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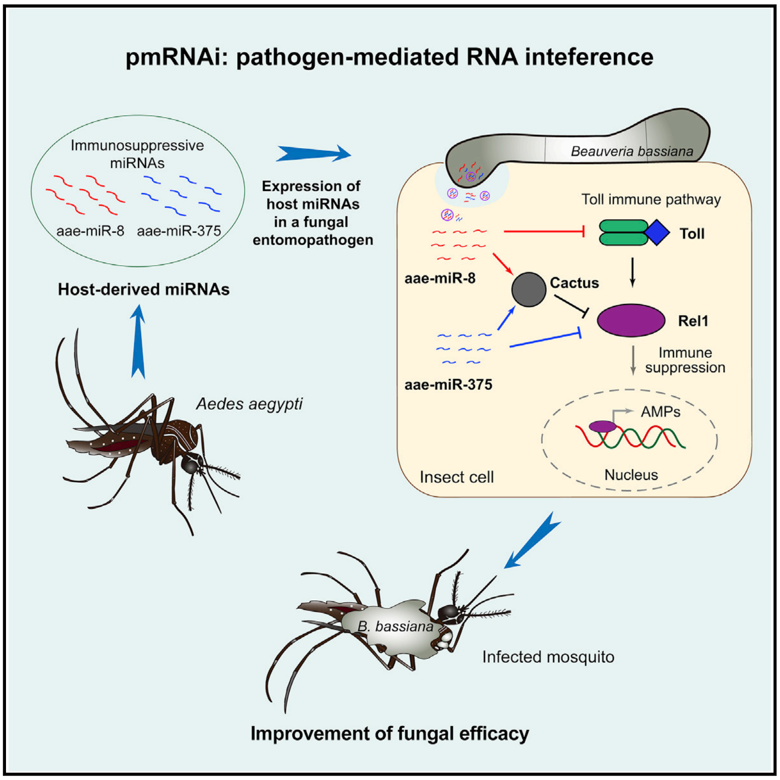
Expression of mosquito miRNAs in entomopathogenic fungus induces pathogen-mediated host RNA interference and increases fungal efficacy

Graphical abstract



Highlights

- miR-8 and miR-375 negatively regulate the Toll immune response in *Ae. aegypti*
- Expression of host miRNAs in *B. bassiana* induces cross-kingdom RNAi in insects
- Fungi expressing the host miRNAs suppress insect immunity and facilitate infection
- Pathogen-mediated RNAi provides an effective strategy to increase fungal efficacy

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In brief

Cui et al. report an approach for increasing efficacy of fungal insecticides by engineering fungal entomopathogen *Beauveria bassiana* to produce host immunosuppressive miRNAs, which could activate cross-kingdom RNAi during infections. The engineered fungal strains could suppress host immunity and display increased virulence toward mosquitoes and the greater wax moth.

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Report

Expression of mosquito miRNAs in entomopathogenic fungus induces pathogen-mediated host RNA interference and increases fungal efficacy

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SUMMARY

The growing threat of insecticide resistance prompts the urgent need to develop additional tools for mosquito control. Entomopathogenic fungi provide an eco-friendly alternative to chemical insecticides. One limitation to the use of mycoinsecticides is their relatively low virulence. Here, we report an approach for suppressing mosquito immunity and increasing fungal virulence. We engineered *Beauveria bassiana* to express *Aedes* immunosuppressive microRNAs (miRNAs) to induce host RNA interference (RNAi) immune responses. We show that engineered strains can produce and deliver the miRNAs into host cells to activate cross-kingdom RNAi during infection and suppress mosquito immunity by targeting multiple host genes, thereby dramatically increasing fungal virulence against *Aedes aegypti* and *Galleria mellonella* larvae. Importantly, expressing host miRNAs also significantly increases fungal virulence against insecticide-resistant mosquitoes, creating potential for insecticide-resistance management. This pathogen-mediated RNAi (pmRNAi)-based approach provides an innovative strategy to enhance the efficacy of fungal insecticides and eliminate the likelihood of resistance development.

INTRODUCTION

Aedes mosquitoes transmit numerous devastating viral infectious diseases, such as dengue fever, yellow fever, Zika, and Chikungunya fever, which are becoming major global health burden (Bhatt et al., 2013; Grubaugh et al., 2017; Petersen et al., 2016). Due to the lack of available treatments and vaccines, vector control remains the most effective way to prevent arboviral diseases (Cirimotich et al., 2011). However, progress in disease control is being threatened by the wide spread of mosquito insecticide resistance (Koekemoer et al., 2011; Labbe et al., 2007; Lima et al., 2011), which severely limits our ability to counteract this intolerable burden. To tackle the growing risk of *Aedes*-borne arboviral diseases, the World Health Organization recently announced the launch of the Global Arbovirus Initiative (Launch of the WHO Global Arbovirus Initiative, 2022), strengthening the urgent need for development of more sustainable vector-control tools that can overcome insecticide resistance.

Entomopathogenic fungi such as *Beauveria bassiana* offer an eco-friendly alternative to chemical insecticides. The insecticidal fungi are the only insect pathogens that can infect their host by directly penetrating the exoskeletons and are especially suitable for controlling blood-sucking mosquitoes and sap-sucking pests (Blanford et al., 2005; Bukhari et al., 2011; Scholte et al., 2005, 2007). However, fungal biopesticides kill insects slower than chemical insecticides due to host immune defenses (Blanford et al., 2005; Fang et al., 2012; Scholte et al., 2005), limiting their widespread application. Genetic engineering has become a powerful tool to improve fungal virulence and biocontrol efficacy (Lovett et al., 2019; Lovett and St Leger, 2018; Wang and Wang, 2017). Over-expression of cuticle-degrading proteins, toxins, peptide hormones, or neuropeptides could enhance fungal pathogenicity and increase lethality (Fan et al., 2012; Fang et al., 2005; Wang and St Leger, 2007; Wang et al., 2011). However, the use of insecticidal proteins to control insect pests may promote selection for resistance. Numerous studies have reported that both field- and laboratory-reared mosquitoes could evolve resistance to mosquitocidal *Bacillus* Cry toxic proteins (Wirth, 2010). Cotton bollworm *Helicoverpa armigera* has also developed resistance to Cry toxins produced by *Bacillus thuringiensis* (Bt) cotton (Zhang et al., 2011). A recent study showed that a field population of oriental armyworm *Mythimna separata* (Walker) rapidly evolved high resistance to the insecticidal protein Vip3Aa, reaching more than 3,000-fold resistance after eight generations compared with the unselected insects (Quan et al., 2021).

