A fungal pathogen deploys a small silencing RNA that attenuates mosquito immunity and facilitates infection

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Insecticidal fungi represent a promising alternative to chemical pesticides for disease vector control. Here, we show that the pathogenic fungus *Beauveria bassiana* exports a microRNA-like RNA (bba-miR1) that hijacks the host RNA-interference machinery in mosquito cells by binding to Argonaute 1 (AGO1). bba-miR1 is highly expressed during fungal penetration of the mosquito integument, and suppresses host immunity by silencing expression of the mosquito Toll receptor ligand Spätzle 4 (Spz4). Later, upon entering the hemocoel, bba-miR1 expression is decreased, which avoids induction of the host proteinase CLIPB9 that activates the melanization response. Thus, our results indicate that the pathogen deploys a cross-kingdom small-RNA effector that attenuates host immunity and facilitates infection.
Malaria remains one of the world’s deadliest infectious diseases, with 435,000 global deaths and 219 million new cases in 2017, up from 217 million cases in 2016. Because mosquitoes are obligatory vectors for malaria transmission, vector control represents the front-line intervention tool for preventing malaria transmission. However, vector control efforts are being undermined by increasing insecticide resistance, and alternative ecologically sound approaches to control mosquitoes are urgently needed. The insect pathogenic fungus Beauveria bassiana (Cordycipitaceae) offers a promising environment-friendly alternative to chemical pesticides, which has hampered their widespread application. Thus, better understanding of molecular interactions between the fungus and the host mosquito is of particular importance for improvement of its efficacy.

Insects employ innate immune responses to fight pathogenic infection. Toll is the principal antifungal pathway by directing the production of antifungal peptides. Melanization is another important facet of the mosquito immune defense against fungi. To cause infection, pathogens must counteract their host immune responses. In case of fungi, the molecular mechanism of host immunity has not been described, although the metabolites secreted by insect fungal pathogens have been implicated in this phenomenon. Plant pathogenic fungi are known to deliver cell-entering effectors to suppress host immunity. However, to our knowledge, no examples of such effector molecules have been described for insect pathogenic fungi.

Small silencing RNAs (sRNAs) are short noncoding RNAs that regulate gene expression by binding to Argonaute (AGO) proteins and directing the RNA-induced silencing complex (RISC) to RNAs with complementary sequences. miRNAs are a class of sRNAs of ~22 nt, and are important modulators of various biological processes, including development and host–microorganism interactions.

Here, we report that the insect fungal pathogen B. bassiana produces a microRNA-like sRNA bba-miR1 that acts as a host cell-entering effector to modulate the expression of mosquito immune genes, demonstrating the fungal pathogen has adapted the cross-kingdom RNA interference-mediated defense mechanism during the evolutionary arms race with the insect hosts.

**Results**

*Beauveria bassiana* expresses miRNA-like small RNAs. To explore possible roles of miRNAs in mosquito–fungus interactions, we profiled sRNA libraries generated from *Beauveria bassiana*-infected *Anopheles stephensi* mosquitoes collected at 36, 60, and 84 h post fungal topical inoculation. We identified four miRNA-like small RNAs (milRNAs) whose sequences cannot be mapped to the *A. stephensi* genome, but perfectly matched the *B. bassiana* genome (Supplementary Fig. 1a). These 4 milRNAs (bba-miR1, bba-miR2, bba-miR3, bba-miR4) had predicted miRNA-like precursor stem-loop structures (Supplementary Fig. 1b), suggesting that *B. bassiana* may generate miRNA-like small RNAs during host infection. Fungi have diverse miRNA biogenesis pathways, and some miRNAs are Dicer independent. *B. bassiana* has two Dicer proteins Dicer1 and Dicer2. To investigate whether *B. bassiana* miRNAs are produced by Dicer, we generated Dicer1 deletion mutant (∆Dcl1), Dicer2 deletion mutant (∆Dcl2), and double-mutant ∆Dcl1/∆Dcl2 by homologous replacement (Supplementary Fig. 2a, d–f). We found that bba-miR1 could not be detected in ∆Dcl2 and ∆Dcl1/∆Dcl2, leading sRNA libraries generated from fungal mycelium. WT and two mutant strains miR1-KO 1# and miR1-KO 2# following topical application of a spore suspension (10⁷ conidia/ml). Mosquitoes sprayed with sterile 0.01% Triton X-100 were used as a negative control (Triton). Survival of female adult mosquitoes infected with the wild-type (WT) ARSEF252 and two mutant strains miR1-KO 1# and miR1-KO 2# following topical application of a spore suspension (10⁷ conidia/ml). Mosquitoes sprayed with sterile 0.01% Triton X-100 were used as a negative control (Triton). Survival of female adult mosquitoes following topical application of a suspension of 10⁷ conidia/ml of WT and two overexpression strains bba-miR1-OV 1# and bba-miR1-OV 2#. The statistical significance of survival curves was analyzed with the Log-rank test. Expression of bba-miR1 during *Beauveria bassiana* infection (Supplementary Fig. 1a). These 4 milRNAs (bba-miR1, bba-miR2, bba-miR3, bba-miR4) had predicted miRNA-like precursor stem-loop structures (Supplementary Fig. 1b), suggesting that *B. bassiana* may generate miRNA-like small RNAs during host infection. Fungi have diverse miRNA biogenesis pathways, and some miRNAs are Dicer independent. *B. bassiana* has two Dicer proteins Dicer1 and Dicer2. To investigate whether *B. bassiana* miRNAs are produced by Dicer, we generated Dicer1 deletion mutant (∆Dcl1), Dicer2 deletion mutant (∆Dcl2), and double-mutant ∆Dcl1/∆Dcl2 by homologous replacement (Supplementary Fig. 2a, d–f). We found that bba-miR1 could not be detected in ∆Dcl2 and ∆Dcl1/∆Dcl2, leading sRNA libraries generated from fungal mycelium.

