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Epigenetic Regulation in Insect–Microbe Interactions

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Abstract

Insects have evolved diverse interactions with a variety of microbes, such as pathogenic fungi, bacteria, and viruses. The immune responses of insect hosts, along with the dynamic infection process of microbes in response to the changing host environment and defenses, require rapid and fine-tuned regulation of gene expression programs. Epigenetic mechanisms, including DNA methylation, histone modifications, and noncoding RNA regulation, play important roles in regulating the expression of genes involved in insect immunity and microbial pathogenicity. This review highlights recent discoveries and insights into epigenetic regulatory mechanisms that modulate insect–microbe interactions. A deeper understanding of these regulatory mechanisms underlying insect–microbe interactions holds promise for the development of novel strategies for biological control of insect pests and mitigation of vector-borne diseases.

INTRODUCTION

Insects, including agricultural pests, disease vectors, and pollinators, have a great impact on crop agriculture and human health. They have evolved diverse interactions with a variety of microbes, including pathogenic fungi, bacteria, and viruses. Entomopathogenic microbes, which specifically infect insects, offer a promising environmentally friendly alternative for controlling insect pests and insect-borne diseases (70). Meanwhile, insect vectors harbor highly diverse and dynamic symbiotic bacteria that hold potential for preventing pathogen transmission (23). A deeper understanding of the underlying mechanisms governing insect–microbe interactions is crucial for better controlling insect pests and vector-borne diseases, as well as for enhancing the efficacy of entomopathogenic microbes and symbiotic bacteria.

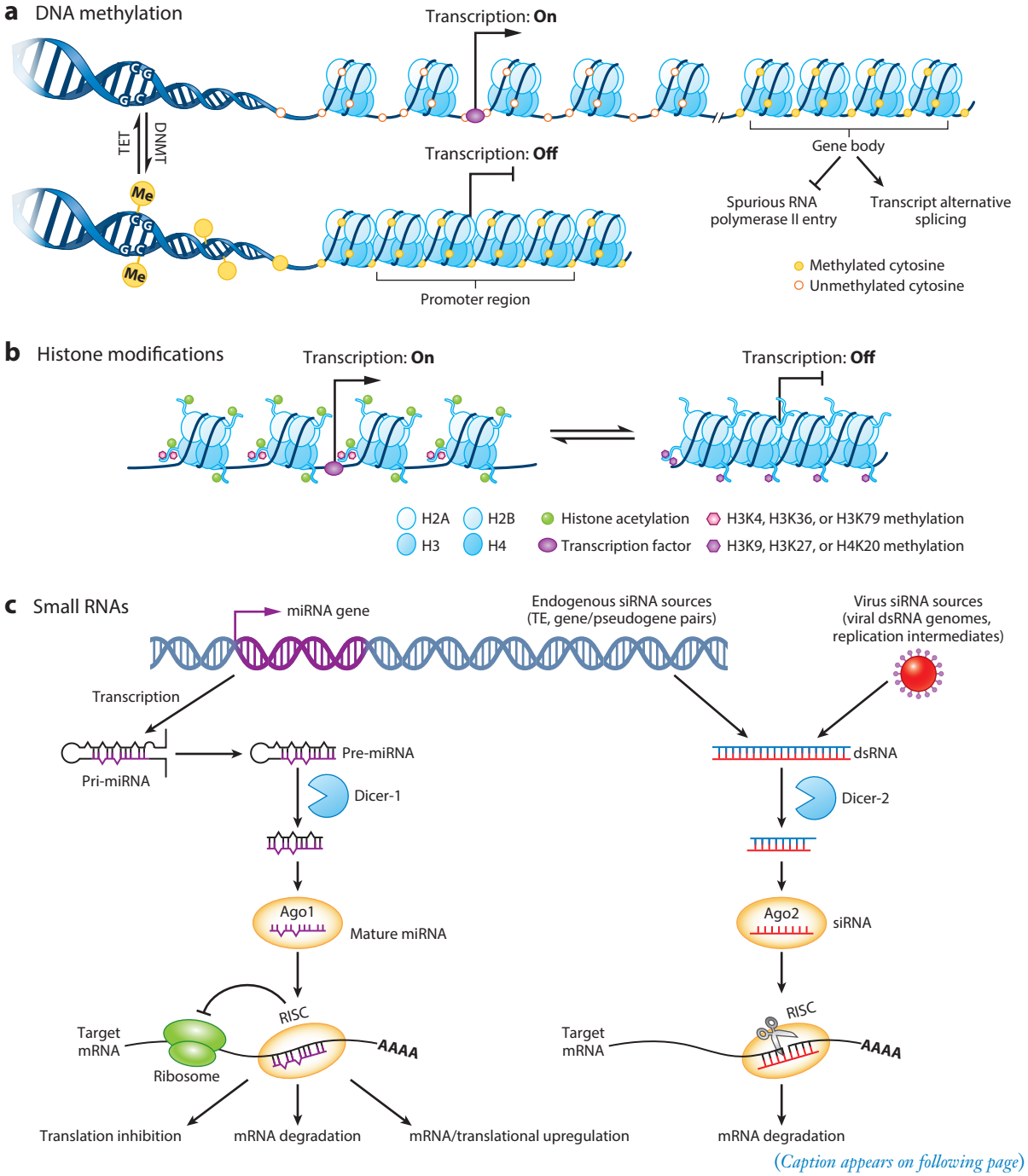
To counteract microbial infections, the insect host activates and manipulates its innate immune system via signaling pathways to produce effectors capable of inhibiting microbe replication. Activation of the defense relies on the insect's capacity to extensively reprogram its gene expression upon recognition of microbes (77). Conversely, to successfully infect and colonize the host insect, microbes have evolved intricate strategies to invade, colonize, and replicate in the host that involve morphological and developmental changes, secretion of virulence factors, and assimilation of host nutrients (31, 52). The dynamic infection process also requires rapid and fine-tuned regulation of microbial gene expression programs in response to the changing host environment and defenses (31).