Small RNAs (sRNAs), including microRNAs (miRNAs) and small interfering RNAs (siRNAs), are a class of small noncoding RNAs that function as important posttranscriptional regulators of gene expression in eukaryotes. They play an important role in the intricate interaction between insect hosts and fungal pathogens, either by regulating their own genes or inducing gene interference in the counter party for their own benefit (Cui et al., 2019; Wang et al., 2021b; Weiberg et al., 2013; Zhang et al., 2016). We recently discovered that insect pathogenic fungus *B*. *bassiana* delivers a miRNA-like RNA (bba-milR1) into mosquito cells during infection and hijacks host RNA interference (RNAi) machinery by loading its sRNAs into the mosquito AGO1 protein, which triggers cross-kingdom RNAi to suppress host genes (Cui et al., 2019), whereas insect hosts also transport their miRNAs into the fungal pathogen to suppress infection (Wang et al., 2021b). The discovery of cross-kingdom RNAi offers a potential approach to increase fungal efficacy against mosquitoes and other insect pests. Moreover, expression of host miRNAs in fungal strain engineering would minimize the likelihood of resistance development in the target insects.

In this study, we report that host miRNAs with immunosuppressive activities can be exploited to increase fungal virulence. We engineered *B*. *bassiana* to express *Ae*. *aegypti* miRNAs, aae-miR-8 and aae-miR-375, that are negative regulators of the Toll immune signaling pathway, which both upregulated *Cactus* (the inhibitor of Toll pathway) but silenced *Toll5B* and *Rel1A*, respectively. Heterologous expression of the host miRNAs in *B*. *bassiana* can induce cross-kingdom RNAi to suppress host antifungal immune response and increase fungal lethality to laboratory-reared insecticide-susceptible and wild-caught insecticide-resistant *Ae*. *aegypti* mosquitoes. Our study opens up avenues for the development of pathogen-mediated RNAi (pmRNAi)-based approach in more effective and safer biocontrol of mosquitoes and other insect pests.

RESULTS

Host miRNAs miR-8 and miR-375 negatively regulate *Ae*. *aegypti* Toll immune response

To increase the ability of transgenic *B*. *bassiana* to suppress host immunity, we selected two miRNAs, miR-8 and miR-375, that were shown to negatively modulate the Toll immune pathway in *Drosophila melanogaster* and *Ae*. *aegypti*, respectively (Choi and Hyun, 2012; Hussain et al., 2013; Lee and Hyun, 2014). aae-miR-375 was shown to inhibit *Rel1* encoding the nuclear factor κB (NF-κB) transcription factor of the Toll signaling pathway (Bian et al., 2005; Shin et al., 2005) and activates the expression of *Cactus*, a gene encoding an inhibitor of Rel1 in *Ae*. *aegypti* (Bian et al., 2005). To identify target genes of miR-8 in *Ae*. *aegypti*, we firstly predicted aae-miR-8 target genes associated with *Ae*. *aegypti* innate immunity. We found that aae-miR-8 could base pair with immunity genes *Cactus*, *Toll1A*, *Toll1B*, and *Toll5B* (Figure S1). To validate the interaction between aae-miR-8 and the predicted target genes, we conducted target verification using luciferase reporter assays. Dual-luciferase reporter assays revealed that aae-miR-8 significantly suppresses Toll5B but induces Cactus (Figures 1A–1C), whereas mutations of the aae-miR-8 binding sites in the target genes *Toll5B* and *Cactus* abolished the interactions between aae-miR-8 and the two target genes (Figures 1A–1C), demonstrating that aae-miR-8 targets the *Aedes* immune genes *Toll5B* and *Cactus*. *B*. *bassiana* infection induced the expression of *Toll5B* in *Ae*. *aegypti* mosquitoes (Shin et al., 2006). To investigate whether Toll5B is involved in antifungal immune defense in *Ae*. *aegypti*, we knocked down *Ae*. *aegypti Toll5B* expression by systemic injection of *Toll5B* double-stranded RNA (ds*Toll5B*). The transcript level of *Toll5B* was decreased by 66% (Figure 1D). Silencing of *Toll5B* rendered mosquitoes more susceptible to *B*. *bassiana* infection (Figure 1E), and the LT_{50} (time required to kill 50%) values in ds*Toll5B*-treated mosquitoes was dramatically reduced compared with the ds*GFP* control (log rank test, $p < 0.0001$). We further quantified the transcript levels of the downstream antimicrobial peptide (AMP) genes of the Toll pathway, including *Defensin A* (*DefA*), *DefC*, *Diptericin* (*Dip*), *Cecropin A* (*CecA*), *CecD*, *CecE*, and *CecG*, in ds*Toll5B*-treated mosquitoes. The transcription levels of all these AMP genes were markedly reduced in the fat body of ds*Toll5B*-treated mosquitoes (Figure 1F). Taken together, these results show that Toll5B is involved in *Aedes* antifungal Toll immune signaling pathway.

Expression of host miRNAs in *B*. *bassiana* suppresses mosquito immunity

To engineer *B*. *bassiana* to produce the host miRNAs, we transformed *B*. *bassiana* strain ARSEF252 with the expression vector containing a constitutive *Aspergillus nidulans glyceraldehyde phosphate dehydrogenase* (*gpd*) promoter and the nucleotide sequences surrounding mature aae-miR-8 or aae-miR-375 amplified from *Ae*. *aegypti* genomic DNA, thereby generating the transgenic strains Bb-aae-miR-8, Bb-aae-miR-375, and Bb-aae-miR-8+375 (Figure S2A). Heterologous expression of the host miRNAs in the transgenic strains was confirmed by qRT-PCR (Figures 2A, S2B, and S2C). Expression of aae-miR-8 or aae-miR-375 had no impact on fungal growth and development (Figure S2F). Interestingly, the expression level of aae-miR-8 was higher than aae-miR-375 in the fungus (Figures S2B–S2E), which was similar to their expression patterns in the mosquito.

Next, to test whether expression of host miRNAs by *B*. *bassiana* could suppress insect immunity, we examined the expression levels of the corresponding immune genes in mosquitoes after topical infection with wild-type *B*. *bassiana* (Bb-WT), Bb-aae-miR-8, and Bb-aae-miR-375 transgenic strains. Transcription levels of aae-miR-8 targets *Toll5B* and *Cactus* were significantly lower and higher, respectively, in Bb-aae-miR-8-infected mosquitoes than in Bb-WT-infected mosquitoes (Figure 2B). Meanwhile, the expression of the various AMP genes, including *DefA*, *DefC*, *Dip*, *CecA*, *CecD*, *CecE*, and *CecG*, was also dramatically decreased in Bb-aae-miR-8-infected mosquitoes (Figure 2D). In Bb-aae-miR-375-infected mosquitoes, the expression of *Rel1A* and *Cactus* was reduced and induced, respectively, compared with the Bb-WT-infected mosquitoes (Figure 2C). Consequently, the mRNA levels of *DefA*, *DefC*,

