

AGRICULTURE

Epitranscriptomic regulation of insecticide resistance

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N⁶-methyladenosine (m⁶A) is the most prevalent messenger RNA modification in eukaryotes and an important posttranscriptional regulator of gene expression. However, the biological roles of m⁶A in most insects remain largely unknown. Here, we show that m⁶A regulates a cytochrome P450 gene (*CYP4C64*) in the global whitefly pest, *Bemisia tabaci*, leading to insecticide resistance. Investigation of the regulation of *CYP4C64*, which confers resistance to the insecticide thiamethoxam, revealed a mutation in the 5' untranslated region of this gene in resistant *B. tabaci* strains that introduces a predicted m⁶A site. We provide several lines of evidence that mRNA methylation of the adenine at this position, in combination with modified expression of m⁶A writers, acts to increase expression of *CYP4C64* and resistance. Collectively, these results provide an example of the epitranscriptomic regulation of the xenobiotic response in insects and implicate the m⁶A regulatory axis in the development of insecticide resistance.

INTRODUCTION

Herbivorous insects have evolved sophisticated biochemical defense systems to protect themselves against the natural xenobiotics present in their environment. These systems, which include diverse families of detoxifying enzymes, are also frequently recruited in the evolution of resistance to synthetic toxins such as chemical insecticides. One of the most important enzyme superfamilies in this regard are the cytochrome P450s (P450s). Numerous studies have shown that one or more P450 genes are commonly constitutively overexpressed in insecticide-resistant insect strains and/or induced upon insecticide exposure (1, 2). The increased production of P450s in these strains has been shown to most commonly result from gene duplication/amplification or from mutations in cis- or trans-acting factors (3–12). In contrast, examples of post-transcriptional regulation of resistance-associated P450s are extremely rare (13, 14).

RNA modifications represent a critical layer of epitranscriptomic regulation of gene expression. Of these, N⁶-methyladenosine (m⁶A) RNA is among the most abundant chemical modifications present in eukaryotic mRNA. This dynamic and reversible epitranscriptomic mark has been shown to play an important role in RNA-mediated regulation of various cellular processes, including gene expression, alternative splicing, mRNA stability, and microRNA biogenesis (15–20). m⁶A is installed on the mRNA by a complex holoenzyme (~900 kDa) composed of two core methyltransferases: methyltransferase-like 3 (METTL3) and METTL14 (21, 22), and several auxiliary cofactors: Wilms tumor 1-associated protein (WTAP) (23), KIAA1429 (24), RNA binding motif protein 15 (RBM15) (25), and zinc finger CCCH domain-containing protein 13 (ZC3H13) (26). These methyltransferases share a conserved methyltransferase domain, but METTL14 appears to be catalytically inactive and may play a structural role

in substrate recognition (27, 28). In insects, study of the biological functions of m⁶A has been limited to model species. In the fruit fly, *Drosophila melanogaster*, the m⁶A pathway has been shown to play a role in neuronal functions and sex determination (29–31). More recently, transcriptome-wide profiling of m⁶A in the silkworm, *Bombyx mori*, has been used to investigate its role in regulating gene expression, chromosome alignment and segregation, and nucleopolyhedrovirus (BmNPV) infection (32, 33). However, to date, no role has been ascribed for m⁶A in the xenobiotic response of insects to plant allelochemicals and insecticides.

The whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), is a globally distributed, highly damaging pest of agriculture that attacks a wide range of food and commodity crops (34). The intensive use of insecticides to manage this insect pest has led to the evolution of widespread resistance to many of the compounds used for control (35, 36). Investigation of the molecular basis of resistance to neonicotinoid insecticides in this species has implicated the overexpression of several P450 genes in resistance, most notably *CYP6CM1* and *CYP4C64* (37, 38). In the case of *CYP6CM1*, both its causal role in resistance (39) and trans-regulation by the transcription factor CREB (12) have been demonstrated. However, the role of *CYP4C64* in resistance and its regulation is currently unclear.