Among the mechanisms driving this vital transcriptional reprogramming for the insect host and microbes, epigenetic regulatory mechanisms have rapidly emerged as significant players, especially in the regulation of insect–microbe interactions. Epigenetic regulations refer to heritable changes in gene expression that do not involve altering the DNA sequence, including DNA methylation, histone modifications, and noncoding RNA regulation such as small RNA (sRNA)-mediated gene interference (11) (**Figure 1**). Epigenetics plays vital roles in regulating insect and microbe development, reproduction, microbe-induced modulation of insect physiology and immunity, and insect-induced alteration of microbial pathogenicity (77). In the sections that follow, we outline recent advancements in the study of epigenetic regulations, with a focus on the roles of DNA methylation, histone modification, and sRNA regulation in insect–microbe interactions.

DNA METHYLATION IN INSECT–MICROBE INTERACTIONS

DNA Methylation in Insects

DNA methylation occurs mainly as 5-methylcytosine (5mC) in genomic DNA, which is an epigenetic mechanism in eukaryotes. A conserved set of proteins, called DNA methyltransferases (DNMTs), transfer a methyl group from *S*-adenyl methionine onto the C5 position of the cytosine to form 5mC (**Figure 1*a***). In animals, de novo methyltransferase DNMT3 establishes new methylation marks on unmethylated CG sites, whereas the maintenance methyltransferase DNMT1 maintains preexisting methylation patterns by preferentially methylating hemimethylated DNA substrates (27). In contrast, DNMT2, initially misclassified, is now recognized for its role in transfer RNA methylation (28, 71). DNMT3 has been solely identified in insect taxa spanning four orders (Coleoptera, Hymenoptera, Hemiptera, and Blattodea). DNMT1 is found in numerous insect orders except Diptera and has undergone duplication in some groups. This duplication event is proposed to compensate for DNMT3 (8). DNMT2 is the most evolutionarily conserved and is the only known DNMT present in dipterans such as *Drosophila melanogaster*, *Aedes aegypti*, and *Anopheles gambiae* (56). The absence of a complete typical DNA methylation toolkit suggests the potential existence of alternative enzymatic machinery in insects (8). DNA



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Gene regulation by DNA methylation, histone modifications, and small RNAs. (*a*) DNA methylation occurs mainly at the cytosine bases in genomic DNA, which is a reversible process catalyzed by DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) dioxygenases. DNA methylation in the promoter region generally serves as a repressive transcriptional signal, while gene body DNA methylation is associated with active transcription, prevents spurious RNA polymerase II entry, and affects transcript alternative splicing. (*b*) Histone N-terminal tails undergo posttranslational modifications such as acetylation and methylation. Generally, histone methylation of H3K4, H3K36, and H3K79, as well as histone acetylation, promotes open chromatin states that are accessible to the transcription machinery, thus enhancing gene expression. In contrast, histone methylation of H3K9, H3K27, and H4K20, along with histone deacetylation, induces condensed chromatin configurations, restricting DNA accessibility and suppressing gene transcription. (*c*) Biogenesis of small RNAs. For microRNAs (miRNAs), primary miRNA (pri-miRNA) is transcribed and forms stem-loop structures. Subsequently, the stem-loop at the base is cleaved to generate precursor miRNA (pre-miRNA) that is transported into the cytoplasm. The ribonuclease enzyme Dicer-1 removes the hairpin head to produce a short double-stranded RNA (dsRNA) that associates with Argonaute 1 (Ago1) to assemble the RNA-induced silencing complex (RISC). The miRNA passenger strand undergoes degradation, while the guide strand directs the RISC toward target messenger RNAs (mRNAs), initiating mRNA degradation, translation inhibition, and mRNA or translational upregulation. For small interfering RNAs (siRNAs), dsRNA precursors from endogenous [including transposable elements (TEs) and gene/pseudogene pairs] or viral [including viral dsRNA genomes and replication intermediates] sources are processed by Dicer-2 into siRNA duplexes, which are then loaded into Ago2-containing RISC. The passenger strand is degraded, and the guide strand mediates target mRNA degradation (slicing).

methylation is a reversible process. 5mC can be converted into hydroxymethylcytosine through oxidative demethylation catalyzed by ten-eleven translocation (TET) dioxygenases. Insects seem to encode a single TET homolog, as reported for only a few species (18, 85).