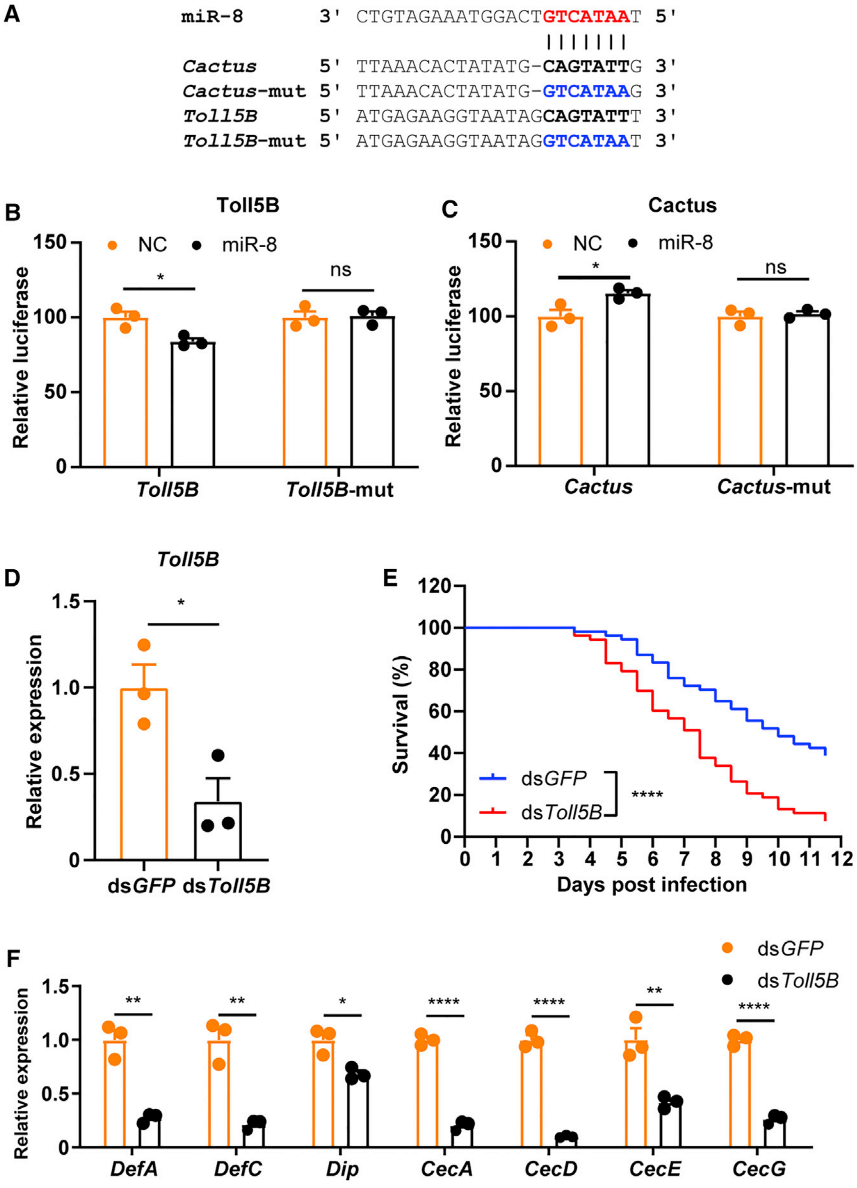

Figure 1. MicroRNA aae-miR-8 negatively regulates *Ae. aegypti* Toll immune response

(A) Sequence alignment of aae-miR-8 with the target genes *Toll5B* and *Cactus* of *Ae. aegypti*. Red characters are the seed region of aae-miR-8. Black bold characters are target sequences. The mutated forms of target genes are shown in blue characters.

(B and C) aae-miR-8 suppresses the expression of *Toll5B* (B) and induces *Cactus* (C) as determined by dual-luciferase reporter assay. $^{*}p < 0.05$, ns, not significant (Student's t test).

(D) Analysis of silencing efficiency of *Toll5B* in adult female *Ae. aegypti* mosquitoes. Systemic injection of *Toll5B* dsRNA (ds*Toll5B*) significantly reduced *Toll5B* transcript levels. The expression values were normalized to ds*GFP*.

(E) Effect of *Toll5B* silencing on the survival of adult *Ae. aegypti* mosquitoes following topical application of a spore suspension of 10^7 conidia/mL of *B. bassiana* ARSEF252. $^{****}p < 0.0001$ (log rank test).

(F) Effect of *Toll5B* silencing on the expression of the effector genes *DefA*, *DefC*, *Dip*, *CecA*, *CecD*, *CecE*, and *CecG*. The expression values were normalized to ds*GFP*. The experiments were repeated three times with similar results. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (Student's t test). The data are presented as the means ± SEM. Number of dissected mosquitoes (n = 10).

CecE, *CecG*, and *Dip* were notably reduced in Bb-aae-miR-375-infected mosquitoes (Figure 2E). These results indicate that *B. bassiana* provides an effective delivery system for host miRNAs, and the engineered *B. bassiana* strains expressing aae-miR-8 or aae-miR-375 strongly attenuate *Ae. aegypti* immune responses during infection by targeting the key immune-related genes.

Expression of host miRNAs increases fungal virulence

To investigate whether expressing aae-miR-8 or aae-miR-375 increases fungal virulence, the insecticide-susceptible Rockefeller strain of adult female *Ae. aegypti* mosquitoes were topically infected with Bb-WT and the transgenic strains. Pathogenicity assays showed that both Bb-aae-miR-8 and Bb-aae-miR-375 transgenic strains were much more potent than the WT against the adult mosquitoes (Figure 3A). Expressing aae-miR-8 or aae-miR-375 resulted in about 20% and 15% reduction in the survival time (LT_{50}) of mosquitoes, respectively (Table S1). These results showed that expression of aae-miR-8 or aae-miR-375 substantially enhance the virulence of *B. bassiana* against *Ae. aegypti* (Rockefeller strain). However, co-expression of miR-8 and miR-375 (Bb-aae-miR-8+375) did not enhance fungal virulence compared with the single miRNA-expressing strains (Figure 3A; Table S1), possibly because miR-8 and miR-375 target the same immune pathway.

To validate the elevated virulence of transgenic fungal strains was due to expression of host miRNAs, we firstly micro-injected *Aedes* mosquitoes with an antagomir to miR-8 or miR-375 (Figures 4A and 4C) and then infected mosquitoes with WT or the transgenic strains. Inhibition of miR-8 or miR-375 function in mosquitoes impaired the increased virulence of the transgenic strains (Figures 4B and 4D), demonstrating the contribution of miR-8 and miR-375 to fungal virulence. To further verify the function of miR-8 and miR-375 in regulating the Toll immunity, we injected the dsRNA of *Rel1A* (Figure 4E) into mosquitoes and infected ds*Rel1A*-treated mosquitoes with WT and transgenic fungal strains expressing miR-8 or miR-375. The mosquitoes with an impaired Toll pathway exhibit similar susceptibility to WT and transgenic strains (Figures 4F–4H), indicating that the inhibitory effect of miR-8 or miR-375 on the Toll pathway mainly contributes to the enhanced fungal virulence.