Here, we functionally demonstrate the causal role of *CYP4C64* in resistance to the neonicotinoid thiamethoxam and investigate the factors driving its overexpression in resistant strains. We uncover the regulation of this P450 by the m⁶A pathway and thus provide an example of insecticide resistance mediated by a previously undescribed epitranscriptomic mechanism.

RESULTS

The *B. tabaci* P450 gene *CYP4C64* confers resistance to thiamethoxam

We have previously shown that resistance to thiamethoxam in a *B. tabaci* MEAM1/B strain, selected with this insecticide in the laboratory, is associated with the overexpression of the cytochrome P450 (*CYP4v2*, partial sequence), *CYP4C64* (40, 41). Further monitoring of *B. tabaci* field populations has also suggested that this gene may be involved in imidacloprid resistance in strains of *B. tabaci*

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Mediterranean (MED) (37, 38). To investigate whether expression of this P450 gene also plays a role in the resistance of *B. tabaci* field populations to thiamethoxam, we first sequenced the partial [370 base pairs (bp)] sequence of *CYP4C64* obtained by a previous mRNA sequencing project (41) and performed 3' and 5' rapid amplification of cDNA ends (RACE) to obtain the complete complementary DNA (cDNA) sequence (fig. S1B). The resulting *CYP4C64* cDNA sequence (GenBank JX144366) contains a 1533-bp open reading frame (ORF) encoding 510 amino acid residues (58,969 Da), a 247-bp 5' untranslated region (UTR), and a 1158-bp 3' UTR (fig. S1B). The encoded protein contains features and conserved domains that are typical for microsomal monooxygenases such as a hydrophobic N-terminal domain that commonly acts as a transmembrane anchor, the WxxxR motif (positions 127 to 131), the oxygen-binding motif (AGxxT, positions 326 to 331), the heme-binding motif (PFxxGxxxCxG, positions 447 to 457), and the conserved PXXFXP sequence (positions 424 to 429) (figs. S1A and S2).

To investigate the molecular basis of thiamethoxam resistance in field strains of *B. tabaci* in China, we collected eight field strains (Fig. 1A and table S1) and investigated their sensitivity to thiamethoxam using full dose-response bioassays. Compared with the reference strain *TH-S*, low resistance ratios (6- to 19-fold) were observed in five strains [*TJ-R* ($R^{#3}$), *WH-R* ($R^{#6}$), *TZ-R* ($R^{#7}$), *LF-R* ($R^{#9}$), and *NK-R* ($R^{#10}$)], and high resistance levels to thiamethoxam were observed

in the *THR* ($R^{#1}$), *HZ-R* ($R^{#2}$), *JZ-R* ($R^{#4}$), *JY-R* ($R^{#5}$), and *CS-R* ($R^{#8}$) strains (47- to 151-fold; table S2).

To examine whether thiamethoxam resistance in a subset of these strains was associated with a mutation of the neonicotinoid target site, the nicotinic acetylcholine receptor (nAChR) and nine nAChR subunit genes (eight α subunit and one β subunit genes) present in the genome of *B. tabaci* MED/Q (42) were cloned and sequenced from the $S^{#2}$, $R^{#2}$, $R^{#5}$, and $R^{#8}$ strains. No nonsynonymous mutations were observed in the sequences obtained that consistently distinguish these strains (data file S1).

To investigate the role of *CYP4C64* in the resistant field strains, we used a rabbit polyclonal antibody raised against a synthetic peptide of *CYP4C64* to estimate the expression of the encoded enzyme in the susceptible and resistant *B. tabaci* strains. *CYP4C64* was found to be strongly overexpressed in the thiamethoxam-resistant strains compared with the susceptible strains, and the correlation between relative protein expression levels (*CYP4C64* expression values versus actin expression values) and resistance levels (RR values; table S2) was positive and highly significant ($P = 1.11 \times 10^{-4}$) (Fig. 1B and fig. S3D). Furthermore, when two resistant strains ($R^{#2}$ and $R^{#8}$) and one susceptible strain ($S^{#2}$) were treated with 1.0 mM thiamethoxam, *CYP4C64* expression increased in a time-dependent manner in the two resistant strains (Fig. 1C), demonstrating that the expression of this P450 is also induced by this compound.