DNA methylation is widespread in insects, but DNA methylation levels are highly variable across insect orders (8). Holometabolous insects, in particular, display reduced DNA methylation levels (85). Within the order Blattodea (cockroaches and termites), there appears to be a potential loss of DNA methylation in eusocial species, whereas solitary species exhibit the highest levels (8). Unlike in vertebrate genomes, which tend to be globally methylated, DNA methylation in insects occurs primarily within gene bodies, particularly in evolutionarily conserved cellular housekeeping genes (3, 8, 92). Generally, DNA methylation at gene promoters, as is observed in mammals and plants, functions as a repressive transcriptional signal, impeding the binding of transcription factors to DNA or recruiting proteins, such as methyl-CpG-binding domain proteins, along with other epigenetic modifications, to suppress gene expressions through changes in chromatin structure, conformation, and stability (54). However, gene body methylation, which is a type of gene methylation found in insects, is often positively associated with active transcription (92). In some cases, methylated DNA can be read and bound by transcriptional antisilencing factors, which in turn recruit other regulatory proteins to enhance gene transcription (33). Intragenic DNA methylation is also negatively associated with transcriptional noise by preventing spurious RNA polymerase II entry and cryptic transcription initiation (82, 117). Moreover, DNA methylation may affect transcript alternative splicing and histone occupancy in insects (26, 66). DNA methylation in insects plays important roles in caste development, longevity, reproduction, embryogenesis, behavior regulation, and insecticide resistance (21, 46, 72, 99, 120, 127).

Insect DNA Methylation Regulates Immune Responses to Microbes

Upon encountering microbes, insects dynamically remodel their DNA methylation patterns, thereby achieving epigenetic control over gene expression. In *Bombyx mori*, infection with the cytoplasmic polyhedrosis virus (BmCPV) induces tissue-specific alterations in genomic DNA methylation patterns and transcriptional profiles, suggesting a potential mediation of gene expression changes via DNA methylation variation (114). Similarly, infection with *B. mori* nucleopolyhedrosis virus (BmNPV) leads to significant changes in both DNA methylation patterns and host gene expression profiles. Inhibition of DNMT activity in *B. mori* reduces the expression

of antiapoptosis family genes, leading to increased cell apoptosis and suppression of BmNPV, indicating a negative role of host DNA methylation in insect immunity against virus (38). However, during viral infection, differentially methylated genes in honey bees (*Apis mellifera*) do not significantly overlap with differentially expressed genes, suggesting that insect transcriptional changes may not be directly governed by DNA methylation in response to viral infection (22).

Accumulating evidence shows that host DNA methylation facilitates optimal proliferation of bacteria in insects, and inhibition of host DNA methylation help the insect to eliminate bacterial infection. DNMTs in *Helicoverpa armigera* and *Antheraea pernyi* are induced upon pathogenic bacterial infection, potentially modulating bacterial proliferation by affecting the expression of antimicrobial peptides (AMPs), crucial components of insect antibacterial immunity (6, 49). Inhibition of DNMTs by 5-azacytidine increases AMP expression levels, attenuates bacterial infection, and improves host survival, suggesting a negative relationship between DNA methylation and insect immunity. Pathogenic and commensal bacteria trigger differential DNA methylation levels in *Galleria mellonella*, along with various transcriptional reprogramming of the host innate immune system, resulting in activation of host immunity by pathogenic bacteria but suppression by commensals (36). However, the direct impact of DNA methylation status on the expression of immunity-related genes, as well as the underlying molecular mechanism by which DNA methylation regulates host immune responses, remains unexplored.

DNA methylation appears to modulate insect immune responses against entomopathogenic fungi as well. *G. mellonella* larvae exhibit differential expressions of DNMTs when infected with three different strains of *Metarhizium anisopliae* (101). Unlike entomopathogenic viruses and bacteria that infect their insect hosts through the intestine, entomopathogenic fungi invade insects directly through the integument by secreting cuticle-degrading enzymes (86, 130). Thus, the insect cuticle serves as the primary physical barrier against fungal infection, and the fat body plays a critical role in systemic immune responses (78). Tissue-specific differences in DNA methylation are implicated in the experimental evolution of resistance against fungi. Initially, global DNA methylation levels are higher in the fat body than in the cuticle of fungus-susceptible *G. mellonella* larvae, but no significant differences persist between these tissues in resistant larvae over multiple generations in response to repeated encounters with *Metarhizium robertsii* (78). In addition, resistant larvae exhibit lower DNA methylation levels than susceptible larvae, indicating a negative regulatory role of DNA methylation in insect immunity against fungal pathogens (78).

Methylation of Microbial DNA Regulates Pathogenicity

Methylation of microbial DNA plays an important role in modulating microbial pathogenicity. Certain enterobacteria belonging to the genera *Photorhabdus* and *Xenorhabdus*, which are symbiotically associated with entomopathogenic nematodes, exhibit pronounced pathogenicity to insects. After invasion of the insect host by the nematodes, bacteria are released into the insect hemocoel, inducing septicemia and ultimately leading to lethality through the deployment of a diverse array of virulence factors (25, 83). Notably, DNA adenine methyltransferase (Dam), a well-characterized orphan DNMT in bacteria, emerges as an important regulator of virulence genes (84). Overexpression of *Dam* in *Photorhabdus luminescens* does not affect bacterial growth but results in the downregulation of flagellar genes, leading to a significant decrease in motility and a delayed virulence after infection in larvae of the lepidopteran *Spodoptera littoralis* (83). Conversely, *dam* overexpression does not modify the virulence properties in *Xenorhabdus nematophila*, indicating that this evolutionarily conserved DNMT may play diverse roles in different pathogenic bacteria (25).

