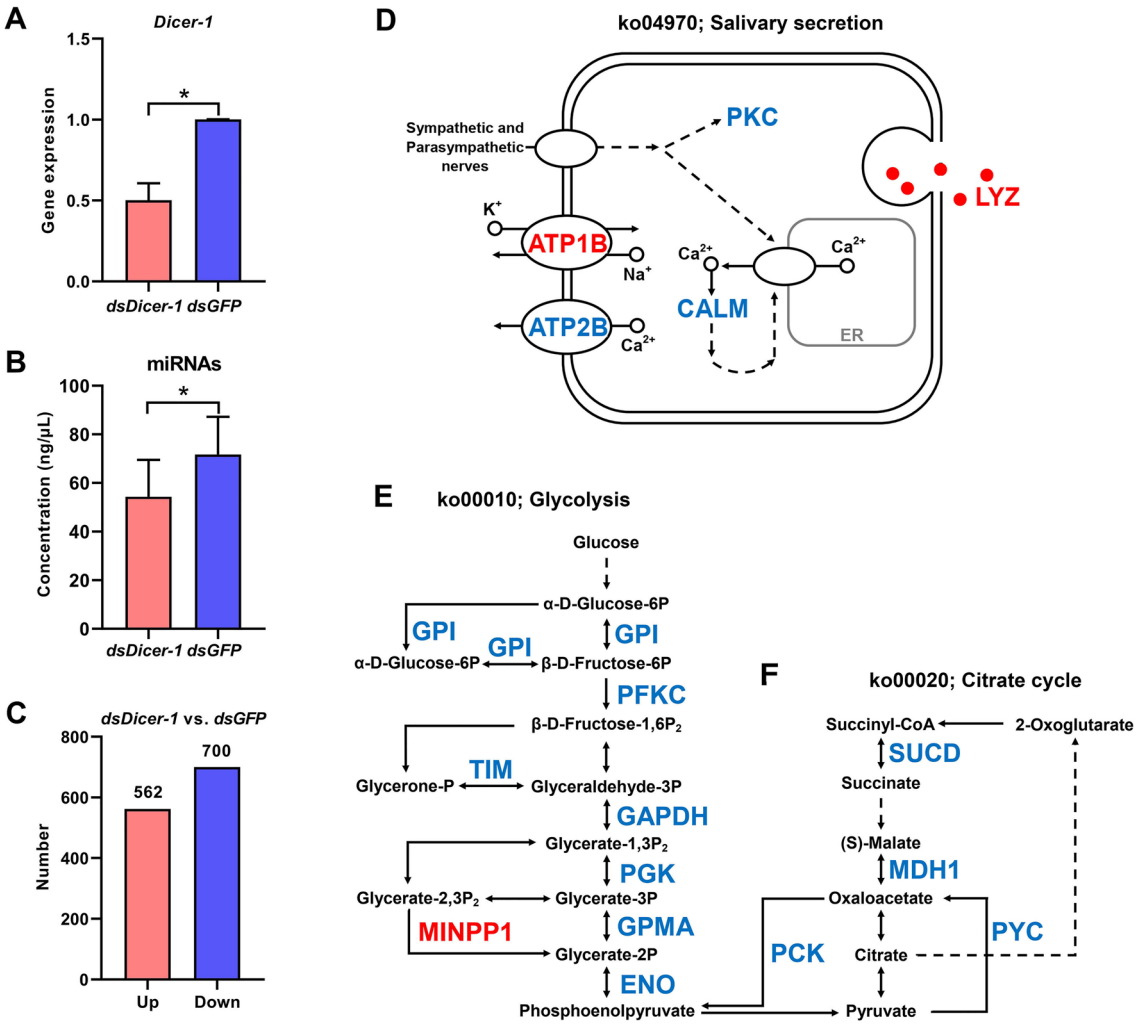

Fig. 1 Gene expression profile in the whole bodies of *Dicer-1*-silenced termites. **A** *Dicer-1* expression. The data are shown as the mean ± SEM. *$p < 0.05$. **B** The concentration of miRNAs. The data are shown as the mean ± SEM. *$p < 0.05$. **C** The number of differentially expressed genes (DEGs). **D** The DEGs mapped to salivary secretion. ATP1B, Sodium/potassium-transporting ATPase subunit beta; ATP2B, Plasma membrane calcium-transporting ATPase 1; PKC, Protein kinase C alpha type; CALM, Calmodulin-like protein; LYZ, Lysozyme C. **E** The DEGs mapped to glycolysis. GPI, Glucose-6-phosphate isomerase; PFKC, ATP-dependent 6-phosphofructokinase; TIM, Triosephosphate isomerase; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PGK, Phosphoglycerate kinase; MINPP1, Multiple inositol polyphosphate phosphatase 1; GPMA, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase; ENO, Enolase. **F** DEGs mapped to citrate cycle. PCK, phosphoenolpyruvate carboxykinase [GTP]; PYC, pyruvate carboxylase; MDH1, malate dehydrogenase; SUCD, succinate–CoA ligase [ADP-forming]. Red letters indicate upregulation; blue letters indicate downregulation