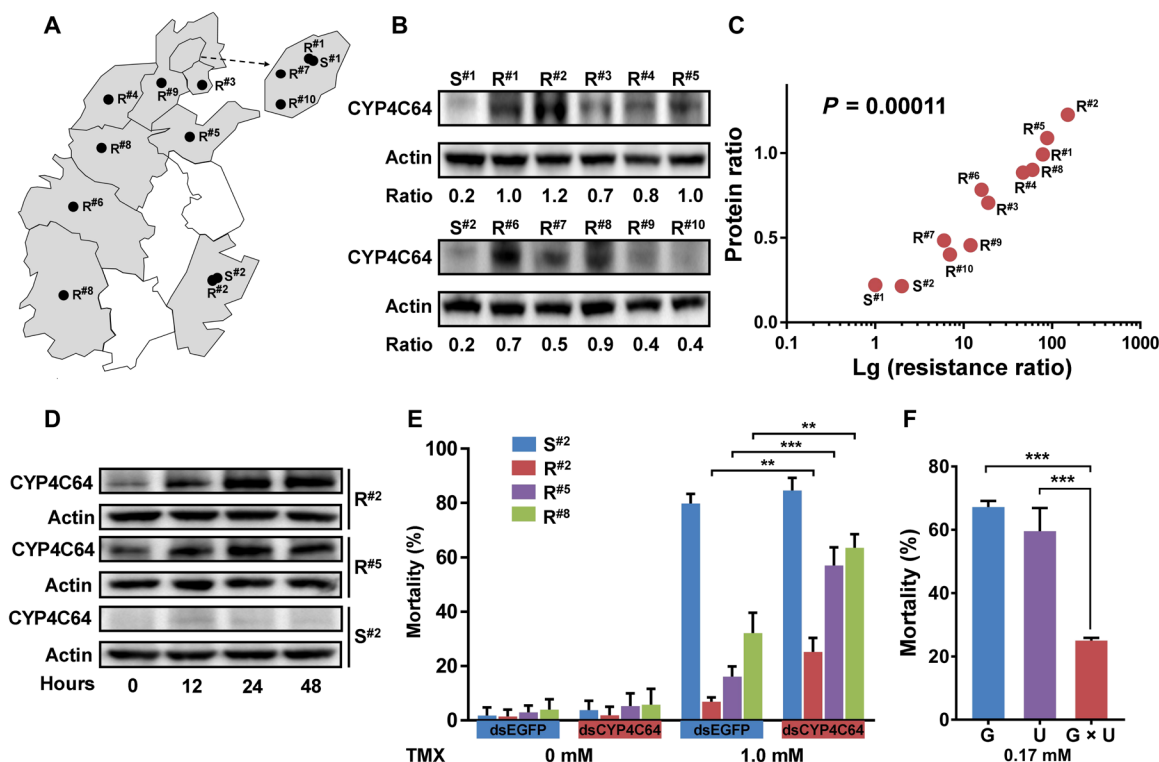


Fig. 1. The P450 gene *CYP4C64* confers resistance to thiamethoxam in *B. tabaci*. (A) Map of China showing the provinces (shaded gray) where field strains of *B. tabaci* were collected. (B) Western blot analysis of the expression levels of *CYP4C64* in 12 strains of *B. tabaci* from China. Relative protein ratio is relative to the loading control (actin). (C) Linear regression analysis between thiamethoxam resistance level and relative *CYP4C64* expression. (D) Expression of *CYP4C64* in one susceptible strain ($S^{#2}$) and two resistance strains ($R^{#2}$ and $R^{#8}$) of *B. tabaci* at three time points (12, 24, and 48 hours) following exposure to 1.0 mM thiamethoxam. Actin was used as a loading control. (E) Sensitivity of one susceptible ($S^{#2}$) and two resistance strains ($R^{#2}$ and $R^{#8}$) of *B. tabaci* to 1.0 mM thiamethoxam (TMX) after RNAi knockdown of *CYP4C64*. Adults fed on *dsEGFP* were used as a negative control [$n = 3$, mean \pm SE; $**P < 0.01$ and $***P < 0.001$, analysis of variance (ANOVA) with Tukey's HSD post hoc test]. (F) Sensitivity of transgenic *Drosophila* expressing *CYP4C64* to thiamethoxam (0.17 mM) compared to the parental lines that do not actively express the transgene (G: *Tub-GAL4⁺* line; U: *UAS-CYP4C64⁺* line; G \times U: *UAS-CYP4C64/Tub-GAL4* line; $n = 3$, mean \pm SE; $***P < 0.001$, ANOVA with Tukey's HSD post hoc test).

To demonstrate the causal role of *CYP4C64* in thiamethoxam resistance, we used RNA interference (RNAi) to knock down its expression, and we examined the effect of this on the survival of thiamethoxam-treated adults of the resistant and susceptible strains. After 36 hours of feeding on a diet containing double-stranded RNA (dsRNA) specific for *CYP4C64*, the mRNA levels of this gene significantly decreased by 49.7% ($P = 1.66 \times 10^{-9}$; figs. S3A and S14A). Knockdown of *CYP4C64* significantly decreased the resistance of adult whiteflies of the $R^{\#2}$, $R^{\#5}$, and $R^{\#8}$ strains, relative to the control [fed enhanced green fluorescent protein (EGFP) dsRNA], when treated with 1.0 mM thiamethoxam ($R^{\#2}$: $P = 4.26 \times 10^{-3}$; $R^{\#5}$: $P = 