In fungi, different DNMTs, including DNMT1/Masc2, Dim-2, RID/Masc1, and DNMT5, have been identified (81). Fungal DNA methylation generally distributes in transposable elements, repeat sequences, gene promoters, and transcribed regions, contributing to transposon silencing

and modulation of gene expression (34). Importantly, DNA methylation in fungi has profound effects on various aspects of fungal biology, including fungal growth, development, secondary metabolism, and pathogenicity. Notably, a global reprogramming of DNA methylation during fungal development in the insect pathogenic fungus *Cordyceps militaris*, albeit without direct association with genes involved in fungal sexual development, has been observed (109). Similarly, a dynamic shift in DNA methylation patterns occurs between conidial and mycelial stages in *M. robertsii* (64). Knockout of *Dim-2* in *M. robertsii* causes a marked reduction in fungal virulence toward insect hosts (108). Moreover, DNA methylation plays a pivotal role in regulating the expression of virulence factors in *M. robertsii*, such as genes encoding the insecticidal metabolite destruxin, and the collagen-like protein MCL1 involved in blastospore coating and immune evasion, particularly in response to tick cuticle exposure. This finding underscores a regulatory role of DNA methylation in orchestrating dynamic gene expressions during the infection process (93).

HISTONE MODIFICATIONS IN INSECT–MICROBE INTERACTIONS

In eukaryotic cells, genomic DNA is wrapped around a histone octamer comprising two copies of each core histone (H2A, H2B, H3, and H4), forming nucleosomes that constitute chromatins. The histone N-terminal tails can undergo posttranslational modifications such as acetylation and methylation to modulate the positive charge density of the core histones, leading to the conformational change in chromatin structure and consequently influencing DNA accessibility (53). Generally, histone methylation of H3K4, H3K36 and H3K79, as well as histone acetylation, forms loose chromatins accessible to the transcription machinery and thus promotes gene expression. In contrast, histone methylation of H3K9, H3K27, and H4K20, as well as histone deacetylation, induces condensed chromatins, thereby restricting DNA accessibility and suppressing gene transcription (53) (**Figure 1*b***).

Histone acetylation and histone methylation represent dynamic and reversible processes regulated by the opposing activities of histone acetyltransferases (HATs)/histone methyltransferases and histone deacetylases (HDACs)/histone demethyltransferases. Maintaining equilibrium between these opposing enzymatic activities is essential for insect growth, development, and metamorphosis (77). A recent study shows that a bacteriocyte symbiont-derived folate can modulate H3K9 trimethylation in whitefly, thereby influencing ovary mitochondrial function and ultimately determining host sex ratio (121).

Histone Modifications Regulate Insect Immunity

The role of histone modifications in modulating insect–pathogen interactions has received limited attention. Pathogen infection can induce histone modification changes in the insect host, subsequently contributing to transcriptional remodeling during microbial infection (91). For instance, in *An. gambiae* mosquitoes, genomic loci exhibiting dynamic histone modifications post pathogen infection correlate with genes involved in immune functions, including AMPs, CLIP proteases, and members of the melanization and complement systems, suggesting that histone modifications play a key role in mediating transcriptional responses to infection (32, 91). Infection with the entomopathogenic fungus *M. anisopliae* or the bacterial pathogen *Listeria monocytogenes* elicits a stronger induction of HDACs than of HATs in *G. mellonella* larvae, suggesting that microbial pathogens preferentially modulate HDACs to suppress the expression of host genes during infection (79). The dysregulation of the delicate balance between HATs and HDACs by pathogens results in aberrant metamorphosis and delayed pupation in insects. In contrast, treatment with HDAC inhibitors accelerates pupation and increases survival by upregulating genes encoding a matrix metalloproteinase for wound healing, an inhibitor of microbial metalloproteinases causing sepsis, and immunity-related signaling kinases (79). These

findings underscore the ability of pathogens to manipulate host histone deacetylation to suppress immunity-related gene expression and facilitate infection.

Conversely, in some cases, histone acetylation in insects is induced to confer resistance to pathogens. Following Zika virus (ZIKV) infection, *Ae. aegypti* exhibits heightened expression of the HAT CREB-binding protein (CBP), leading to upregulation of AMP genes via H3K27ac modification and thereby suppressing ZIKV infection and increasing mosquito survival (2). Treatment of mosquitoes with sodium butyrate, an HDAC inhibitor, results in hyperacetylation of H3K27 and induction of mosquito immune responses (2). A similar effect has been observed in the western honey bee, *A. mellifera*, where inhibition of HDACs by sodium butyrate causes histone hyperacetylation. This hyperacetylation event leads to the activation of genes involved in immune signaling and detoxification, thereby bolstering the honey bee's immune response to viral infection (37). Histone acetylation may also play a pivotal role in immune priming, whereby prior infections enhance subsequent immune response in insects (29). In *An. gambiae*, immune priming is mediated by the systemic release of a hemocyte differentiation factor (HDF), which increases the proportion of circulating granulocytes and enhances cellular immunity (89). Silencing of the HAT Tip60 in *An. gambiae* abolishes immune priming, indicating that it is essential for HDF synthesis to maintain immune priming, although the underlying histone regulatory mechanism is still unknown (30).

Although the activation of immune-related signaling pathways is required for eliminating infectious pathogens via immune responses, hyperactivation of immune responses can have cytotoxic effects (50). Loss of H3K9 methyltransferase G9a in *D. melanogaster* leads to hyperactivation of the Jak/Stat pathway–mediated responses, resulting in early lethality in RNA virus–infected flies, indicating that G9a regulates tolerance to virus infection by modulating the Jak/Stat pathway response (75). Likewise, histone deacetylase 1 (HDAC1) is recruited to inhibit transcription of antimicrobial effector genes to prevent the excessive activation of the NF-κB-mediated immune response in *D. melanogaster* (50, 51, 76). These studies suggest that insect immune responses are under tight regulation by histone modifications to achieve controlled and transient activation for pathogen elimination.