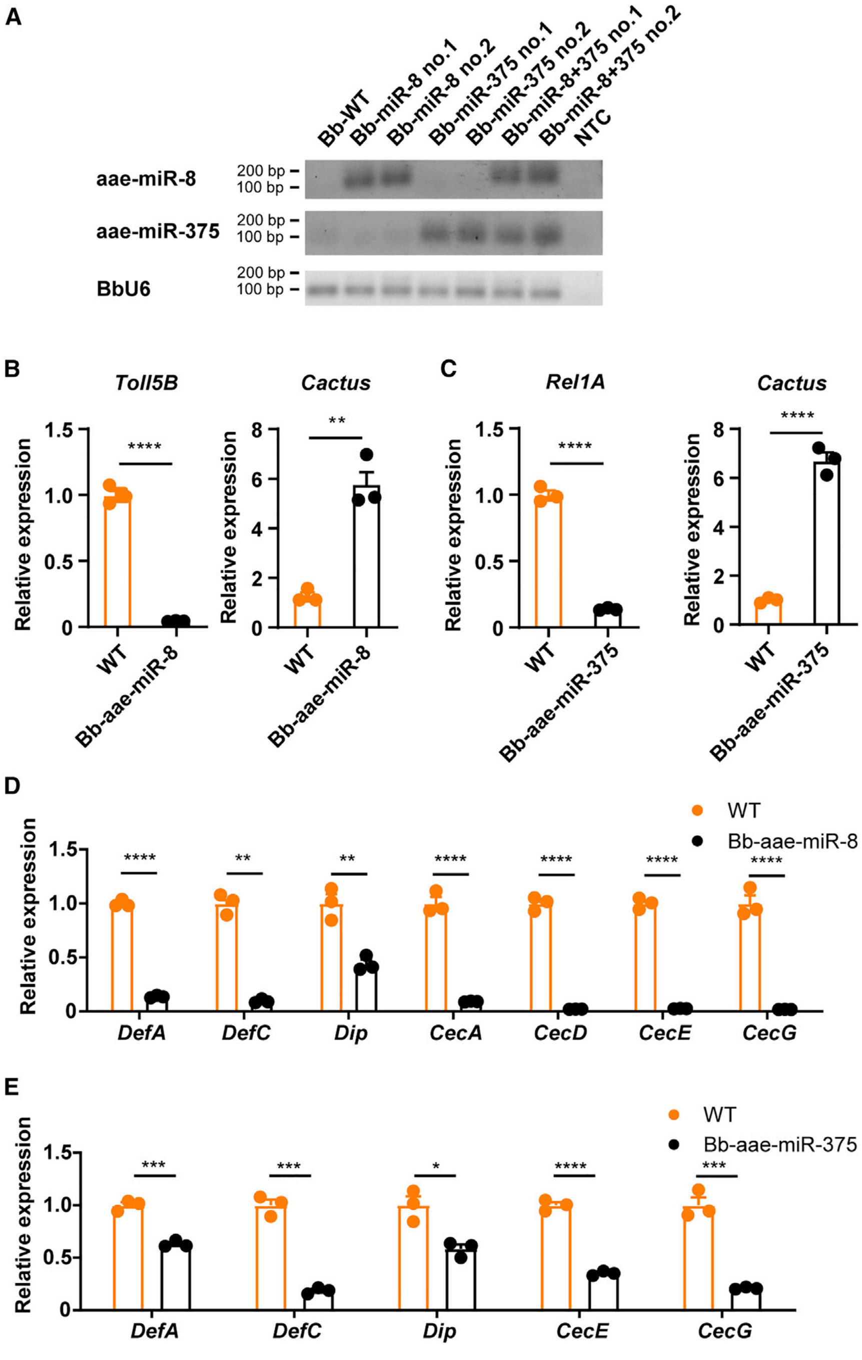

Figure 2. Expression of host miRNAs aae-miR-8 and aae-miR-375 in *B. bassiana* markedly suppresses mosquito immunity during infection

(A) Expression of aae-miR-8 and aae-miR-375 in the WT *B. bassiana* (Bb-WT), Bb-aae-miR-8, Bb-aae-miR-375, and Bb-aae-miR-8+375 strains were determined by RT-PCR. The *B. bassiana* snRNA U6 was used as an internal reference gene. NTC, no template control.

(B) The transcript levels of *Toll5B* and *Cactus* in mosquitoes infected with the Bb-WT or Bb-aae-miR-8 strains.

(C) The transcript levels of *Rel1A* and *Cactus* in mosquitoes infected with the Bb-WT or Bb-aae-miR-375 strains.

(D) The transcript levels of antimicrobial peptide (AMP) genes (*DefA*, *DefC*, *Dip*, *CecA*, *CecD*, *CecE*, and *CecG*) in mosquitoes infected with the Bb-WT or Bb-aae-miR-8 strains.

(E) The transcript levels of AMP genes (*DefA*, *DefC*, *Dip*, *CecE*, and *CecG*) in mosquitoes infected with the Bb-WT or Bb-aae-miR-375 strains. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (Student's t test). The data are presented as the means ± SEM. Number of dissected mosquitoes (n = 10).

Populations of *Ae*. *aegypti* have developed resistance to commonly used insecticides in many locations worldwide, which exacerbates disease-control burden (Ranson and Lissenden, 2016). We next investigated whether expression of the host miRNAs also increases fungal virulence against an insecticide-resistant Jinghong strain of *Ae*. *aegypti*, a field-caught mosquito cohort, which was found to be highly resistant to various chemical insecticides such as permethrin, λ-cyhalothrin, β-cypermethrin, α-alphamethrin, and DDT insecticides (Lan et al., 2019). Expression of aae-miR-8, aae-miR-375, or aae-miR-8 + aae-miR-375 also markedly enhanced *B*. *bassiana* virulence to *Ae*. *aegypti* resistant mosquitoes (Figure 3B), resulting in 30% reduction in the survival time (LT_{50}) of the mosquitoes (Figure 3B; Table S1). These results show that expression of the host miRNAs miR-8 or miR-375 can markedly improve virulence of *B*. *bassiana* against insecticide-resistant mosquitoes.

miR-8 and miR-375 were also present in *Anopheles gambiae* (Biryukova et al., 2014), *Anopheles stephensi* (Mead and Tu, 2008), *D*. *melanogaster* (Lee and Hyun, 2014), and diamondback moth *Plutella xylostella* (Etebari et al., 2013). The sequences of miR-8 and miR-375 are conserved in these insect pests. Moreover, miR-8 and miR-375 also potentially target immunity-related genes in these insects (Figure S3), suggesting that expressing aae-miR-8 and aae-miR-375 in *B*. *bassiana* may enhance fungal virulence against lepidopteran insects that are the most destructive pests in agriculture. To test this hypothesis, a lepidopteran host, the greater wax moth *Galleria mellonella* caterpillar, was used to bioassay the effect of host miRNAs' expression on virulence. Expression of aae-miR-8 or aae-miR-375 by *B*. *bassiana* substantially increased fungal virulence against *G*. *mellonella* larvae (Figure 3C), resulting in about 20% reduction in the LT_{50} values (Table S1). Interestingly, the Bb-aae-miR-8+375 strain co-expressing aae-miR-8 and aae-miR-375 was much more potent than Bb-aae-miR-8