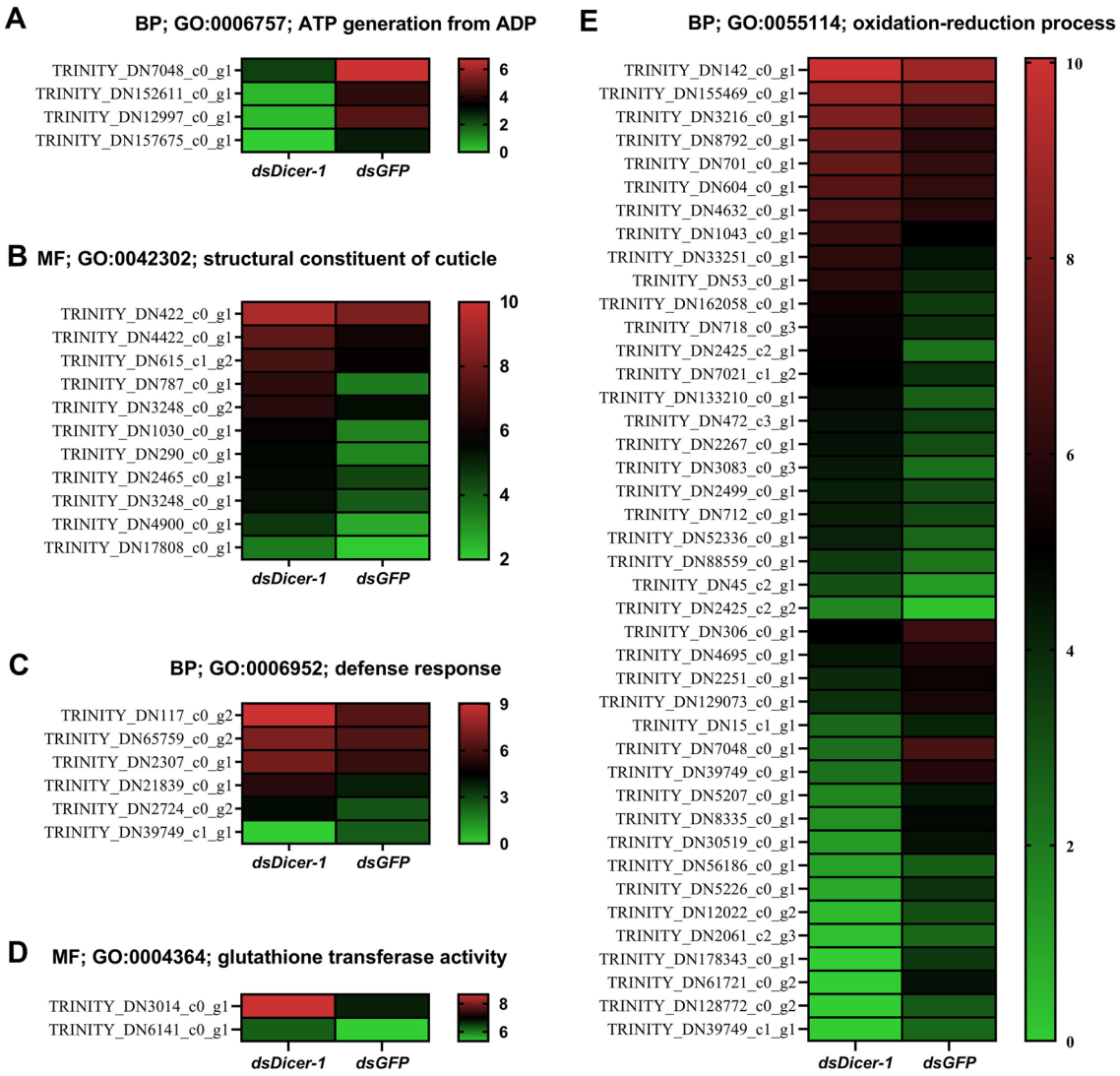

Fig. 2 Heat maps of DEGs clustered into defence-related GO terms. **A** ATP generation from ADP. **B** Structural constituent of cuticle. **C** Defence response. **D** Glutathione transferase activity. **E** Oxidation–reduction process

RT–qPCR verification of DEGs from mRNA-seq

The glycolysis, citrate cycle, and ATP generation from ADP were related to carbohydrate and energy metabolism, in which eight DEGs (*glyceraldehyde-3-phosphate dehydrogenase*, *2,3-bisphosphoglycerate-dependent phosphoglycerate mutase*, *enolase, triosephosphate isomerase*, *ATP-dependent 6-phosphofructokinase*, *phosphoenolpyruvate carboxykinase [GTP]*, *succinate–CoA ligase [ADP-forming] subunit alpha-2*, and *malate dehydrogenase*) were verified to be significantly downregulated after *Dicer-1* silencing (Fig. 3A–H; $n = 6$, $p < 0.05$; Wilcoxon test), suggesting disorders of glycolysis, the citrate cycle, ATP generation, and hence carbohydrate and energy metabolism in the whole bodies of *Dicer-1*-silenced termites.