Histone Modifications Regulate Fungal Pathogenicity

Entomopathogenic fungi undergo significant infection-induced morphological changes and develop an array of infection structures during the course of infection (103). Upon adherence to the hydrophobic insect cuticle, fungal conidia germinate and differentiate into a specialized infection structure called an appressorium. Under the combined action of cuticle-degrading enzymes and turgor pressure derived from accumulating glycerol, the appressorium penetrates the cuticle and breaks into the insect body. The penetrating mycelium further differentiates into hyphal bodies, which evade host immune responses and utilize the nutrient-rich hemolymph for growth. Concurrently, fungi secrete effectors and toxins to suppress host immune defenses. Growing evidence shows that histone modifications play indispensable roles in rapid and fine-tuned regulation of fungal gene expression programs in response to the changing host environment and defenses (53).

Upon cuticle induction, the histone lysine methyltransferase KMT2 in *M. robertsii* is upregulated, activating the transcription factor gene *cre1* via H3K4 trimethylation (H3K4me3) (52). Cre1 further regulates the cuticle-induced gene *hyd4* required for cell hydrophobicity to trigger appressorium formation. This KMT2-Cre1-Hyd4 regulatory pathway was further demonstrated to function in another entomopathogenic fungus, *Beauveria bassiana*, regulating both virulence and stress responses (88). After appressorium formation, another histone methyltransferase, ASH1, is strongly induced and regulates appressorium turgor generation and cuticle penetration by activating the peroxin gene *pex16* via H3K36 dimethylation (H3K36me2) (104). Pex16 is essential for the biogenesis of peroxisomes that promote lipid hydrolysis to produce large

amounts of glycerol for turgor generation in the appressorium. Moreover, deletion of defective in methylation 5 (*dim5*), which is responsible for H3K9 methylation in *B. bassiana*, a hallmark of gene repression, blocks the biosynthesis and secretion of cuticle-degrading enzymes, resulting in loss of fungal insect pathogenicity through cuticle infection (87). These findings indicate that histone methylation plays a crucial role in regulating appressorium formation, turgor generation, cuticle degradation, and fungal pathogenicity.

Histone acetylation mediated by HATs such as Gcn5, Spt10, Sas3, and Rtt109, as well as histone deacetylation mediated by HDACs like Rpd3, Hos2, and Sir2, is also crucial for fungal virulence against insect hosts (53). Histone acetylation usually distributes across the fungal genome and regulates global gene expression. Therefore, single deletion of these enzyme-encoding genes in fungi usually elicits pleiotropic effects on asexual development, stress responses, secondary metabolite biosynthesis, and pathogenicity (53). However, the key target genes of these enzymes and downstream virulence factors contributing to fungal pathogenicity require further investigation. A recent study showed that fungal HAT1 and HDAC1 function in a cascade to regulate the expression of stage-specific genes, facilitating adaptation to distinct cuticular and hemocoel microenvironments. When the fungus *M. robertsii* penetrates into the insect hemocoel, decreased HAT1-mediated H3K4ac downregulates HDAC1, resulting in the derepression of H3K56ac and subsequent activation of the gene encoding the regulatory protein colonization of hemocoel 1 (COH1). COH1 interacts with colonization of hemocoel 2 (COH2) to reduce COH2 stability, which represses cuticle penetration genes and activates genes involved in hemocoel colonization (126).

SMALL RNAs IN INSECT–MICROBE INTERACTIONS

sRNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), are a class of short regulatory noncoding RNAs of 18 to 24 nucleotides that regulate gene expressions at post-transcriptional and translational levels in eukaryotes. Primary miRNA (pri-miRNA) is transcribed by RNA polymerase II and comprises stem-loop structures (1). The nuclear ribonuclease protein Drosha cleaves the stem-loop at the base to generate precursor miRNA (pre-miRNA), which is further transported into the cellular cytoplasm with the help of Exportin-5. Another ribonuclease enzyme, Dicer-1, removes the hairpin head to produce a short double-stranded RNA (dsRNA) that associates with Argonaute 1 (Ago1) to assemble the RNA-induced silencing complex (RISC). Subsequently, the miRNA passenger strand undergoes degradation, while the guide strand directs the RISC toward target messenger RNA (mRNA) (**Figure 1*c***). While most reports have shown that miRNAs usually negatively regulate their target genes, either by suppression of translation or by mRNA degradation (16, 17, 41), recent reports have demonstrated that miRNAs may have a positive regulatory effect on the target by promoting transcription (35, 45), transcript stabilization (42, 73), or translation (98). In contrast, dsRNA precursors originating from endogenous sources (such as transposable elements, gene/pseudogene pairs, structured RNA, and convergent transcripts) or virus sources (viral dsRNA genomes, replication intermediates, structured RNA, and convergent transcripts) are processed into siRNAs by Dicer-2, and the siRNA duplex is loaded into an Ago2-containing RISC (9, 94, 102, 110) (**Figure 1*c***). sRNAs act as key regulators of various biological processes, including development, insect immune responses, pathogen pathogenicity, and communications in insect–microbe interactions (1, 4, 15, 68, 69). Notably, a single mRNA can be targeted by multiple sRNAs, and similarly, individual sRNA can target multiple mRNAs.