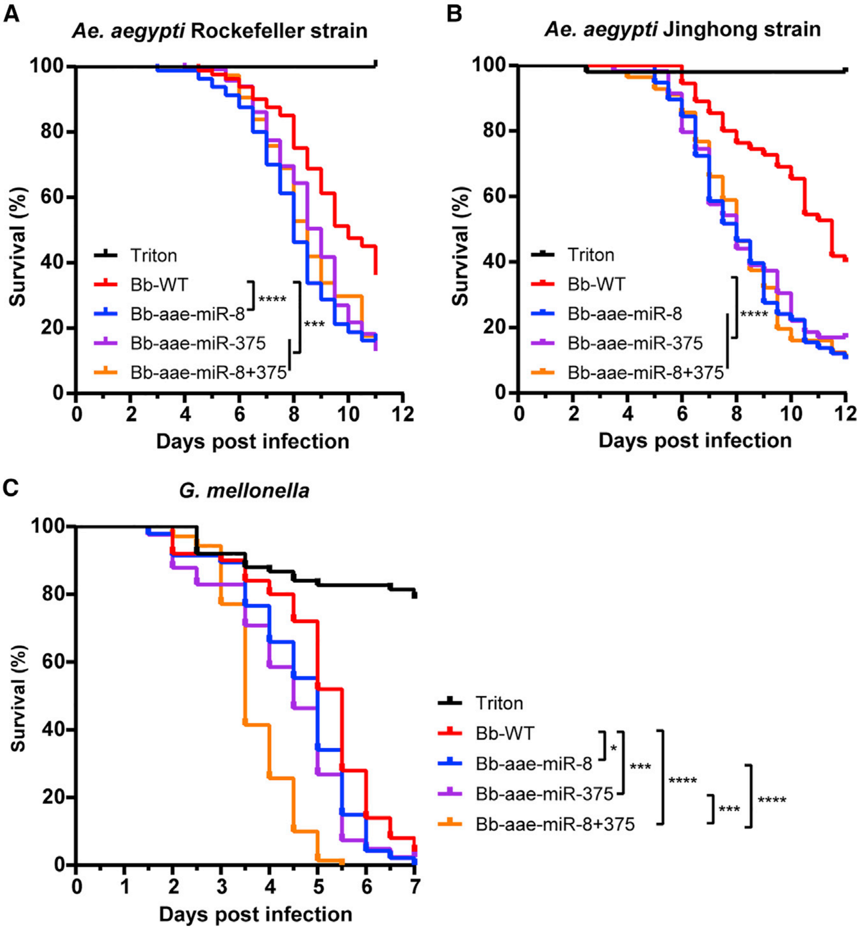

Figure 3. Expressing aae-miR-8 or aae-miR-375 enhances the virulence of *B. bassiana*

(A and B) Survival curves of insecticide-susceptible Rockefeller strain (A) and insecticide-resistant Jinghong strain (B) of adult *Ae. aegypti* female mosquitoes infected with the Bb-WT, Bb-aae-miR-8, Bb-aae-miR-375, or Bb-aae-miR-8+375 strains following topical application of a spore suspension (10^7 conidia/mL).

(C) Survival curves of *G. mellonella* larvae infected with the Bb-WT, Bb-aae-miR-8, Bb-aae-miR-375, or Bb-aae-miR-8+375 strains by immersion in fungal conidial solutions (10^7 conidia/mL). Control insects were treated with 0.01% Triton X-100. Graphs represent percent survival as calculated mean survival rate of each group. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (log rank test).

or Bb-aae-miR-375 strains against *G. mellonella* larvae (Figure 3C), indicating that simultaneous expression of aae-miR-8 and aae-miR-375 by *B. baasiana* has a synergistic effect on fungal virulence against *G. mellonella*. Taken together, these results show that expression of aae-miR-8 or aae-miR-375 substantially increase *B. bassiana* virulence against both insecticide-susceptible and -resistant *Ae. aegypti* mosquitoes, as well as the lepidopteran pest *G. mellonella*.

DISCUSSION

The entomopathogenic fungus *B. bassiana* is currently under intensive study as a biocontrol agent for the control of a variety of insect pests such as mosquitoes and agricultural pests. Insects have evolved refined innate immune mechanisms to defend against pathogenic infection (Dimopoulos, 2003). To successfully cause infection, pathogenic fungi must attenuate the host immune responses. The insect Toll signaling pathway is a principal antifungal defense mechanism via inducing the expression of AMPs (Lemaitre et al., 1996). Here, we report an approach termed pmRNAi for insect control in which expression of host immunosuppressive miRNAs in a fungal pathogen is exploited for attenuation of insect immunity and enhancement of fungal virulence. Expression of two host miRNAs, aae-miR-8 and aae-miR-375, in *B. bassiana* can induce cross-kingdom RNAi to interfere with the expression of host genes involved in the Toll immune pathway, thereby suppressing mosquito immunity and increasing fungal virulence.

miRNAs regulate gene expression posttranscriptionally and are important modulators of insect physiology and development. miR-8 and miR-375 were shown to target multiple genes in various insects. In *D. melanogaster*, miR-8 modulates immune homeostasis by targeting multiple immune genes *Toll*, *U-shaped* (*Ush*), and *Dorsal* (Lee and Hyun, 2014). In addition, miR-8 regulates body growth by suppressing its target *Ush* to regulate insulin signaling and fly body growth (Hyun et al., 2009). miR-8 downregulates the Toll pathway and PPO cascade by upregulating the transcript level of serine protease inhibitor gene *Serpin 27* in *P. xylostella* (Etebari and Asgari, 2013). In female *Ae. aegypti*, miR-8 regulates reproductive process by targeting the Wingless signaling pathway (Lucas et al., 2015), and miR-375 induced upon blood feeding can regulate the transcription of *Rel1* and *Cactus* (Hussain et al., 2013). In this study, we showed that miR-8 can upregulate *Cactus* and downregulate *Toll5B* in *Ae. aegypti*.