![Fig. 1](https://example.com/figure1.png) **Fig. 1** Roles of *B. bassiana* bba-miR1 on fungal pathogenicity against mosquitoes. a) bba-miR1 cannot be detected in *B. bassiana* Dicer2 deletion mutant (∆Dcl2) and Dicer1/Dicer2 double-mutant (∆Dcl1/∆Dcl2), as revealed by RT-PCR. RNA was extracted from fungal mycelium. b) Survival of female adult mosquitoes infected with the wild-type (WT) ARSEF252 and two mutant strains miR1-KO 1# and miR1-KO 2# following topical application of a spore suspension (10⁷ conidia/ml). Mosquitoes sprayed with sterile 0.01% Triton X-100 were used as a negative control (Triton). c) Survival of female adult mosquitoes following topical application of a suspension of 10⁷ conidia/ml of WT and two overexpression strains bba-miR1-OV 1# and bba-miR1-OV 2#. The statistical significance of survival curves was analyzed with the Log-rank test. d) Expression of bba-miR1 during *B. bassiana* ARSEF252 infection. RNA was extracted from fungus-infected mosquitoes. The *B. bassiana* U6 small nuclear RNA (U6) was used as an internal reference in qRT-PCR assays. The expression values are normalized to time 0. The data represent three biological repeats with three technical replicates and are shown as mean ± s.e.m. Source data are provided as a Source Data file.
mutants (Fig. 1a), demonstrating that bba-milR1 is Dicer dependent, whereas bba-milR2, bba-milR3, and bba-milR4 are still expressed in Dcl1 or Dcl2 single- and double-mutants (Supplementary Fig. 1c).