Roles of sRNAs in Insect Immunity

Diverse host sRNAs play important roles in fine-tuning insect immunity. In *Drosophila* spp., miR-8, miR-317, miR-958, and miR-959–962 cluster members negatively regulate the Toll signaling

pathway by directly targeting mRNAs of the key components of the Toll signaling pathway, such as the transmembrane receptor Toll protein and the transcription factors Dif and Dorsal (55, 57, 58, 60). Additionally, miR-310 family members and miR-964 negatively regulate the Toll pathway by targeting *Drosomycin* mRNA (62, 65). In *Ae. aegypti*, aae-miR-375, which is induced by a blood meal, increases RNA levels of *cactus* (the inhibitor of the Toll pathway) and decreases RNA levels of *REL1* (the NF-κB transcription factor of the Toll pathway) (45). In contrast, in *Plutella xylostella*, miR-8 positively regulates the expression of serine protease inhibitor Serpin-27, thereby inhibiting activation of the Toll pathway and maintaining a low level of AMPs in the absence of infection (20). Furthermore, miR-959–962 cluster members are upregulated at the late stage of bacterial infection, suggesting their potential role in attenuating an exaggerated immune response and maintaining immune homeostasis (58).

miRNAs are also important regulators of the IMD and Jun N-terminal kinase (JNK) signaling pathways. Let-7, miR-9a, miR-981, and miR-317, for example, directly inhibit the expression of the AMP Diptericin or the Imd receptor PGRP-LC, thereby negatively modulating the IMD pathway (24, 61, 128). Moreover, the transcription factor Myc activates miR-277 to inhibit the expression of the *imd/Tab2* gene, revealing a new Myc–miR-277–*imd/Tab2* axis involved in immune homeostasis maintenance (59). Conversely, miR-34 activates antibacterial innate immunity by directly repressing genes encoding the septate junction protein Dlg1 and nuclear hormone family transcription factor Eip75B, indirectly increasing Diptericin expression (118). In the pea aphid, *Acyrthosiphon pisum*, miR-184 negatively regulates JNK, thus affecting the JNK signaling pathway (74).

Microbial infections induce changes in insect miRNA expression, consequently altering host immune responses. Upon exposure to *Bacillus thuringiensis* (Bt), several conserved miRNAs, such as miR-1, miR-10, miR-184, miR-275, and Let-7, are upregulated to negatively modulate the immunity of *G. mellonella* (80). Specifically, upregulated miR-2b-3p inhibits trypsin expression, which is crucial for insect defense mechanism (63). In Bt-susceptible insects, certain miRNAs are highly expressed and positively regulate the expression of Bt receptors such as aminopeptidase N and cadherin-like protein (119). These findings indicate that pathogens interfere with insect immunity by inducing host miRNAs to facilitate their replication. Conversely, some insect miRNAs are downregulated to activate immune responses. For instance, upon BmCPV infection, downregulation of bmo-miR-278-3p leads to upregulation of its target gene, *IBP2*, encoding for insulin-related peptide binding protein 2, enhancing host immune responses (115). Likewise, in *P. xylostella* miR-8 level is reduced upon infection, leading to decreased Serpin-27 expression, which activates the Toll pathway (20). In *A. pisum*, miR-184 is remarkably downregulated after bacterial infection, which induces the JNK pathway and inhibits bacterial proliferation (74).

sRNAs in Insect–Bacterial Symbiont Interactions

Growing evidence shows that the intracellular bacterial endosymbionts alter the insect miRNA profile to facilitate colonization in the host. In the sweetpotato whitefly, *Bemisia tabaci*, novel-m0780-5p is reduced upon infection by the symbiont *Portiera*, resulting in upregulation of its target gene *panBC* for the synthesis of pantothenate that mediates the coordination of whitefly and symbiont fitness (97). In *Wolbachia*-infected *Ae. aegypti*, the host miRNA aae-miR-2940 is highly induced to upregulate the expression of genes encoding a host metalloproteinase and a protein arginine methyltransferase, ArgM3, which are critical for efficient maintenance of the endosymbiont (42, 122). *Wolbachia* induces aae-miR-981, which downregulates importin β4 to block the translocation of Ago1 to the nucleus in *Ae. aegypti*, leading to alteration in epigenetic effects (43). Furthermore, the manipulation of host miRNA expression has been proposed to contribute

to *Wolbachia*-mediated virus blocking. *Wolbachia* also manipulates aae-miR-2940 to suppress the expression of a host DNMT gene *Dnmt2*, contributing to dengue virus (DENV) inhibition in *Ae. aegypti* (123).

Cross-Kingdom RNAi in Insect–Fungus Interactions

sRNAs in filamentous fungal pathogens have been characterized for their involvement in virulence and developmental processes. sRNAs exhibit distinct expression patterns during conidiogenesis in *M. anisopliae* (129). While a large number of sRNAs regulate gene expression endogenously, recent studies have revealed that some sRNAs can transport between fungal pathogens and hosts to induce gene interference in the interacting organism in *trans* (17, 106, 107), a mechanism termed cross-kingdom RNA interference (RNAi) or cross-species RNAi (105, 111, 112) (**Figure 2**). A fungal pathogen, *B. bassiana*, delivers a miRNA-like RNA (bba-milR1) to mosquito cells, where bba-milR1 hijacks the host RNAi machinery by binding to mosquito Ago1 and suppresses mosquito immunity by silencing the host Toll receptor ligand gene Spätzle 4 (*Spz4*) (17). Moreover, host insects can also deliver their miRNAs into pathogens (**Figure 2**). Upon fungal infection, mosquitoes increase expression of their miRNAs, including Let-7 and miR-100, which are exported to the invading fungus and suppress fungal virulence genes (107). These findings demonstrate that cross-kingdom RNAi is bidirectional in insect–fungus interactions. Based on this discovery, a recent study introduced a novel approach termed pathogen-mediated RNAi for enhancing fungal virulence. This approach involves genetically modifying fungal pathogens to express specific mosquito miRNAs that suppress the host immune response, thereby increasing fungal efficacy (16). This study provides an innovative strategy for exploiting a wealth of pest endogenous miRNAs to augment the efficacy of microbial control agents (5).