Trans-kingdom movement of sRNA has been reported to occur between various hosts and their interacting organisms, which induces cross-species or cross-kingdom RNAi (Zhu et al., 2021). miRNAs or sRNAs can be transported from sickle cell erythrocytes into the malaria parasite (LaMonte et al., 2012), from plants to fungal pathogens (Koch et al., 2013; Nowara et al., 2010), from an insect to a fungal pathogen (Wang et al., 2021b), and from plants to insects (Mao et al., 2007; Nunes and Dean, 2012). Such sRNA signal transfer has been exploited for practical purposes. Plants were engineered to produce dsRNA or sRNAs to silence insect or pathogen genes, an approach termed host-induced gene silencing (HIGS), and has shown promise for the control of various crop-damaging insects and

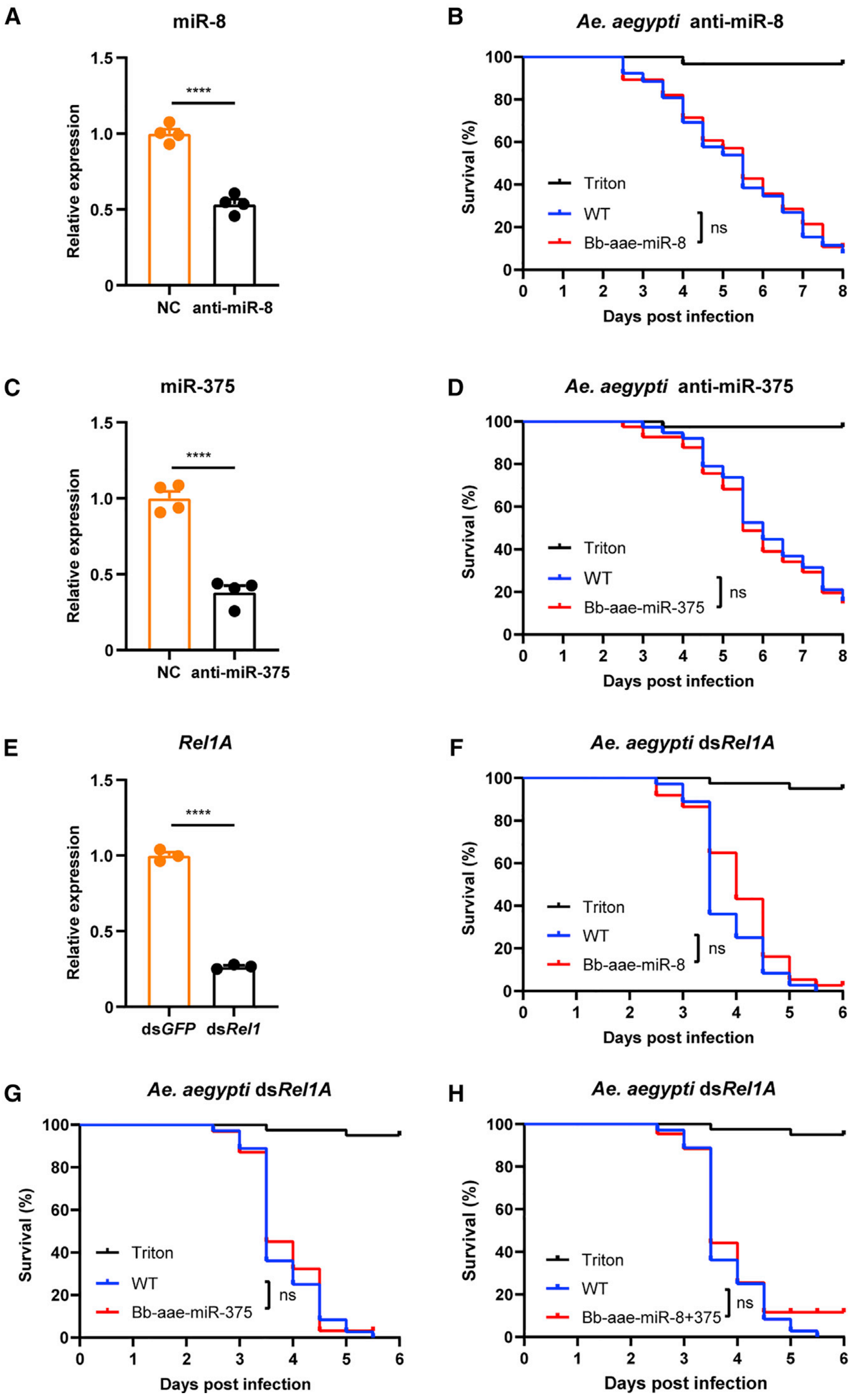

Figure 4. Inhibition of aae-miR-8, aae-miR-375, or mosquito Toll immunity compromises the virulence of the miRNA-expressing fungal strains

(A) Inhibition of aae-miR-8 expression in *Ae. aegypti* Rockefeller strain by injecting antagomir (anti-miR-8). The expression values were normalized to negative control antagomir.

(B) Survival curves of aae-miR-8 antagomir-injected *Ae. aegypti* Rockefeller strain (*Ae. aegypti* anti-miR-8) infected with the Bb-WT or Bb-aae-miR-8 strains following topical application of a spore suspension (10^7 conidia/mL).

(C) Inhibition of aae-miR-375 expression in *Ae. aegypti* Rockefeller strain by injecting antagomir (anti-miR-375). The expression values were normalized to negative control antagomir.

(D) Survival curves of aae-miR-375 antagomir-injected *Ae. aegypti* Rockefeller strain (*Ae. aegypti* anti-miR-375) infected with the Bb-WT or Bb-aae-miR-375 strains following topical application of a spore suspension (10^7 conidia/mL).

(E) *Ae. aegypti* Rockefeller strain was injected with dsRNA of *Rel1A* (ds*Rel1A*) to inhibit the Toll immunity. The expression values were normalized to ds*GFP*.

(F–H) Survival curves of the ds*Rel1A*-injected *Ae. aegypti* mosquitoes infected with the Bb-WT, Bb-aae-miR-8 (F), Bb-aae-miR-375 (G), or Bb-aae-miR-8+375 (H) strains following topical application of a spore suspension (10^7 conidia/mL). Control insects were treated with 0.01% Triton X-100. Graphs represent percentage of survival of each group. ****$p < 0.0001$ (Student's t test). The data are presented as the means ± SEM. Number of dissected mosquitoes (n = 10). ns, not significant (log rank test).

pathogens (Baum et al., 2007; Koch et al., 2013; Mao et al., 2007; Nowara et al., 2010). Reversely, various pathogens and parasites can also deliver sRNAs into their host cells and trigger silencing of host immune related genes to suppress host defense. For example, plant fungal pathogens *Botrytis cinerea* and *Verticillium dahlia* transfer sRNAs into host cells and silence host immune-related genes (Wang et al., 2016; Weiberg et al., 2013). We recently discovered that *B. bassiana* also delivers a miRNA-like RNA (bba-milR1) into mosquito cells during infection and induces cross-kingdom RNAi to silence host immune-related genes and facilitates infection (Cui et al., 2019). In this study, we engineered *B. bassiana* to produce two *Ae. aegypti* miRNAs, aae-miR-8 and aae-miR-375, which suppress mosquito antifungal immune response by interfering with the multiple target genes related to the Toll signaling pathway. The transgenic *B. bassiana* strains displayed greatly increased virulence toward adult *Aedes* mosquitoes and the lepidopteran pest *G. mellonella*. Notably, the host miRNA-expressing fungal strains are highly effective against the insecticide-resistant *Ae. aegypti* mosquitoes, demonstrating that expressing host miRNAs by the insecticidal fungus creates potential for managing insecticide resistance. Moreover, co-expression of miR-8 and miR-375 by *B. baasiana* has synergistic effect on fungal virulence against the lepidopteran host *G. mellonella*. This indicates that expressing host miRNAs has great potential in improving fungal virulence. To reduce the impact of the transgenic fungus on nontarget, beneficial insects like honeybees, strains with high virulence to mosquitoes but low virulence to beneficial insects, or sRNAs specifically targeted to mosquito genes, could be selected. In addition, chemical attractants (Wang et al., 2021a) can be used to increase the chance of mosquitoes getting infected with the fungal insecticide.