**bba-milR1 facilitates fungal infection.** To investigate whether these miRNAs are involved in fungal pathogenicity, we generated deletion mutants by separately disrupting the precursor sequences of the four miRNAs in *B. bassiana* ARSEF252 by homologous replacement (Supplementary Fig. 2a, g–i). Pathogenicity assays against *A. stephensi* showed that deletion of bba-milR1 resulted in a significant reduction in fungal virulence to mosquitoes relative to wild-type *B. bassiana* ARSEF252 (P < 0.0001, Log-rank test) (Fig. 1b). However, deletion of bba-milR2, bba-milR3, or bba-milR4 did not alter fungal virulence (Supplementary Fig. 3). We next generated the transgenic strains that overexpress bba-milR1, bba-milR2, bba-milR3, or bba-milR4 under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter. For each miRNA, a fragment of ~400 bp primary miRNA centered on the genome sequence of *B. bassiana* ARSEF252 was amplified and cloned into the fungal expression vector. The overexpression vectors were separately transformed into ARSEF252 (Supplementary Fig. 2b). We validated that expression level of the four miRNAs was significantly higher in transgenic strains compared with wild-type (Supplementary Fig. 4a). These results further confirm that these miRNAs are transcribed from the genome of *B. bassiana*. Overexpression of the bba-milR2, bba-milR3, or bba-milR4 did not affect fungal virulence (Supplementary Fig. 4b), whereas overexpression of bba-milR1 (milR1-OV) resulted in significant decrease in virulence to adult *A. stephensi* mosquitoes (P < 0.0001, Log-rank test) (Fig. 1c) and had no obvious negative impact on fungal development (Supplementary Fig. 2c), suggesting that constitutive expression of bba-milR1 impacts fungal pathogenicity. To further investigate the function of bba-milR1 in fungus–mosquito interactions, we quantified its expression pattern during *B. bassiana* infection of *A. stephensi*. bba-milR1 was induced by ~30-fold at 36 h post infection (hpi), and then declined to very low levels as the fungus enters the host’s hemocoel at about 60 hpi (Fig. 1d). To investigate whether bba-milR1 is expressed by other *B. bassiana* strains, 5-day-old female *A. stephensi* mosquitoes were infected with ARSEF252 and two other *B. bassiana* strains ARSEF2860 and Bb-bm01. We found that bba-milR1 is also detected in mosquitoes infected with these two strains (Supplementary Fig. 5), suggesting that bba-milR1 is conserved among *B. bassiana* strains.

**bba-milR1 binds to mosquito Argonaute 1.** Recent studies showed that microorganism-encoded miRNAs can act as cross-kingdom regulators of host gene expression19,21,22. Given the high expression level of bba-milR1 at early stages of infection, we hypothesized that it may play a role in the interaction between fungus and host. To investigate whether *B. bassiana* bba-milR1 can enter mosquitoes, we incubated *Aedes albopictus* C6/36 cells with Cy3-labeled bba-milR1 and tracked RNA localization. As shown in Fig. 2a, bba-milR1 was loaded in vesicles that were present outside the cells, on the cell surface, and was transported into insect cells. These results suggest that bba-milR1 enters host cells via vesicles. Similar results were observed in *Drosophila* S2 cells (Supplementary Fig. 6). To examine the transport of miRNA in vivo, Cy3-labeled bba-milR1 was injected into the mosquito hemocoel and found that Cy3-labeled bba-milR1 was present in the cytoplasm of fat body cells (Fig. 2b).

In insects, miRNAs are preferentially assembled with Argonaute 1 (AGO1) to form an RNA-induced Silencing Complex (RISC) that modulates gene expression24. To investigate whether bba-milR1 associates with RISC, we performed RNA immunoprecipitation (RIP) assays using an *A. stephensi* AGO1 (AsAGO1) antibody and assayed for AGO1-bound miRNAs using Reverse Transcription PCR (RT-PCR). As a positive control, *A. stephensi* derived mirR-10-5p and mirR-2940-3p were detected in AsAGO1-RIP samples (Fig. 2c). We found that *B. bassiana* milR1, milR2, milR3, and milR4 were specifically detected in AGO1-RIP samples (Fig. 2c). These results indicate that miRNAs produced by *B. bassiana* are exported into host cells and bind to mosquito AGO1 to hijack the RNA interference (RNAi) machinery.

**bba-milR1 targets the mosquito host genes Spz4 and CLIPB9.** To investigate whether bba-milR1 targets mosquito genes, three stringent target prediction softwares (microTar, PITA, and miRanda) were used to predict potential targets in the 3’UTR, 5’UTR and coding sequences of the *A. stephensi* genome (Supplementary Table 1; Fig. 3a). We found that bba-milR1 could base pair with some mosquito immunity genes, including genes encoding theioste-containing protein, CLIP-domain serine protease, serine protease inhibitor, and Spätzle-like cytokine (Supplementary Table 1). To validate the interaction between bba-milR1 and the predicted immune-related target genes, we performed target verification tests ex vivo and in vivo. To verify these targets ex vivo, we cloned the DNA sequences surrounding predicted target sites using *A. stephensi* cDNA as templates into psiCheck2 vectors downstream Renilla luciferase stop codon. The engineered vectors and bba-miR1 mimics were co-transfected into HEK293T cells, and measured luciferase activity. Dual-luciferase reporter assays showed that bba-milR1 can suppress Spz4 and induce CLIPB9 expression (Fig. 3b, c). When the regions homologous to the seed sequence of bba-milR1 were mutated in the Spz4 and CLIPB9 reporter constructs, the luciferase activity was not affected by bba-milR1 (Fig. 3a–c). To confirm that the regulation of the two targets was indeed triggered by bba-milR1 in vivo, we injected bba-milR1 agomir, a type of chemically modified double-stranded microRNA, into adult *A. stephensi* mosquitoes, and examined the transcript level of the target genes by qRT-PCR. We found that the transcript levels of Spz4 and CLIPB9 were significantly suppressed and induced in agomir-treated groups, respectively (Fig. 3d, e). These results show that the host genes Spz4 and CLIPB9 are targeted in the coding regions and are suppressed and induced by *B. bassiana* bba-milR1, respectively. However, ex vivo and in vivo results for other predicted targets were contradictory (Supplementary Fig. 7).