DEGs (*defensin* and *terminicin*) in the defence response encoded AMPs. Expression of these genes was significantly increased in *Dicer-1*-silenced termites compared to that in *GFP*-treated termites (Fig. 3I, J; $n = 5$ or 6, $p < 0.05$; Wilcoxon test). In the oxidation–reduction process, *cytochrome P450 9e2* and *peroxiredoxin-4* were significantly altered after *Dicer-1* silencing in termites (Fig. 3K, L; $n = 6$, $p < 0.05$; Wilcoxon test). These results implied an important effect of *Dicer-1* on the immune response and oxidation–reduction reaction in whole-body of the termites.

The effect of *Dicer-1* on behavioural disease defences in termite groups

To determine the effect of *Dicer-1* on the behavioural response to fungal contamination, we tested four important

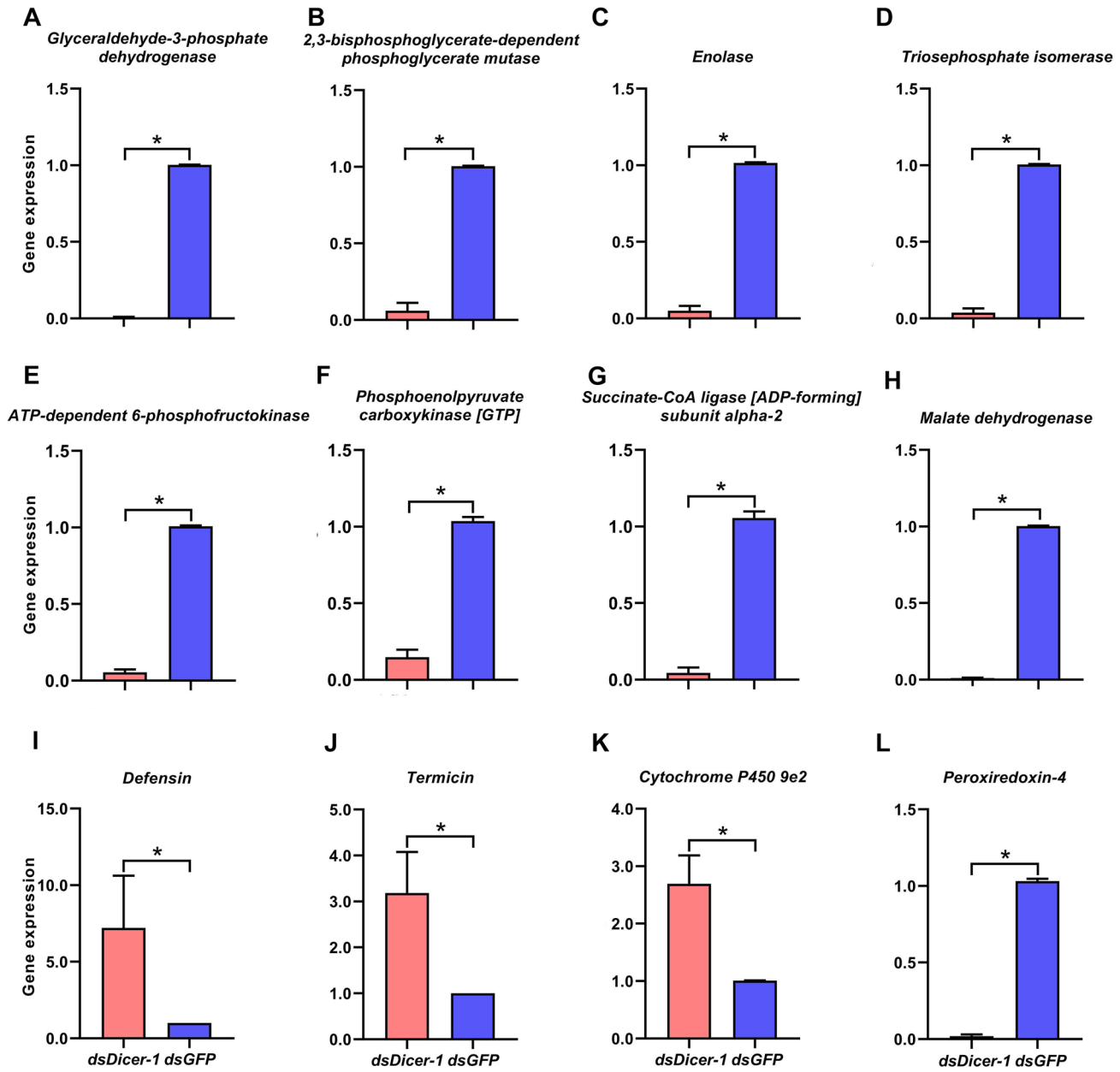

Fig. 3 RT-qPCR verification of DEGs from mRNA-seq. **A**–**H** Genes associated with carbohydrate and energy metabolism. **I**–**J** Genes associated with immune response. **K**–**L** Genes associated with oxidation–reduction reactions. The data are shown as the mean ± SEM. *$p < 0.05$