How sRNAs travel between hosts and pathogens remains understudied. Compelling evidence suggests that host cells can send sRNAs in encapsulated extracellular vesicles (EVs) to the interacting organisms (10, 13). EVs are membrane-enclosed nanoparticles released by various cells to the extracellular environment for delivery of proteins, lipids, DNAs, RNAs, metabolites, and other molecules (7, 12, 14, 100). EVs are increasingly recognized as important mediators of intercellular communication. EVs are also the vital channel for communication in insect and fungal pathogen interactions. In *B. bassiana*, sRNA bba-milR1 is transported into insect cells by being loaded into vesicles (17). This finding supports the view that EV-mediated transport is certainly one of the major sRNA delivery machineries between interacting organisms (**Figure 2**).

Cross-Kingdom RNAi in Insect–Virus Interactions

RNAi by cellular miRNAs is the important mechanism utilized by insect hosts to curb viral infection. Several insect cellular miRNAs can bind to the viral genomic coding region and repress its expression. For instance, cellular Hz-miR24 from *Helicoverpa zea* is upregulated during late stages of ascovirus infection and suppresses the expression of viral DNA-dependent RNA polymerase II RPC2 and its β subunits that are required for the expression of late genes involved in the production of mature virions (39). *Ae. aegypti* microRNA miR-2944b-5p interacts with the 3′ untranslated region (UTR) of chikungunya virus, a positive-sense single-stranded RNA virus, to restrict viral replication within mosquitoes (19). In *B. mori*, bmo-miR-390 inhibits the expression of BmNPV *cg30*, which is involved in the formation of occlusion bodies (48). Similarly, miR-34-5p encoded by *Spodoptera frugiperda* inhibits the replication of and infection by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) by directly targeting AcMNPV genes (47).

Conversely, some viruses can suppress or utilize insect miRNAs to escape host surveillance and promote their replication. In *B. mori*, bmo-miR-2819, which targets BmNPV immediately early