7.41 \times 10^{-4}$; $R^{\#8}$: $P = 3.85 \times 10^{-3}$; Fig. 1E and fig. S3B) and 5.1 mM thiamethoxam ($R^{\#2}$: $P = 3.99 \times 10^{-4}$; $R^{\#5}$: $P = 8.41 \times 10^{-3}$; $R^{\#8}$: $P = 2.56 \times 10^{-3}$). To provide additional evidence that *CYP4C64* confers tolerance to thiamethoxam in vivo, we created a transgenic *Drosophila* line expressing this P450. Flies expressing the *CYP4C64* transgene displayed significant resistance to thiamethoxam compared with control flies that do not express the transgene in insecticide bioassays (0.17 mM: {UAS-*CYP4C64*^{+/+}} versus {UAS-*CYP4C64*/*Tub-GAL4*}, $P = 3.80 \times 10^{-5}$; {*Tub-GAL4*^{+/+}} versus {UAS-*CYP4C64*/*Tub-GAL4*}, $P = 9.36 \times 10^{-3}$; 0.34 mM: {UAS-*CYP4C64*^{+/+}} versus {UAS-*CYP4C64*/*Tub-GAL4*}, $P = 1.18 \times 10^{-4}$; {*Tub-GAL4*^{+/+}} versus {UAS-*CYP4C64*/*Tub-GAL4*}, $P = 2.51 \times 10^{-4}$; Fig. 1F and fig. S3C). Together, these results provide unequivocal evidence that *CYP4C64* confers resistance to thiamethoxam.

A thiamethoxam resistance-associated mutation in the 5'UTR of *CYP4C64*

To examine whether mutations in the mRNA sequence of *CYP4C64* are associated with resistance, the ORF, 5'UTR, and 3'UTR were polymerase chain reaction (PCR) amplified and sequenced from six pools of cDNA derived from three thiamethoxam-resistant and one susceptible *B. tabaci* strains. While a few single-nucleotide polymorphisms were observed at certain positions in the ORF and 3'UTR region, these did not consistently distinguish sequences from the resistant and susceptible strains (data files S2 and S3). However, a polymorphism (T to A at position—206 bp; Fig. 2A) was observed in the 5'UTR of *CYP4C64* that was observed at much greater frequency in the thiamethoxam-resistant strains compared with the susceptible strain (data file S4). Further sequencing of the 5'UTR region from additional resistant and susceptible strains ($n = 112$ to 143 individuals sequenced; female:male = 1:1) confirmed that this mutation is consistently found at much higher frequency in thiamethoxam-resistant strains (0.86 to 0.91), compared with susceptible strains (0.08 to 0.10) (Fig. 2B, table S3, and data file S4).