**bba-milR1 manipulates host immunity to facilitate infection.** The Toll pathway in mosquitoes and other insects operates as a primary immune defense against fungal infection12,25. The activation of the Toll pathway requires the binding of the cytokine Spätzle (Spz) to the Toll receptor, an interaction that transduces extracellular immune signals into cells. There are six members of the Spz family in the *A. stephensi* genome, of which Spz4 has the highest expression in the integument and fat body (Supplementary Fig. 8). To confirm that Spz4 can be targeted by bba-milR1 during infection, we further examined the expression levels of Spz4 in mosquitoes after infection with wild-type *B. bassiana* (WT) and bba-milR1 overexpression strain (milR1-OV). Expression of Spz4 was significantly lower in milR1-OV-infected mosquitoes than in WT-infected mosquitoes (Fig. 4a; Supplementary Fig. 9). Meanwhile, the mRNA levels of the major antifungal effector genes encoding Cecropin 1 (CEC1) and Defensin 1 (DEF1) were also reduced in milR1-OV-infected mosquitoes (Fig. 4a; Supplementary Fig. 9), indicating that bba-milR1 can suppress Spz4 and immune response during mosquito infection. To determine whether Spz4 is involved in the mosquito
Toll immune signaling pathway, we silenced Spz4 by systemic injection of Spz4 double-stranded RNA (dsSpz4). The mRNA levels of CEC1 and DEF1, which are antimicrobial peptide genes downstream of the Toll pathway, were reduced by ∼50% in dsSpz4-treated mosquitoes compared with the dsGFP-treated controls (Fig. 4b). The CEC1 and DEF1 transcript levels were decreased by ∼70% in agomir-injected mosquitoes compared with negative controls, which provides further evidence for Spz4 repression and inhibition of the Toll signaling pathway by bba-miR1 (Fig. 4c). To define the contribution of Spz4 in the mosquito immune defense against B. bassiana infection, we knocked down Spz4 by injecting dsSpz4 and then infected mosquitoes with B. bassiana. At 60 h after topical infection of B. bassiana, the formation of hyphal bodies, a proxy for fungal invasion efficiency, was significantly more abundant in dsSpz4-treated mosquitoes than the dsGFP control (Fig. 4d). The fungal biomass in dsSpz4-treated mosquitoes was 2.2-fold greater than the dsGFP controls (Fig. 4e). Moreover, silencing of Spz4 rendered mosquitoes more susceptible to the B. bassiana infection than the dsGFP-treated control, and the survival rate was significantly lower (Fig. 4f).

These results indicate that Spz4, a Toll receptor ligand, regulates the expression of the antifungal peptide genes to protect mosquitoes from fungal infection. We conclude that bba-miR1 acts as a fungal effector molecule to suppress mosquito immune response by targeting host Spz4.