behavioural defences, locomotion, grooming, cannibalism, and burial, in *Dicer-1*-silenced termites. Our results showed that locomotion (Fig. 4A, t = 7.013, df = 8, $p < 0.01$; paired t test; Supplementary material 1-Fig. S3 and Video S1), grooming (Fig. 4B, t = 5.949, df = 8, $p < 0.01$; paired t test; Supplementary material 1-Fig. S4 and Video S2), cannibalistic (Fig. 4C, $n = 9$, $p < 0.05$; Wilcoxon test; Supplementary material 1-Fig. S1 and Video S3) and burial (Fig. 4D; $n = 9$, $p < 0.05$; Wilcoxon test; Supplementary material 1-Fig. S2 and Video S4) behaviours were significantly reduced in *Dicer-1*-silenced termites compared to *GFP*-treated termites, suggesting an important role of *Dicer-1* in driving behavioural disease defences in termite colonies.

The effect of *Dicer-1* and miR-71-5 on antifungal abilities in termites

To determine the effect of miRNAs on the physiological response to fungal contamination, we tested the total antifungal activities of *Dicer-1*-silenced termites. Our results showed that the antifungal activity was significantly decreased (Fig. 5A; $t = -3.046$, df = 5, $p < 0.05$; paired t test)

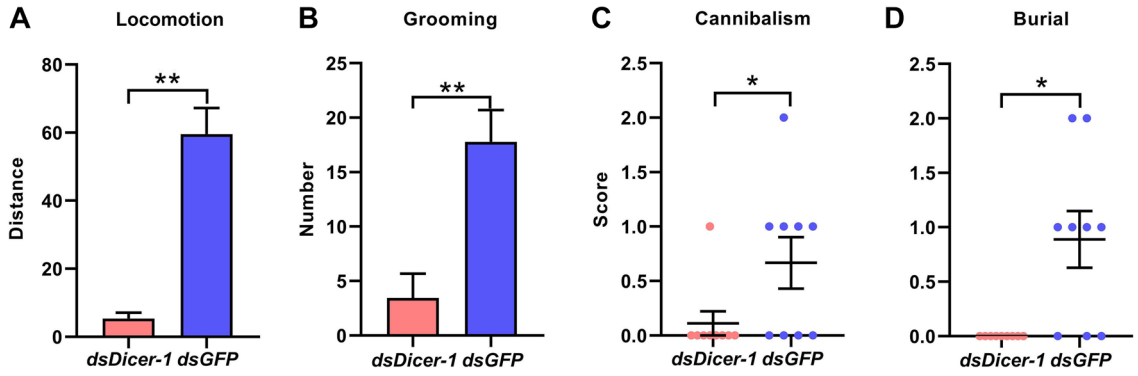

Fig. 4 Effect of miRNA dysregulation on defensive social behaviours in termite groups. **A** Locomotion, **B** grooming, **C** cannibalistic and **D** Burial behaviours of *Dicer-1*-silenced versus *GFP*-treated termite groups. The data are shown as the mean ± SEM. *$p < 0.05$, **$p < 0.01$

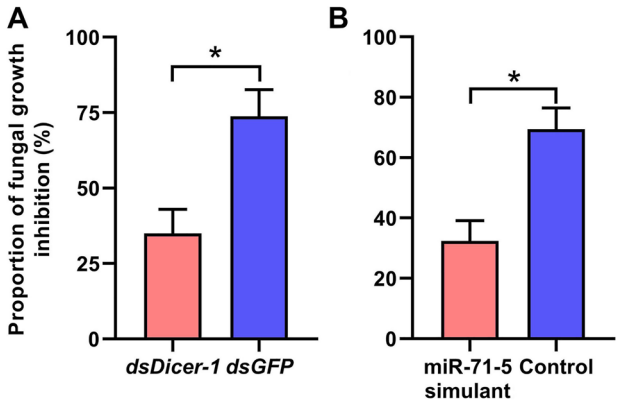

Fig. 5 Effect of miRNA dysregulation on physiological defence in termite individuals. **A** Antifungal activity of *Dicer-1*-silenced vs. *GFP*-treated termites. **B** Antifungal activity of miR-71-5 versus control simulant-treated termites. The data are shown as the mean ± SEM. *$p < 0.05$

in *Dicer-1*-silenced termites. Furthermore, miR-71-5 was selected for further testing to determine its role in physiological defence, which was chosen according to the comparative profiling of miRNAs and mRNAs in fungus-contaminated versus naive termites and miRNA-mRNA analysis (data unpublished). We found that the antifungal activity (Fig. 5B; $t = -4.000$, $df = 5$, $p < 0.05$; paired t test) was significantly reduced in termites treated with miR-71-5 simulants compared to those treated with simulant controls. These results suggested that miRNAs played an important role in driving physiological disease defences in termites.