Intriguingly, the T-206A transversion is predicted to introduce a sequence (CGACA) that closely resembles an N^6 -methyladenine (m^6A) consensus sequence. To examine whether this polymorphism affects the expression of *CYP4C64*, we individually cloned the 5'UTR sequences of thiamethoxam-resistant and thiamethoxam-susceptible whiteflies into a vector containing a minimal promoter and

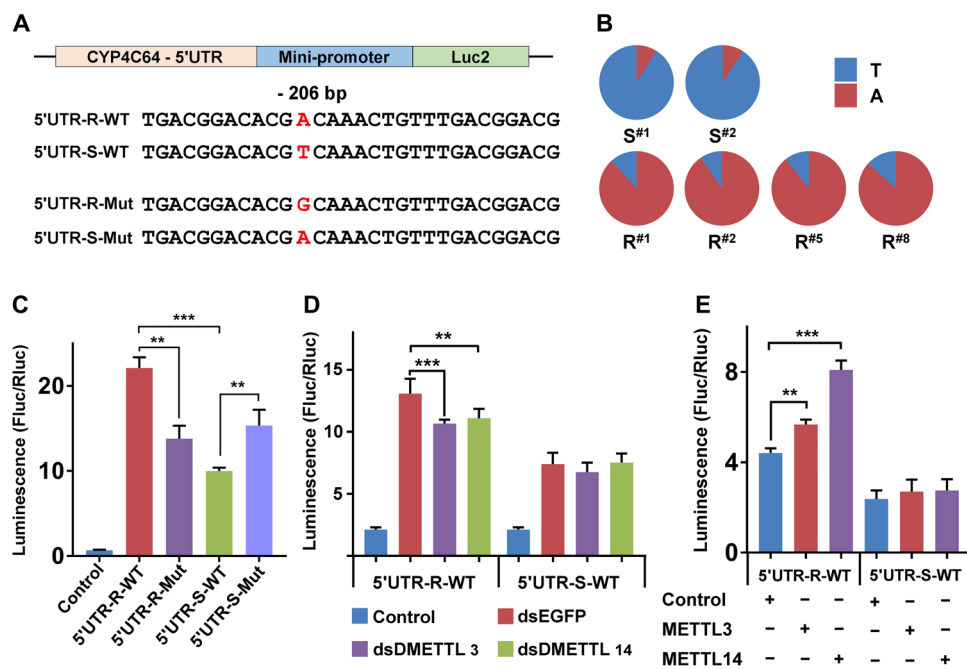


Fig. 2. A 5'UTR mutation affects the expression of *CYP4C64*. (A) A single mutation in the 5'UTR consensus sequence of *CYP4C64* (T-206A) is observed in thiamethoxam-resistant *B. tabaci*. (B) Frequency of T-206A in two thiamethoxam-susceptible strains and four resistance strains ($n = 112$ to 143 individuals sequenced). (C) Identification of the functional role of T-206A in the 5'UTR of *CYP4C64* on gene expression using dual-luciferase reporter assays. pGL4.26-*CYP4C64*^(5'UTR) and pGL4.73 were cotransfected into S2 cells. The 5'UTR of *CYP4C64* derived from susceptible (5'UTR-S-WT) and resistant (5'UTR-R-WT) *B. tabaci* and two mutant variants of these sequences (5'UTR-S-Mut and 5'UTR-S-Mut; see main text for details) were analyzed ($n = 6$, mean \pm SE; $**P < 0.01$ and $***P < 0.001$, ANOVA with Tukey's HSD post hoc test). (D) RNAi knockdown of two *D. melanogaster* m^6A writer genes, *METTL3* and *METTL14*, modulates *CYP4C64* expression in S2 cells. The 5'UTR of *CYP4C64* (pGL4.26-*CYP4C64*^(5'UTR)) derived from resistant or susceptible (pGL4.26-*CYP4C64*^(5'UTR-S-WT)) whiteflies were transfected into S2 cells together with dsRNA for either *METTL3* or *METTL14* ($n = 3$, mean \pm SE; $**P < 0.01$ and $***P < 0.001$, ANOVA with Tukey's HSD post hoc test). (E) Overexpression of *B. tabaci* *METTL3* and *METTL14* in S2 cells enhances expression of pGL4.26-*CYP4C64*^(5'UTR-R-WT) ($n = 6$, mean \pm SE; $**P < 0.01$ and $***P < 0.001$, ANOVA with Tukey's HSD post hoc test).