Melanization, another important immune mechanism of arthropods, also plays an important role in the innate immune response to encapsulate and retard invasive B. bassiana growth.
and dissemination in mosquitoes\textsuperscript{13}. Melanization is regulated by activation of prophenoloxidase (PPO) into phenoloxidase (PO), and involves an extracellular proteinase cascade and serpin inhibitors (Fig. 5a). The clip domain serine proteinase CLIPB9 acts as a PPO-activating proteinase to regulate melanization in mosquitoes\textsuperscript{26}. To confirm whether CLIPB9 is indeed modulated by bba-miR1 during infection, we examined the transcript levels of CLIPB9 in mosquitoes after infection with WT, milR1-OV, and milR1-KO strains. The results showed that there was no significant difference in the transcript levels of CLIPB9 between mosquitoes at 36 h post infection with WT, milR1-OV, and milR1-KO (Fig. 5b; Supplementary Fig. 10a), indicating that bba-miR1 does not interact with CLIPB9 during the early stages of infection when fungus penetrating the mosquito integument. However, the expression of CLIPB9 was significantly higher and lower in the mosquitoes infected by \textit{B. bassiana} milR1-OV and the milR1-KO mutant, respectively, than the WT-infected mosquitoes at late stages of infection (84 hpi) (Fig. 5c; Supplementary Fig. 10b). To test the role of CLIPB9 in the defense against \textit{B. bassiana} infection, we silenced CLIPB9 by systemic injection of CLIPB9 dsRNAs. The dsCLIPB9 silencing reduced CLIPB9 mRNA levels by 83% (Supplementary Fig. 11a). Then, we measured the hemolymph PO activity in dsGFP-treated and dsCLIPB9-treated mosquitoes at 3 days post injection. The PO activity was significantly lower in dsCLIPB9-injected mosquitoes than dsGFP-injected mosquitoes (Supplementary Fig. 11b). Similarly, infection of milR1-OV and milR1-KO resulted in significant increase and decrease in \textit{A. stephensi} hemolymph PO activity compared with WT at 84 hpi, respectively (Fig. 5d). As a consequence of CLIPB9 knock down, the number of \textit{B. bassiana} hyphal bodies was higher than in dsGFP-treated controls (Fig. 5e). qPCR analysis demonstrated that fungal load was significantly higher in dsCLIPB9-treated mosquitoes compared with the dsGFP-treated controls (Fig. 5f). Moreover, silencing of CLIPB9 rendered mosquitoes more susceptible to \textit{B. bassiana} infection than dsGFP-treated controls (Fig. 5g). Taken together, these results show that \textit{B. bassiana} markedly reduces bba-miR1 expression during late stages of infection to elaborately avoid induction of CLIPB9, and in this way circumvent the melanization response (Fig. 6).
Beauveria bassiana has been at the forefront of efforts to develop biocontrol alternatives to the use of chemical insecticides for vector control. This fungus is also widely used as a model for studies of fungal pathogenicity and fungus–invertebrate host molecular interactions. Insects possess a refined innate immune system capable of recognizing fungal pathogens. To develop in insects, fungal pathogens must be able to attenuate their host immune responses. How this happens remains elusive. Here, we show that the fungal pathogen produces a sRNA effector that is translocated into host insect cells to attenuate host immunity and achieve its infection, which is an example of the evolution of a cross-kingdom RNA-mediated defense mechanism.

**Fig. 4** bba-milR1 attenuates host immunity by suppressing Toll receptor ligand Spätzle 4. **a** The relative transcript levels of Spz4, cecropin 1 (CEC1), and defensin 1 (DEF1) in mosquitoes at 48 h post infection (hpi) with WT and bba-milR1-overexpressing strain milR1-OV 1#. The expression values are normalized to WT. **b** Effect of Spz4 silencing on the expression of CEC1 and DEF1. The expression values are normalized to dsGFP. **c** The relative transcript levels of Spz4, CEC1, and DEF1 in the mosquitoes injected with bba-milR1 agomir (200 nM) or control agomir (200 nM). The expression values are normalized to control. **d** Effect of Spz4 silencing on fungal hyphal body formation in the mosquito hemocoel at 60 hpi. Black arrows point to hyphal bodies, and the red arrow points to a mosquito hemocyte. Scale bars, 10 μm. **e** qPCR-based quantification of fungal load in mosquitoes injected with dsGFP or dsSpz4 at 60 hpi. Fungal levels are expressed as that of fungal 18S rRNA relative to A. stephensi ribosomal protein S7 (AsS7) DNA. **f** Effect of Spz4 silencing on the survival of mosquitoes following topical application of a suspension of 10^7 conidia/ml of B. bassiana ARSEF252 (Log-rank test). Values are mean ± s.e.m. The experiments were repeated three times with similar results. *P < 0.05, **P < 0.01, ***P < 0.001. P-value < 0.05 was regarded as statistically significant (Student’s t test). Source data are provided as a Source Data file.