Dicer-1 and miR-71-5 influenced the survival of fungus-contaminated termites

As both *dsDicer-1* and miR-71-5 simulants led to reduced antifungal activity of termites, we tested their potential for the biological control of termites. We found that *Dicer-1*-silenced termites contaminated with fungus exhibited significantly decreased survival compared to that of other treated termites (*dsDicer-1* + fungus vs. *dsGFP* + fungus: $\chi^2 = 81.839$, $p < 0.001$; *dsDicer-1* + fungus vs *dsDicer-1* + Tween 80: $\chi^2 = 63.070$, $p < 0.001$; *dsDicer-1* + fungus vs *dsGFP* + Tween 80: $\chi^2 = 65.474$, $p < 0.001$). *GFP*-treated termites contaminated with fungus showed significantly decreased survival compared to the termites without fungal contamination (*dsGFP* + fungus versus *dsDicer-1* + Tween 80: $\chi^2 = 58.636$, $p < 0.001$; *dsGFP* + fungus versus *dsGFP* + Tween 80: $\chi^2 = 68.849$, $p < 0.001$). There was no significant difference between *dsDicer-1*- and *dsGFP*-treated termites without fungal contamination ($\chi^2 = 2.037$, $p = 0.154$) (Fig. 6A). Additionally, the survival of fungus-contaminated termites treated with the miR-71-5 simulant was significantly decreased compared to that of other treated termites (miR-71-5 + fungus vs. control + fungus: $\chi^2 = 63.593$, $p < 0.001$; miR-71-5 + fungus vs. miR-71–5 + Tween 80: $\chi^2 = 60.585$, $p < 0.001$; miR-71-5 + fungus vs. control + Tween 80: $\chi^2 = 67.129$, $p < 0.001$). The survival of fungus-contaminated termites treated with the simulant control was significantly decreased compared to that of the termites without fungal contamination (control + fungus vs. miR-71-5 + Tween 80: $\chi^2 = 61.065$, $p < 0.001$; control + fungus vs. control + Tween 80: $\chi^2 = 68.064$, $p < 0.001$). There was no significant difference in survival between termites treated with the miR-71-5 simulant and simulant control without fungal contamination (miR-71-5 + Tween 80 vs. control + Tween 80: $\chi^2 = 1.000$, $p = 0.317$) (Fig. 6B). These results suggested a good effect of miRNAs coupled with entomopathogens in biologically controlling termites.

Discussion

miRNAs perform multiple functions, such as regulating carbohydrate and energy metabolism, the immune response, oxidation–reduction reactions, and other life processes

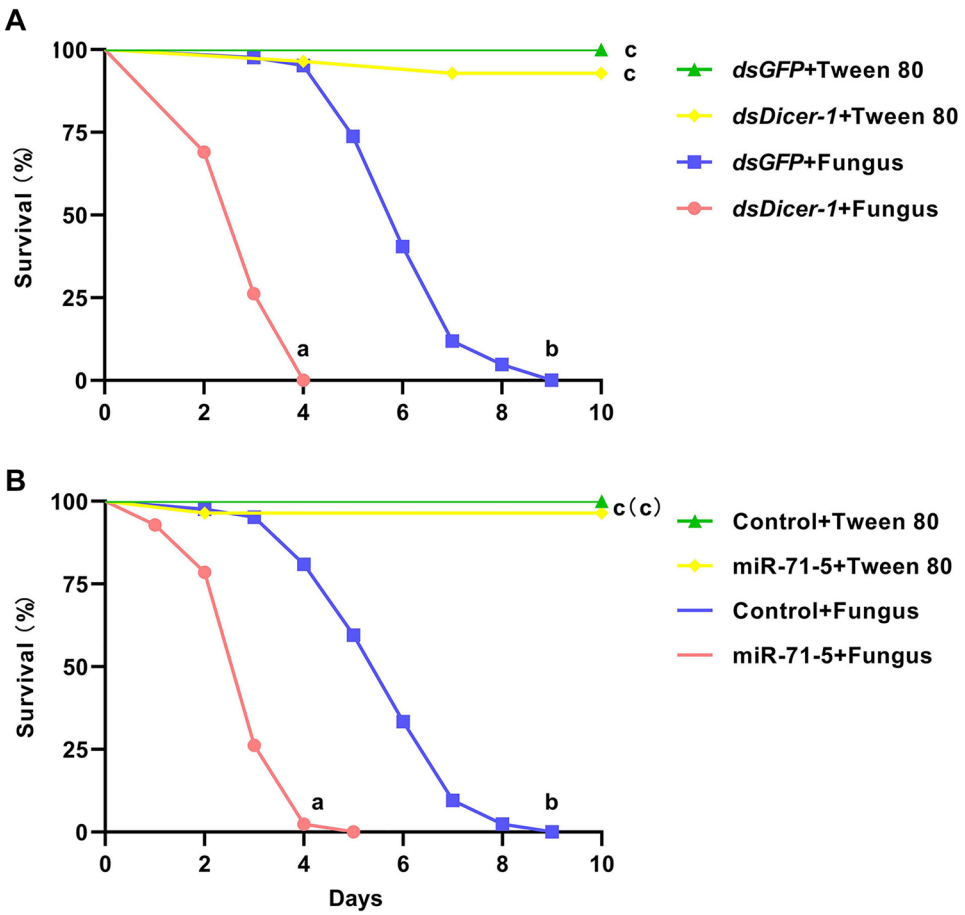

Fig. 6 Effect of miRNA dysregulation on the survival of fungus-contaminated termite groups. **A** Survival of *Dicer-1*-silenced vs. *GFP*-treated termites with or without fungal contamination; **B** Survival of miR-71-5 versus control simulant-treated termites with or without fungal contamination. Different letters indicate significant differences, $p < 0.05$