Expression of an insecticidal protein to control insect pests may promote the development of resistance (Quan et al., 2021; Wirth, 2010; Zhang et al., 2011). In contrast, use of the host-derived miRNAs in genetic engineering for strain improvement would eliminate the likelihood of the development of resistance because insects do not explicitly force selection for resistance to their endogenous miRNAs that are critical for host immunity and health. Thus, our approach offers a solution to allay the concerns from stakeholders. Moreover, the increase in fungal virulence may be tailored to be insect specific depending on the host miRNA chosen. Furthermore, contrary to the insecticidal proteins, a single miRNA may have multiple target genes in one or more pathways to exert its biological functions, while the expression of a given target gene also be regulated by more than one miRNA, suggesting that miRNAs have combinatorial effects on the target gene. In theory, multiple host miRNAs can be expressed in the same fungal strain to manipulate robust regulation of target genes or pathways important for insect immunity or other physiological processes and enable further increase the efficacy of fungal insecticides. Our study paves the avenue to exploit a wealth of host miRNAs in the genetic engineering of entomopathogens for generating more powerful and safer biopesticides for sustainable control of mosquitoes and other insect pests.

Limitations of the study

In this work, we report that an entomopathogenic fungus can be genetically engineered for potential delivery of host miRNAs into mosquitoes to attenuate their immune system and facilitate fungal infection, which is an example of utilization of cross-kingdom RNAi to control insect pests. This pmRNAi-based technology provides an innovative approach to enhance the efficacy of fungal insecticides and aid in managing insecticide resistance. Our study utilizes two host miRNAs that target the same mosquito immune pathway, and, as a result, co-expression of the two miRNAs had no synergistic effect on fungal virulence against *Aedes* mosquitoes. In future study, it could be considered to express different sets of miRNAs targeting different genes or pathways important for insect immunity or other physiological processes to further increase fungal efficacy. In this study, we used a strong promotor, the *A. nidulans gpdA* promoter, to drive constitutive expression of host miRNAs, which might result in fitness cost. Thereby, efficient control of miRNA expression requires improvement. The infection-stage-specific promotors driving the expression of host miRNAs are preferred. Under control of these promoters, host miRNAs can be expressed in a spatial and temporal manner, which could eliminate unnecessary energy waste during fungal growth and infection and would make the pmRNAi-based approach more effective and safer for the biocontrol of mosquitoes and other insect pests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.111527.

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AUTHOR CONTRIBUTIONS

S.W. and J.L. conceived the study. S.W., C.C., and J.L. designed the experiments. C.C. performed the majority of experiments. Y.W. and C.C. constructed fungal modification. C.C., Y.L., and P.S. reared mosquitoes. C.C. and P.S. analyzed and verified target genes. C.C. synthesized and injected dsRNAs. C.C., Y.W., and Y.L. conducted insect bioassays. J.J. and H.Z. collected and maintained the insecticide-resistant mosquitoes. C.C., Y.W., and J.L. analyzed the data. C.C., J.L., and S.W. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
RNAiso Plus	Takara	Cat#9109
MEGAscript RNAi kit	ThermoFisher	Cat#AM1626
PrimeScript^{TM} RT reagent Kit	Takara	Cat#RR047A
AceQ qPCR SYBR Green Master Mix	Vazyme	Cat#Q111
miRcute miRNA First-Strand cDNA Synthesis Kit	Tiangen	Cat#KR211
miRcute miRNA qPCR detection kit	Tiangen	Cat#FP411
Experimental models: Organisms/strains		
Beauveria bassiana ARSEF252	This paper	N/A
Aedes aegypti Rockefeller strain	This paper	N/A
Aedes aegypti Jinghong strain	This paper	N/A
Oligonucleotides		
miR-8 mimics, sense strand 5′-UA AUACUGUCAGGUAAAGAUGUC-3′, antisense strand 5′-GACAUCUUU ACCUGACAGUAUUA-3′	Sangon Biotech	N/A
Negative Control miRNA Mimics, sense strand 5′-UUGUACUACACA AAAGUACUG-3′, antisense strand 5′-GUACUUUUGUGUAGUACAAUU-3′	Sangon Biotech	N/A
miR-8 Antagomir, 5′-GACAUCUUU ACCUGACAGUAUUA-3′	Sangon Biotech	N/A
miR-375 Antagomir, 5′-UAACUCGA GCCAAACGAACAAA-3′	Sangon Biotech	N/A
Negative Control Antagomir, 5′-CAGUA CUUUUGUGUAGUACAA-3′	Sangon Biotech	N/A
Cell lines		
HEK293T	ATCC	Cat: CRL-3216
Software and algorithms		
Microsoft Excel	Microsoft	N/A
GraphPad Prism 5.0	GraphPad Prism	N/A
Adobe Illustrator	Adobe	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sibao Wang (sbwang@cemps.ac.cn).

Materials availability

This study did not generate new unique reagents. Requests for resources will be fulfilled by the lead contact with a completed Materials Transfer Agreement.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. The study did not generate any unique code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mosquitoes

The insecticide-susceptible *Aedes aegypti* (Rockefeller strain) and insecticide-resistant *Ae. aegypti* (Jinghong 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. Adult mosquitoes were maintained on 10% (wt/vol) sucrose. The 3-day-old female mosquitoes were applied to experiments.

Fungi

Beauveria bassiana ARSEF252 and transgenic fungal strains were cultured and maintained on Sabouraud dextrose agar plus yeast extract (SDAY; BD Difco) at 26°C.

Cells

HEK293T cells, which were originally isolated from human embryo kidney tissue, were purchased from ATCC. HEK293T cells were grown in DMEM/HIGH GLUCOSE medium (HyClone) containing 10% (vol/vol) heat-inactivated FBS (Gibco) and 1×antibiotic-antimycotic (Gibco) at 37°C under 5% CO_2.