**Discussion**

*Beauveria bassiana* has been at the forefront of efforts to develop biocontrol alternatives to the use of chemical insecticides for vector control. This fungus is also widely used as a model for studies of fungal pathogenicity and fungus–invertebrate host molecular interactions. Insects possess a refined innate immune system capable of recognizing fungal pathogens. To develop in insects, fungal pathogens must be able to attenuate their host immune responses. How this happens remains elusive. Here, we show that the fungal pathogen produces a sRNA effector that is translocated into host insect cells to attenuate host immunity and achieve its infection, which is an example of the evolution of a cross-kingdom RNA-mediated defense mechanism.
Small RNAs play critical roles in regulating gene expression in many organisms. In insects, miRNAs associate with AGO1 to mediate gene regulation. Our immunoprecipitation assays demonstrated that the bba-miR1 can enter the insect cells where it associates with mosquito AGO1 and in this way hijacks the insect RNAi machinery to selectively modulate host defense genes. The bba-miR1 was found in exosome-like extracellular vesicles (EVs) from where it is transported into mosquito and fruit fly cells and fat body tissues. Recent studies suggest that EV-mediated export of sRNA represents a conserved universal mechanism for intra-kingdom and inter-kingdom gene regulation.

In insects, the Toll is activated early by recognizing fungal structures while traversing the external cuticle. We found that A. stephensi Spz4 is highly expressed in the mosquito integument at the point of fungus penetration. Interestingly, we found that the bba-miR1 is highly induced and expressed in B. bassiana during cuticle penetration, so that it can efficiently silence Spz4 and suppress Toll-mediated mosquito immunity.

Once in the hemocoel, fungal hyphae encounter host cellular and humoral defenses. To counter these defenses, the invasive filamentous cells switch to a yeast-type proliferation strategy to form hyphal bodies that possess fewer carbohydrate epitopes, allowing fungi to avoid recognition by the host immune system. The other target of bba-miR1 is A. stephensi CLIPB9, which regulates PPO activation in the melanization response. Although bba-miR1 can induce expression of CLIPB9, we found that B. bassiana reduces bba-miR1 expression to very low level after entering the hemocoel, thus elaborately avoiding direct induction of CLIPB9. If bba-miR1 is overexpressed under the control of a constitutive promoter, it impairs fungal pathogenicity.

In this study, we demonstrate that bba-miR1 is a conserved mechanism that regulates host defense responses to fungal infection in insects. This mechanism represents a novel perspective for the development of host defense strategies against mosquito-borne diseases.

**Methods**

**Mosquito rearing.** Anopheles stephensi (Dutch strain) mosquitoes were maintained at 27 °C with 70 ± 5% relative humidity under 12 h/12 h day–night cycle. Larvae were fed on cat food pellets and ground fish food supplement. Adult mosquitoes were maintained on 10% (wt/vol) sucrose.

**Fungal culture and infection bioassay.** Beauveria bassiana strains ARSEF252, ARSEF2860, and Bb-bm01 were grown and maintained on Sabouraud dextrose agar plus yeast extract (SDAY; BD Difco) at 26 °C. Conidia suspensions were prepared in 0.01% (vol/vol) Triton X-100 and filtered through layers of sterile glass wool to remove hyphal fragments. To conduct fungal infection, 5-day-old female A. stephensi adult females were injected with 10^7 conidia/ml of B. bassiana ARSEF252 (Log-rank test). Values are mean ± s.e.m. Similar results were obtained in three biological repeats. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. P-value < 0.05 was regarded as statistically significant (Student’s t test). Source data are provided as a Source Data file.

**Fungal biomass quantification.** Fungal biomass was standardized to the quantity of S7 and quantified using a NanoDrop 1000 spectrophotometer. The relative quantification of fungal biomass among the groups at this point was significantly higher in the dsCLIPB9 group than in the dsGFP group. The interaction of bba-miR1 with the target gene CLIPB9 activates the Toll immune pathway, which is the principal insect antifungal mechanism.
mosquitoes were sprayed with fungal conidial suspension (10^7–10^9 conidia/mL). Mosquitoes sprayed with sterile 0.01% Triton X-100 were used as a negative control. The treated mosquitoes were subsequently maintained at 26 ± 1 °C and 80 ± 5% relative humidity, with a 12-h/12-h day–night cycle, until mosquitoes died or were collected for sample preparation at the indicated time points. Each treatment was replicated three times with 50 mosquitoes per replicate, and the infection bioassays were repeated three times. Mortality was recorded every 12 h, and cadavers were transferred to moisturized filter paper to monitor the emergence of fungal hyphae.