in whole bodies of the termites. As expected, ingested *dsDicer-1* in termites led to *Dicer-1* silencing and then reduced the miRNA concentration, suggesting the successful induction of miRNA dysregulation. Transcription analysis showed that *Dicer-1* silencing was associated with 32 KEGG pathways and 175 GO terms. In social insect colonies, the salivary secretion can function as external disinfectants and operate in conjunction with social behaviour such as trophallaxis, grooming and nesting to care for offspring, nestmates and colonies (Bulmer et al. 2009; Hamilton et al. 2011; Hamilton and Bulmer 2012). The glycolysis, citrate cycle, and ATP generation from ADP are involved in carbohydrate and energy metabolism. As mounting immune responses and performing social behaviours are energetically costly, disorders of carbohydrate and energy metabolism have negative impacts on these processes (Liu et al. 2020; Hassan et al. 2021a, b; Xu et al. 2021a, b). The structural constituent of cuticle associates with insect cuticles, forming the first barrier to prevent pathogens from invading the haemocoel (Syazwan et al. 2021). Several DEGs involved in the defence response, glutathione transferase activity and oxidation–reduction process are able to encode antimicrobial peptides (AMPs), detoxification proteins, and antioxidant enzymes to increase the resistance and tolerance of insects against pathogens (Liu et al. 2015, 2019a). These defence-related functions are likely to contribute to social immunity in termite colonies. The rest of the KEGG pathways and GO terms were involved in the nervous system, digestive system, longevity, development, etc. (Supplementary material 2-Table S3 and S4). In addition, some of DEGs (e.g. carbohydrate and energy metabolism genes) in *Dicer-1* silenced termites were from microbes (Supplementary material 2-Table S2), which were possibly the gut symbionts and might be important components participating in the life processes of the hosts. Therefore, miRNAs are likely to have a global impact on whole body of the termite.

Carbohydrate and energy metabolism, immune response, and oxidation–reduction reactions are important for miRNAs in driving social immunity. Animal behaviour is an energy-dependent process, and hence, some energy inhibitors cause animals to move slowly and even die (Davidson 1930; Schuler and Casida 2001). In termite colonies, disorders of glycolysis or the citrate cycle result in decreased ATP levels and inhibit locomotion behaviour (Hassan et al. 2021a, b; Xu et al. 2021a, b). Disorders of the citrate cycle also reduce the antifungal activity of termites, leading to increased infection and mortality (Liu et al. 2020). In addition, as symbionts in termite guts were reported to participate in host disease

defences by providing enzyme (Rosengaus et al. 2014) and metabolites (Inagaki and Matsuura 2018), metabolic disorders in the gut symbionts were likely to reduce the symbiont-derived immunocompetence and hence diminished termite disease defences. In our study, eight metabolic genes were verified to be significantly downregulated, suggesting disorders of glycolysis and the citrate cycle in the whole bodies of *Dicer-1*-silenced termites. We suggested that miRNA-mediated carbohydrate and energy metabolism was an important biochemical mechanism in driving social immunity and that disruption of this process was likely to diminish behavioural and physiological disease defences in termite colonies. The insect immune system induces a large number of effector molecules to combat pathogens in vivo. Defensins are compact, cysteine-rich and protease-resistant AMPs with broad-spectrum activity against bacteria, fungi and other parasites (Weber 2021). Termicin is a termite-specific antifungal defensin whose silencing results in decreased antifungal activity and hence increased mortality of fungus-contaminated termites (Hamilton and Bulmer 2012). Insect detoxification and antioxidant systems often function in conjunction with the immune system, which effectively reduces the risk of toxins and ROS during host–pathogen interactions (Syazwan et al. 2021). Cytochrome P450 monooxygenases are responsible for catalysing the oxidation and metabolism of pathogen toxins and producing free radicals or ROS (Shankar and Mehendale 2014). The peroxiredoxin family includes antioxidative enzymes that catalyse the reduction of ROS (Xu et al. 2021a; b). Our results verified that *Dicer-1* silencing significantly altered the gene expression of *defensin*, *termicin*, *cytochrome P450 9e2* and *peroxiredoxin-4*, indicating that miRNAs played important roles in regulating the immune response and oxidation–reduction reactions in whole body of the termite. Therefore, we suggest that carbohydrate and energy metabolism, the immune response, and oxidation–reduction reactions may participate in the driving mechanism of miRNA-shaped social immunity, and their disruption may negatively impact social immunity in termite colonies.