luciferase reporter gene. In the absence of a commercially available whitefly cell line, constructs were expressed in *Drosophila* S2 cells. The cell line expressing the 5'UTR from resistant whiteflies (5'UTR-R-WT) exhibited significantly ($P = 6.03 \times 10^{-10}$) higher reporter gene activity than the line expressing the susceptible 5'UTR sequence (5'UTR-S-WT) in dual-luciferase assays (Fig. 2C). Notably, exposure of cell lines to thiamethoxam did not affect the level of expression of constructs containing the 5'UTR of *CYP4C64* (fig. S14B), suggesting that any xenobiotic response elements for this compound lie outside the region tested. To further confirm the role of this polymorphism in modulating gene expression, we generated constructs where the m⁶A consensus sequence observed in thiamethoxam-resistant whiteflies was changed from CGAC to CGGC (5'UTR-R-Mut). A second construct was generated where the consensus thiamethoxam-susceptible 5'UTR sequence was changed from CGTC to CGAC (5'UTR-S-Mut). Cell lines expressing the 5'UTR-R-Mut construct exhibited a significant ($P = 1.47 \times 10^{-6}$) reduction in reporter gene activity compared with those expressing the 5'UTR-R-WT construct in reporter gene assays (Fig. 2C). In contrast, reporter gene activity of the cell line expressing the 5'UTR-S-Mut construct was significantly ($P = 3.93 \times 10^{-5}$) increased compared with the line expressing the wild-type 5'UTR-S-WT construct (Fig. 2C). While the use of *Drosophila* cell lines in these experiments may not fully recapitulate the cellular environment of native *B. tabaci*, taken together, these results provide evidence that the T-206A mutation observed in resistant whiteflies influences gene expression in vitro.

Recent work on the silkworm, *B. mori*, has suggested that m⁶A RNA methylation can positively regulate gene expression in insects (33). Thus, to investigate the potential role of methylation of the m⁶A site observed in the 5'UTR of thiamethoxam-resistant whiteflies in modulating the expression of *CYP4C64*, we used RNAi to knock down two *D. melanogaster* orthologous m⁶A writer genes that install the m⁶A modification on target RNAs, namely, methyltransferase-like 3 (*METTL3* and *ime4*) and *METTL14* (CG7818) in S2 cells. The constructs carrying the 5'UTRs of resistant and susceptible whiteflies were then transfected into these cells, and reporter gene assays were conducted. Knockdown of *METTL3* and *METTL14* significantly (*METTL3*: $P = 7.87 \times 10^{-4}$; *METTL14*: $P = 8.52 \times 10^{-3}$) decreased the activity of constructs carrying the 5'UTR of resistant whiteflies but not those with the susceptible 5'UTR sequence (Fig. 2D). The finding that knockdown of genes involved in the methylation of m⁶A directly influences the expression of constructs carrying the m⁶A consensus sequence suggests that methylation of the adenine at this position plays a role in regulating the expression of *CYP4C64* in thiamethoxam-resistant *B. tabaci*.