Small RNA library construction and sRNAs sequencing. Mosquito samples were collected at 36, 60, and 84 h after topical infection with B. bassiana ARSEF252. For each time point, about 50 infected mosquitoes collected from two biological replicates were pooled. The fungus-infected mosquito samples were homogenized with beads in RNAiso Plus (TaKaRa). The total RNA was extracted using Directzol RNA Miniprep Kit (Zymo Research Corporation) and treated with DNase I with beads in RNAiso Plus (TaKaRa). The total RNA was extracted using Directzol RNA Miniprep Kit (Zymo Research Corporation) and treated with DNase I following the manufacturer’s instructions. Illumina microRNA sequencing was performed at Shanghai Biotechnology Corporation.

miRNA sequence analysis. The raw reads from sequencing data were filtered by removing poor quality reads, adaptor pollution reads and reads less than 18 nt. The clean reads of small RNAs were aligned to the reference B. bassiana genome (http://www.bassiana.org) and PITA (http://pita.weizmann.ac.il/index.html) and microTar (http://tiger.dbs.nus.edu.sg/microtar) were jointly used to predict miRNA targets in mosquito Anopheles stephensi. Thresholds were set to a score of ≥140 for miRanda (default), ddG ≤ 0 for PITA, and energy ≥ 0.5 for microTar.

miRNA target prediction. Three miRNA prediction softwares miRanda (http://www.microrna.org) and PITA (http://pita.weizmann.ac.il/index.html) and microTar (http://tiger.dbs.nus.edu.sg/microtar) were jointly used to predict miRNA targets in mosquito Anopheles stephensi. Thresholds were set to a score of ≥140 for miRanda (default), ddG ≤ 0 for PITA, and energy ≥ 0.5 for microTar.

Fungal small RNAs deletion and overexpression. For targeted deletion of miRNAs (bba-miR1, bba-miR2, bba-miR3, or bba-miR4), the 5′ and 3′ flanking regions of the miRNAs were amplified by PCR from B. bassiana genomic DNA, and then subcloned into the XbaI and EcoRV sites of the binary vector pBarGFP, respectively. The gene disruption vectors were then transformed into Agrobacterium tumefaciens ARSEF252. For targeted gene disruption in B. bassiana genome to disrupt B. bassiana ARSEF252 by homologous recombination.

To overexpress miRNAs (bba-miR1, bba-miR2, bba-miR3, or bba-miR4) in B. bassiana ARSEF252, the ~400 bp fragment surrounding primary miRNA was amplified by PCR from B. bassiana genomic DNA, and then subcloned into EcoRI and XbaI sites of the binary vector pBarGFP-PgdA downstream of a constitutive Aspergillus nidulans gpdA promoter (PgdA), to generate microRNA expression vectors. The sequence of the microRNA amplicon was confirmed by sequencing. The vectors were separately transformed into wild-type B. bassiana ARSEF252 using Agrobacterium tumefaciens-mediated transformation to generate microRNA overexpression strains.

Generation of Dicer mutants of B. bassiana. To generate Dicer1, Dicer2 single knockout mutant, the 5′ and 3′ flanking regions of the genes open-reading frame were amplified by PCR from B. bassiana ARSEF252 genomic DNA, and then subcloned into the binary vector pBarGFP. Dcl1 and Dcl2 deletion mutants were generated by using homologous recombination and the Agrobacterium tumefaciens-mediated transformation system. To double-knockout Dcl1 and Dcl2, the 5′ and 3′ flanking regions of Dcl1 ORF were subcloned into the binary vector

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**Fig. 6** Model of Beauveria bassiana deploying bba-miR1 to modulate mosquito immunity. During early stages of infection, B. bassiana bba-miR1 is highly expressed, and translocated into the mosquito cells to attenuate immune responses by suppressing the expression of the key activator gene Spz4. At this early stage in the integument, the bba-miR1 is not accessible to circulating hemocytes, the site of melanization. The unannotated sRNA sequences were aligned to the reference B. bassiana genome to find precursor sequences for novel miRNAs. Novel miRNAs were predicted by miRcat2 (http://srna-workbench.cmp.uea.ac.uk/mircat2/) with stem-loop structure. The R package DESeq software was used to analyze differentially expressed miRNAs.
pSurGFP. The resulting vector was applied to A. tumefaciens-mediated transformation in a ΔGFP mutant strain as described above. The sequences of the primers are given in Supplementary Data 1.