Indeed, miRNA dysregulation has negative impacts on movement and hence behavioural defence in termite colonies. Unlike solitary insects, social insects have evolved a rich repertoire of behavioural defences, collectively detecting the presence of pathogens, limiting pathogen spread, and reducing the infection risk of individuals (Van Meyel et al. 2018; Liu et al. 2019a). Older workers perform dangerous out-of-nest tasks and are easily contaminated. When they are contaminated, nestmates tend to coalesce towards and groom contaminated workers (Liu et al. 2019b). Both contaminated workers and their caregivers increase the distance away from the rest of the naïve nestmates. Nurse workers take broods deeper into the depth of the colony to increase the distance away from the contaminated workers (Stroeymeyt et al. 2018). These results show that an increase in physical distance between colony members is an effective strategy to limit pathogen transmission [termed 'organizational immunity' (Liu et al. 2019a)], and locomotion should be an important social behaviour, as it adaptively adjusts the distance between contaminated individuals and naïve nestmates. Hassan and colleagues found that termites significantly enhanced their locomotion during fungal contamination, further demonstrating locomotion in response to pathogens (Hassan et al. 2021a, b). Additionally, grooming, cannibalistic and burial behaviours are well known in the management of contaminated individuals and corpses in social insect colonies. In termites contaminated with fungi, grooming behaviour occurs immediately, before germination (Liu et al. 2019b). In contrast, cannibalistic behaviour only occurs after contaminated individuals become visibly ill (Davis et al. 2018). For corpse management, fresh corpses are often regarded as nutritional rewards and induce cannibalism by producing an early death cue. In contrast, decayed corpses are regarded as infection risks and induce burial by producing late death cues (Sun et al. 2016). Here, we found that *Dicer-1* silencing significantly inhibited locomotion and other movements, such as grooming, cannibalistic and burial behaviours, suggesting that miRNA dysregulation exhibited a general effect on termite movement and consequently affected behavioural defences against pathogens, pathogen-contaminated nestmates and pathogen-contaminated corpses in termite colonies. For the driving mechanism, miRNA-mediated carbohydrate and energy metabolism could be considered one of the most important biochemical factors (see the above discussion). Therefore, miRNAs could shape the behavioural defences of termites.

miRNA dysregulation also had negative impacts on the physiological defences of termites. Termites normally increase the antifungal ability by upregulation of immune genes to enhance the physiological defences (Liu et al. 2015). The gut symbiont-derived antifungal substances provide termites the ability to inhibit fungal growth to enhance the physiological defences (Rosengaus et al. 2014). Therefore, the total antifungal activity was an effective indicator of physiological disease defences of the termites. Recent studies showed that not only immune genes, but also carbohydrate metabolism and antioxidant genes were reported to be important components affecting the antifungal activity of termites (Liu et al. 2020; Zhao et al. 2020; Zhou et al. 2021). Defaunated termites were more vulnerable to fungal contamination (Rosengaus et al. 2014). Although increased the immune gene expressions such as *defensin* and *termicin*, *Dicer-1*-mediated miRNA dysregulation decreased the expression of carbohydrate metabolism and antioxidant genes within termites or gut symbionts and hence significantly reduced the total antifungal activity of termites. Additionally, miR-71-5 simulant-treated termites also exhibited