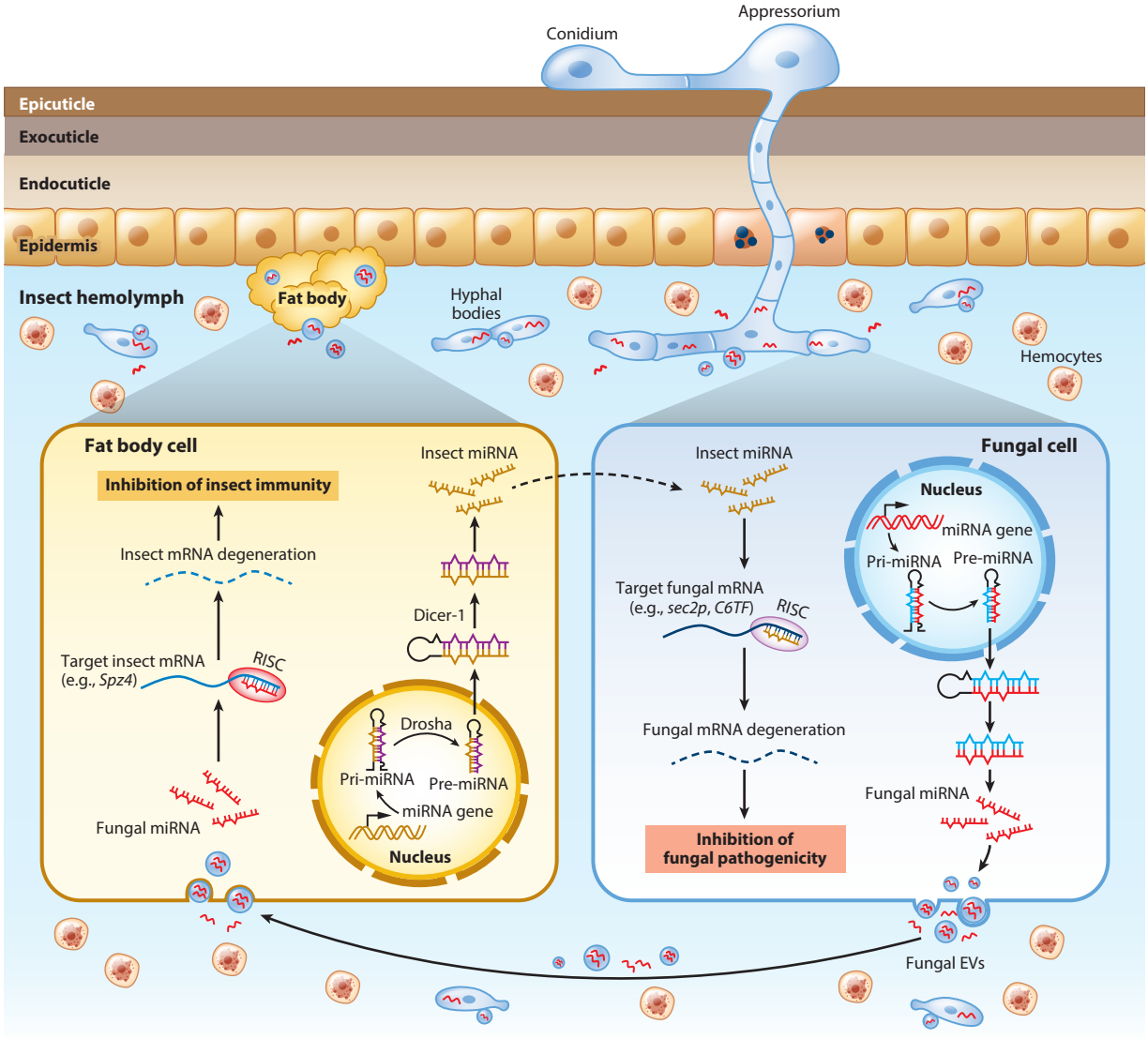


Figure 2

Cross-kingdom sRNA communication between insects and pathogenic fungi. During insect infection, the pathogenic fungus delivers miRNA-like sRNAs to insect cells, where they associate with the host Ago1 protein and silence the expression of immune-related genes such as the mosquito Toll receptor ligand gene *Spz4*, thereby attenuating the host immune response. Conversely, host insects can also export miRNAs into the invading fungus and suppress the expression of virulence-related genes, thus enhancing antifungal defense. Abbreviations: Ago1, Argonaute-1; EV, extracellular vesicle; miRNA, microRNA; mRNA, messenger RNA; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; sRNA, small RNA; *Spz4*, Spätzle 4.

1 gene (*ie-1*), is downregulated upon BmNPV infection (116). bmo-miR-274-3p, which targets BmCPV nonstructural protein 5 (NS5), is also suppressed in the BmCPV-infected midgut (113). In the virus-infected small brown planthopper, *Laodelphax striatellus*, miR-315-5p is upregulated to promote virus infection by downregulating a melatonin receptor required for melatonin-mediated immune responses (124).

On the contrary, viral miRNAs can regulate virus replication by changing the expression of virus or host genes. During early infection stages, the BmNPV-encoded miRNA BmNPV-miR-3 suppresses viral P6.9, a late-expressing and conserved baculovirus DNA-binding protein required for infectious virion formation (96). Downregulation of P6.9 helps maintain a lower viral load to evade host immune surveillance. DENV can encode functional sRNAs such as DENV viral small RNA 5 (DENV–vsRNA-5), which interacts with NS1 sequences in the virus genome, thereby negatively regulating virus replication (40). Both BmNPV and BmCPV can modulate host sRNA-mediated defense by employing vial miRNAs to downregulate host Exportin-5 cofactor Ran, thereby suppressing host miRNA biogenesis (67, 95). Furthermore, West Nile virus encodes a microRNA-like sRNA named KUN-miR-1 in its 3′ UTR that directly upregulates the expression of mosquito GATA4, which is required for virus replication in mosquito cells (44).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Epigenetic mechanisms, such as DNA methylation, histone modifications, and sRNAs, have been recognized as pivotal regulators in modulating insect–microbe interactions by regulating insect immune responses and microbial pathogenicity. Nevertheless, several unresolved issues persist in the realm of epigenetic research.

1. The precise mechanisms by which the insect host detects microbes and transmits signals to induce epigenetic modifications for the regulation of downstream gene expressions remain elusive. It is suggested that Toll-like receptors detect microbial pathogens, leading to subsequent epigenetic alterations that modulate the expressions of genes encoding AMPs and cytokines (36). Moreover, hormone signaling pathways have been implicated in the regulation of insect miRNAs that are in turn involved in the cross talk regulation between 20E and juvenile hormone pathways (125). Certain studies indicated that effector molecules and toxins released by pathogens target host epigenetic mechanisms to subvert host innate immune responses.
2. Epigenomes that include DNA methylation and histone modifications of host insects are altered by the interacting microbes, concomitant with changes in gene expression. However, the direct modulation of gene expression by epigenetic changes remains unclear, underscoring our limited understanding of the interplay between epigenetics and transcriptomic changes. The application of new technologies, such as CRISPR-Cas9 gene editing, holds promise for elucidating the functional roles and identifying target genes of epigenetic enzymes in insects in response to microbial infections.
3. In contrast to DNA methylation and histone modifications, which function endogenously, sRNAs represent unique epigenetic modulators capable of traversing into interacting organisms to target mRNAs crucial for host defense or microbial proliferation. Therefore, sRNAs may serve as novel trafficking effectors to mediate bidirectional cross-kingdom RNAi in the interacting organism. The mechanisms underlying the selective packaging of sRNAs into EVs remain to be explored. Furthermore, exploring additional mechanisms involved in sRNA trafficking beyond EVs presents an intriguing avenue for future investigation.
4. In addition to sRNAs, other noncoding RNAs (ncRNAs) like long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), RNA modifications, and ncRNA-mediated chromatin modifications may play roles in insect–microbe interactions that require further exploration. LncRNAs are emerging as key regulators of gene expression and immune responses in insects, potentially influencing host–pathogen dynamics. CircRNAs, though less studied in insects, have been implicated in immune regulation in other organisms and

may similarly affect insect–microbe interactions. Additionally, RNA modifications such as methylation and editing can modulate RNA stability and function, suggesting they could play roles in regulating host responses to microbial infections. Understanding the functions of these ncRNAs beyond sRNAs is crucial for unraveling the complex molecular mechanisms underlying insect immunity and microbial pathogenesis, with implications for pest management and disease control strategies.

5. DNA methylation, histone modifications, and ncRNAs can interact with each other to collectively regulate gene expression (90). Understanding the landscape of their coordinated regulation is important for gaining deeper insights into the intricate and fine-tuned modulation of gene expression that shapes insect–microbe interactions.

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