An m⁶A methylase is involved in *B. tabaci* resistance to thiamethoxam

The full-length genes *METTL3* (GenBank MN685829) and *METTL14* (GenBank MN685830) in *B. tabaci* encode proteins of 582 and 391 amino acids, respectively, with high pairwise amino acid similarity to members of the *METTL* family from other organisms (figs. S4 and S5). To explore whether these two genes play a role in the regulation of *CYP4C64* expression, they were individually cloned into the pAC5.10b expression vector and cotransfected into S2 cells with pGL4.10-*CYP4C64*^{5'UTR-R-WT} and pGL4.10-*CYP4C64*^{5'UTR-S-WT}. In reporter gene assays, cells expressing the 5'UTR from resistant whiteflies in combination with either whitefly *METTL3* or *METTL14* exhibited significantly (*METTL3*: $P = 1.82 \times 10^{-3}$; *METTL14*: $P =$

7.72×10^{-5} ; Fig. 2E) higher reporter gene activity compared with cells expressing the 5'UTR-R-WT construct alone. To explore global m⁶A abundance in thiamethoxam-susceptible and thiamethoxam-resistant *B. tabaci* strains, we used dot blot and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to compare m⁶A levels in the thiamethoxam-susceptible strain (*S*^{#2}) and two resistant strains (*R*^{#2} and *R*^{#8}), which exhibit high levels of thiamethoxam resistance and *CYP4C64* expression. Global m⁶A levels were significantly higher in both resistant strains (*R*^{#2}: $P = 0.0099$; *R*^{#8}: $P = 0.0038$) compared with the susceptible strain (Fig. 3A and fig. S7, A and B). Furthermore, analysis of *CYP4C64* and m⁶A levels in the *S*^{#2} and *R*^{#2} strains 24 and 48 hours after exposure to 1.0 mM thiamethoxam revealed that both *CYP4C64* and global m⁶A levels increased over time in the resistant strain but not in the susceptible strain (Fig. 3B). To explore the methylation levels of *CYP4C64* mRNA in resistant and susceptible *B. tabaci* strains, we used a gene-specific m⁶A quantitative PCR (qPCR) assay. This revealed a significantly higher level of m⁶A methylation across the 5'UTR region of the *CYP4C64* mRNA of resistant whitefly strains compared with a susceptible strain (*R*^{#2}: $P = 1.5 \times 10^{-3}$; *R*^{#5}: $P = 2.1 \times 10^{-2}$; *R*^{#8}: $P = 1.56 \times 10^{-2}$; Fig. 3C). In contrast, no difference in m⁶A methylation in the coding sequence (CDS) or 3'UTR was observed between strains.

To examine the expression of *METTL3* and *METTL14* in thiamethoxam-resistant and thiamethoxam-susceptible *B. tabaci* strains, homo antibodies were used in Western blots of the *S*^{#2}, *R*^{#2}, *R*^{#5}, and *R*^{#8} strains (antibody immunogen analysis is shown in fig. S6). *METTL3* was overexpressed in all three resistant strains compared with the susceptible strain, while no difference in the expression of *METTL14* between strains was observed (Fig. 3D). We further extended these analyses to other components of the m⁶A methyltransferase complex by cloning the auxiliary cofactors: *WTAP*, *RBM15*, and *KIAA1429* (fig. S11), and comparing their expression in the thiamethoxam-resistant strains *R*^{#2} and *R*^{#8} with the susceptible strain *S*^{#2}. This revealed that *WTAP* and *KIAA1429* are significantly overexpressed in the resistant strains (*WTAP*: *R*^{#2}, $P = 0.003$; *R*^{#8}, $P = 1.49 \times 10^{-5}$; and *KIAA1429*: *R*^{#2}, $P = 0.001$; *R*^{#8}, $P = 0.04$), suggesting that these accessory proteins may also play a role in enhancing m⁶A levels in the resistant *B. tabaci* strains.