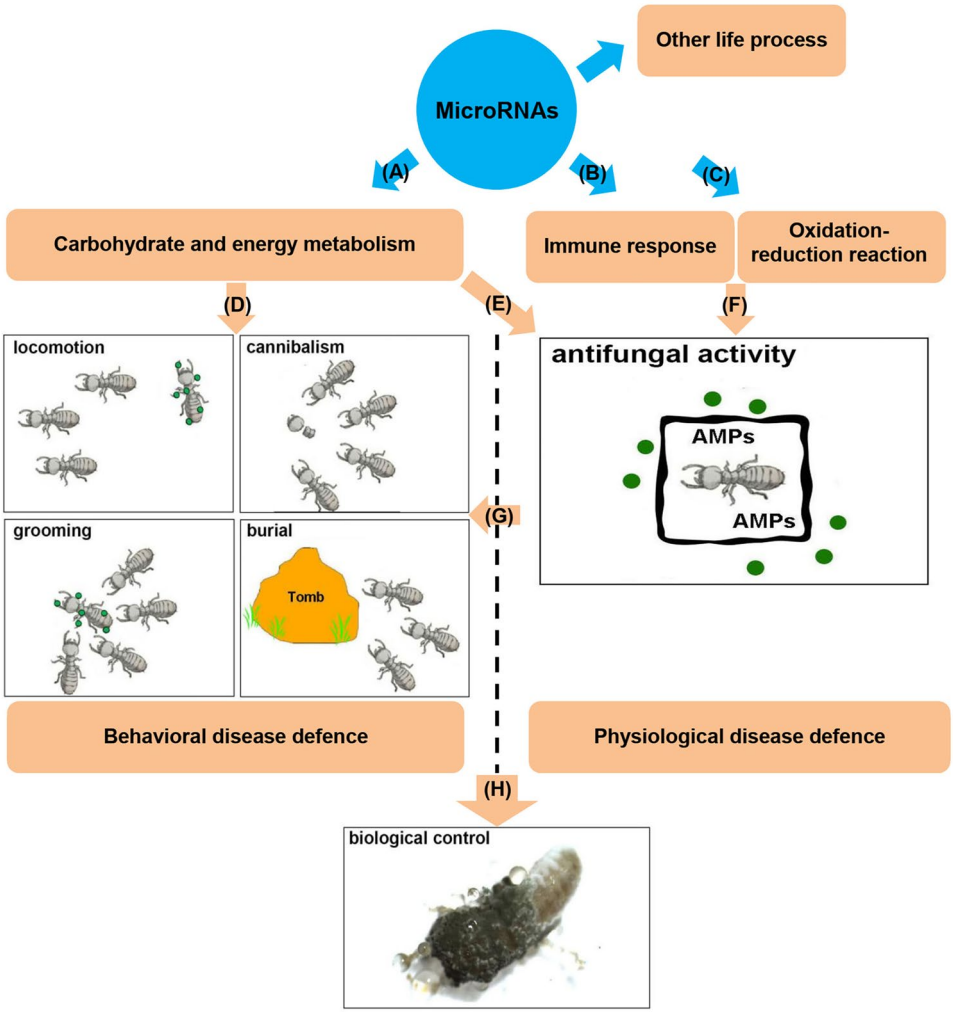

Fig. 7 Potential mechanisms underlying social immunity in termite colonies and novel targets for biological pest control. miRNAs widely affect the whole bodies of termites, including: **A** carbohydrate and energy metabolism, **B** immune response, **C** oxidation–reduction reactions, etc. **D** Carbohydrate and energy metabolism were reported to play important roles in regulating both behaviour and antifungal activity in termites. Therefore, miRNA-mediated carbohydrate and energy metabolism was likely to be one of the genetic and biochemical factors driving behavioural (such as locomotion, grooming, cannibalism, and burial) and physiological (antifungal activity) disease defences in termite colonies. **E** The immune response helped termites inhibit the replication and dissemination of pathogens in the body cavity. The oxidation–reduction reaction protected termites from the damage caused by toxins and ROS from "host–pathogen" interactions. Therefore, the miRNA-mediated immune response and oxidation–reduction reaction play an important role in driving physiological disease defence in termites. **F** miRNAs shaped social immunity by driving behavioural and physiological disease defences in termite colonies. By targeting miRNAs, we could artificially increase the susceptibility of termite colonies to entomopathogens and consequently enhance the biological control of termites

a decrease in the total antifungal activity, further suggesting the negative impact of miRNA dysregulation on physiological defences in termites. Furthermore, the termite groups fed either *dsDicer-1* or miR-71-5 simulant were more vulnerable to fungal contamination, showing an increase in infection mortality. Therefore, miRNAs could shape social immunity by driving both behavioural and physiological disease defences in termite colonies.

To our knowledge, this is the first report of the potential mechanism of miRNAs underlying social immunity in termite colonies. miRNAs can affect salivary secretion, carbohydrate and energy metabolism, cuticle generation, immune responses, oxidation–reduction reactions, etc. Among them, carbohydrate and energy metabolism is likely an important biochemical factor in driving behavioural defences such as locomotion, grooming, cannibalism, and burial in termite colonies. In addition, carbohydrate and energy metabolism, the immune response, and oxidation–reduction reactions are likely to participate in driving termite physiological defences, such as antifungal activity.

Therefore, miRNA-shaped social immunity can influence the susceptibility of termite colonies to entomopathogenic fungi; hence, miRNAs may be effective targets for the biological control of termites (Fig. 7). Numerous studies have proven that RNAi-mediated silencing of target genes could lead to insect death, with considerable potential for insect pest control. In termite pest control, Zhou et al. (2008) first targeted the *endoglucanase* and *hexamerins* genes through dsRNA feeding, demonstrating the feasibility of RNAi-based termiticides. To enhance the lethal effect in termite colonies, RNAi-based termiticides coupled with infectious entomopathogenic fungi have been employed. RNAi-mediated silencing of *termicin*, *GNBP2*, *TG*, *IDH* and other coding genes coupled with *Metarhizium* could significantly increase the susceptibility of nestmates to socially transferred fungal conidia and hence lead to an increased infection mortality rate (Hamilton and Bulmer 2012; Liu et al. 2020; Zhao et al. 2020; Zhou et al. 2021). In our study, we demonstrated the feasibility of targeting small noncoding RNAs through feeding to optimize the biological control of termites using *Metarhizium*. Compared to dsRNAs specifically targeting a gene, the miRNAs were able to target a number of genes with the same or different functions, hence having a broad-spectrum impact on termite life processes. Therefore, by targeting miRNAs, termite groups exhibited diminished behavioural and physiological disease defences at both the group and individual levels, suggesting broad-spectrum inhibition of termite social immunity. By targeting miRNAs, termite groups consequently exhibited a high level of mortality during fungal contamination, suggesting that miRNAs could be employed as termiticides to enhance the biological control of termites by diminishing their social immunity. Therefore, miRNAs could be considered potential novel targets for insect pest control.

Authors' contributions

LL, FMY, QYH and QBT conceived and designed research. LL, CCZ, and LJS conducted experiments. LL, FMY, and CCZ analysed data. LL, QYH and QBT wrote the paper. All authors read and approved the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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