METHOD DETAILS

Construction of miRNA expression vector

To express host miRNAs in *B. bassiana* ARSEF252, the ~400-bp fragment surrounding mature aae-miR-8 (Figure S4A) was amplified by primers Pmir8-F and Pmir8-R (Table S2) using *Ae. aegypti* genomic DNA as templates. The fragment was then subcloned into *Eco*RI and *Eco*RV restriction sites of the binary vector (pBarGFP-PgpdA) under control of a constitutive *Aspergillus nidulans glyceraldehyde phosphate dehydrogenase* promoter (PgpdA) to obtain pBarGFP-PgpdA-miR-8. To construct miR-375 expression vector, the primer pairs Pmir375-F and Pmir375-R were used to amply ~400-bp fragment surrounding mature aae-miR-375 (Figure S4B). The DNA fragment was homologously recombinated into pBarGFP-PgpdA digested by *Eco*RI and *Eco*RV using ClonExpress® II One Step Cloning Kit (Vazyme) to generate pBarGFP-PgpdA-miR-375. For co-expression of miR-8 and miR-375, the miR-375 fragment was subcloned into pBarGFP-PgpdA-miR-8 mentioned above. In details, the primer pairs Pmir375-1s-F and Pmir-375-1s-R were used to amply miR-375 fragment. Then, the miR-375 fragment was homologously recombinated into pBarGFP-PgpdA-miR-8 digested by *Eco*RV using ClonExpress® II One Step Cloning Kit (Vazyme) to obtain pBarGFP-PgpdA-miR-8-miR-375.

Fungus genetic transformation

The miRNA expression vectors were separately transformed into *B. bassiana* ARSEF252 by *Agrobacterium tumefaciens*-mediated transformation to generate host miRNA-expressing strains as previously described (Wang et al., 2011).

dsRNA-mediated gene silencing in adult *Aedes* mosquitoes

To produce the double-stranded RNA of the gene *Toll5B* (ds*Toll5B*), a 489 bp fragment in the coding region of the *Toll5B* gene was PCR-amplified from *Ae. aegypti* cDNA with forward and reverse primers containing the T7 promoter sequence at their 5′ ends (5′-TAATACGACTCACTATAGGG-3′) (Table S2). The PCR products were purified with Cycle-Pure Kit (OMEGA) and used as the template to synthesize dsRNA *in vitro* using the MEGAscript RNAi kit (ThermoFisher). The synthesized dsRNA was purified using the purification column supplied with the kit, eluted with nuclease-free water, and concentrated to 3 μg/μL using a Microcon YM-100 filter (Millipore). A 497 bp dsRNA of *Rel1A* was synthesized as described above. An enhanced green fluorescent protein (eGFP)-derived double-stranded RNA (ds*GFP*) was synthesized to use as a negative control. For dsRNA microinjection, 3-day-old female mosquitoes were injected with 69 nL dsRNA solution (3 ng/μL) into the mosquito hemocoel using Nanoject II microinfector (Drummond). The injected mosquitoes were allowed to recover for 2 to 3 days before performing fungal infection.

miRNA antagomir injection

miRNA Antagomir is a chemically modified single-strand oligonucleotides. The 3-day-old female mosquitoes were microinjected using Nanoject II microinfector (Drummond) into the thorax with 69 nL antagomir (50 μM). Control mosquitoes were injected with negative control Antagomir (50 μM). aae-miR-8, aae-miR-375 and control antagomir were synthesized by Sangon Biotech (Shanghai, China). Mosquitoes were allowed to recover for 2–3 d before fungus infection.

RNA isolation and qRT-PCR

Total RNA was extracted from mosquito fat body or fungal hypha by using RNAiso Plus (TaKaRa) and treated with Recombinant DNase I (TaKaRa) according to the manufacturer's instruction. For mRNA, cDNAs were synthesized from 1 μg total RNA by using PrimeScript^{TM} RT reagent Kit (TaKaRa). Quantitative reverse transcription PCR (qRT-PCR) reactions were carried out by using the AceQ qPCR SYBR Green Master Mix (Vazyme). For miRNAs, cDNAs were reverse transcribed by using miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen), and qRT-PCR was conducted using miRcute miRNA qPCR detection kit (Tiangen) according to the manufacturer's instruction. Each sample was performed in triplicate, and relative expression was calculated using the 2-ΔΔCt method through normalized with housekeeping gene Actin and U6 snRNA for mRNA and miRNA, respectively.

Dual luciferase reporter assay

The ∼400-bp fragment surrounding the predicted miRNA target sites in *Toll5B* was cloned into the psiCheck-2 vector (Promega) using the *Xho*I and *Not*I sites, and *Cactus* was cloned into the *Nhe*I site of the psiCheck-2 vector. Mutagenesis PCR was conducted to introduce point mutations at the miRNA target sites to construct psiCheck-2-mut vectors. The HEK293T cells were transfected with 100 ng of psiCheck-2 reporters with 100 nM of synthetic miRNA Mimics (miR-8, sense strand 5′-UAAUACUGUCAGGUAAAGAUGUC-3′, antisense strand 5′-GACAUCUUUACCUGACAGUAUUA-3′, Sangon Biotech) or Negative Control miRNA Mimics (sense strand 5′-UUGUACUACACAAAAGUACUG-3′, antisense strand 5′-GUACUUUUGUGUAGUACAAUU-3′) using Attractene Transfection Reagent (Qiagen). Cells were collected and lysed at 48 h after transfection, and luciferase activities were measured using the dual luciferase reporter assay system (Promega). Each sample was performed in triplicate and transfection was repeated three times.

Fungal infection bioassay

The virulence of wild type and the host miRNA-expressing *B*. *bassiana* strains Bb-aae-miR-8, Bb-aae-miR-375 and Bb-aae-miR-8+375 were evaluated against female adult *Ae*. *aegypti* and *G*. *mellonella* larvae. *B*. *bassiana* conidia were obtained from 10-day-old SDAY cultures. Conidia suspensions were prepared in 0.01% (vol/vol) Triton X-100 and filtered through layers of sterile glass wool to remove hyphal fragments. For mosquito infection, three-day-old female *Ae*. *aegypti* adult mosquitoes were cold anesthetized, transferred onto filter paper and sprayed with fungal conidial suspension (10^7 conidia/mL). The treated mosquitoes were maintained in cardboard paper cup with 10% glucose at 28°C and 80% relative humidity. Moisturized filter paper was placed on the top of cup to induce spore germination for 48 h. The mortalities were recorded every 12 h and cadavers were transferred to moisturized filter paper to monitor the emergence of fungal hyphae. For negative control, mosquitoes were sprayed with 0.01% Triton X-100. The bioassays were repeated three times, and each treatment was replicated three times with 50 adult mosquitoes per replicate. For *G*. *mellonella* larva infection, the larvae were inoculated by topical immersion in fungal spore suspension (10^7 conidia/mL) or 0.01% Triton X-100 (control) for 30 s. Mortality was recorded every 12 h. Each treatment was replicated three times with 25 *G*. *mellonella* larvae per replicate, and the bioassays were repeated three times.

QUANTIFICATION AND STATISTICAL ANALYSIS

The details of statistical methods are listed in the figure legends. The statistical significance of the survival data from fungal infection bioassays and Triton X-100 treated insects (control) was analyzed with a log rank (Mantel-Cox) test. Other statistical significance between two treatments was determined by Student's t test for unpaired comparisons. The p-value of <0.05 was regarded as statistically significant. All statistics were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software).