Mosquito RNA isolation and qPCR. The total RNA was extracted from mosquitoes using RNeasy Plus (Qiagen) and treated with Recombinant DNase I (TaKaRa) according to the manufacturer’s instruction. For mRNA, DNAs were synthesized from the total RNA using a PrimeScript® RT reagent Kit (TaKaRa). Quantitative reverse transcription PCR (qRT-PCR) reactions were performed using SYBR Green MasterMix Kit (Vazyme). cDNAs for miRNAs were reverse transcribed using miRcute mRNA First-Strand cDNA Synthesis Kit (Tiangen), and qRT-PCR reactions were performed using miRcute miRNA qPCR detection kit (Tiangen) according to the manufacturer’s protocol. Each sample was performed in triplicate. The housekeeping gene RP57 (AS7) and snRNA U6 were used as endogenous control for mRNA and miRNA, respectively.

Luciferase reporter assays. For high-transfection efficiency and low background expression of bba-miR1, the mammalian HEK293T cell line (ATCC) was used for luciferase reporter assay37. The HEK293T cells were grown in the DMEM/HIGH GLUCOSE medium (HyClone) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS, Gibco) and 1× antibiotic-antimycotic (Gibco) at 37°C under 5% CO2. The ~400 bp sequences surrounding the predicted bba-miR1 target sites in CLIP98 and Spz4 were separately cloned into the XhoI and NotI sites of the psiCheck-2 vector (Promega). Mutagenesis PCR was performed to introduce point mutations at the bba-miR1 target sites to construct psiCheck-2-mut vectors. The HEK293T cells were transfected with 100 ng of psiCheck-2 reporters with 100 nM (final concentration) of synthetic bba-miR1 Mimic (sense strand 5′-GCUGCGCGCGUGUAUUGCUCC-3′, anti-sense strand 5′-UGACAGUAAAUCAGGCGAUAUC-3′, BioBio) or Negative Control miRNA Mimic (micrON mimic NC #24, RiboBio) using Attractene Transfection Reagent (Qiagen). Cells were collected and lysed by 48 h after transfection. The activities of firefly and Renilla luciferases were measured using the Dual-Luciferase Reporter Assay System (Promega). Each sample was performed in triplicate, and transfections were repeated three times.

miRNA Agomir injection. bba-miR1 Agomir is a chemically modified double-strand stable bba-miR1 (sense strand 5′-UGACAGUAAAUGCUCCGUG-3′, anti-sense strand 5′-UCUGCGUACCCUGAUAUAUA-3′) (Suzhou GenScript) and 3-day-old female mosquitoes were microinjected using Nanoject II microinfector (Drummond). Injected mosquitoes were allowed to recover for 2 days. For dsRNA microinjection, cold-anesthetized 3-day-old female mosquitoes derived double-stranded RNA (dsGFP) was synthesized and used as a negative control. For dsRNA microinjection, 100 μl of L-DOPA solution (20 mM) was injected into female mosquitoes as described above. The mosquito fat bodies were collected in 1× PBS at 24 h post injection. The tissues were further fixed with 4% paraformaldehyde (PFA) for 10 min. Then, cells were washed with 1× PBS for three times and kept in 1× PBS. For confocal observation, cells were mounted with VECTASHIELD Antifade Mounting Medium containing DAPI (Vector Laboratories, H-1200) for 5 min and fluorescence signals were visualized with Leica SP8 confocal microscope system.

To examine the translocation and localization of bba-miR1 in mosquito for in vivo analysis, 20 nl Cy3-labeled bba-miR1 (20 μM) was injected into female mosquitoes as described above. The mosquito fat bodies were collected in 1× PBS at 24 h post injection. The tissues were further fixed with PFA, stained with DAPI, and observed with a confocal microscope as described above.

Statistical analyses. The statistical significance of the survival data from fungal infection bioassays and Tuxon X-100 treated mosquitoes (control) was analyzed with a log-rank (Mantel–Cox) test. Other statistical significance was determined by Student’s t test for unpaired comparisons between two treatments. P-value of <0.05 was regarded as statistically significant. Except for when specified in the context, the results are expressed as mean ± s.e.m. All statistics were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software).

Data availability
The RNA sequencing datasets have been deposited in the National Center for Biotechnology Information Sequence Read Archive with accession number PRJNA517599. The source data underlying Figs. 1a–c, 2a–c, 3b–d, 4a, 5a, 7a–c, 8, 9, 10a, 11a, b, 12 and 13 are provided as a Source Data file.

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