To investigate the functional role of members of the m⁶A methyltransferase complex in regulating *CYP4C64* in vivo, we focused on the core methyltransferases *METTL3* and *METTL14*. RNAi was used to knock down both genes in adult whiteflies of the resistant strain *R*^{#2}. After 48 hours of feeding on a diet containing dsRNA specific for *METTL3* or *METTL14*, the mRNA levels of the two genes decreased by 43, and 49%, respectively (figs. S7, C and D, and S12, C and D), and this corresponded with a significant decrease in global m⁶A levels (*METTL3*: $P = 0.006$; *METTL14*: $P = 0.007$; fig. S12B). Moreover, a corresponding significant decrease (*METTL3*: 34%, $P = 0.03$; *METTL14*: 51%, $P = 6.64 \times 10^{-5}$) in the transcript levels of *CYP4C64* was observed (fig. S7, C and D). This was correlated with a significant decrease in protein levels of *CYP4C64* (Fig. 3E).

We also explored the effect of RNAi knockdown of *METTL3* and *METTL14* on the expression of other whitefly genes that have been previously implicated in xenobiotic resistance, encompassing nine P450 genes, two glutathione S-transferase (GST) genes, and one adenosine triphosphate (ATP)-binding cassette subfamily G member 3 gene (ABCG) gene (37, 38, 43–45). Of these, four P450s were significantly decreased following knockdown of both *METTL3* and *METTL14* (*METTL3*: *CYP6DZ4*, 64%, $P = 3.44 \times 10^{-9}$; *CYP6DZ7*,

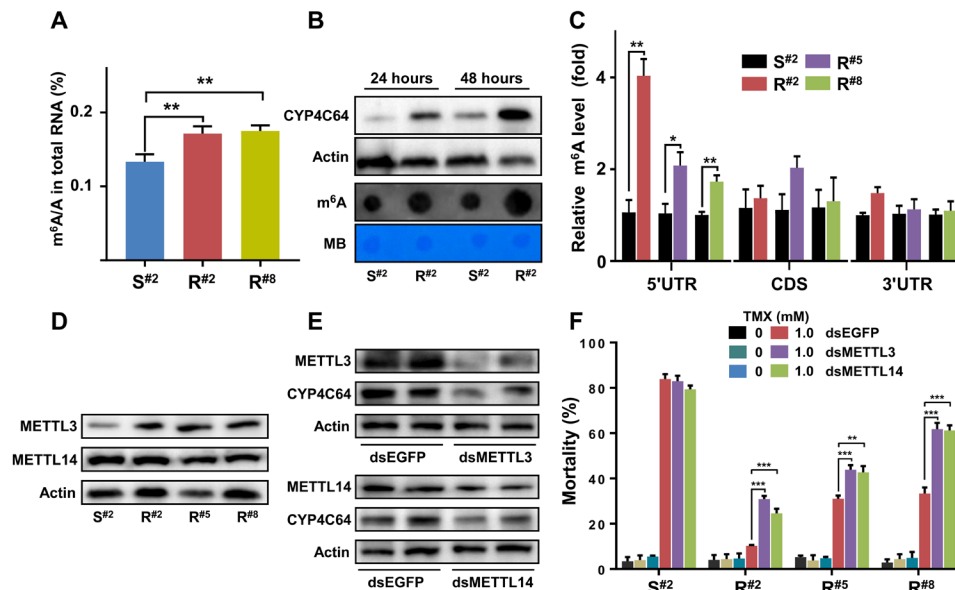


Fig. 3. m⁶A levels are higher in thiamethoxam-resistant *B. tabaci* strains than in a susceptible strain. (A) The m⁶A/A ratio of total RNA derived from thiamethoxam-resistant strains and a thiamethoxam-susceptible strain ($n = 3$, mean \pm SE; $**P < 0.01$, two-tailed Student's t test). (B) *CYP4C64* protein and global m⁶A levels in susceptible and resistant *B. tabaci* strains 24 and 48 hours after exposure to 1.0 mM thiamethoxam. Actin was used as a loading control. MB: methyl blue. (C) Gene-specific quantification of m⁶A in three regions (5'UTR, CDS, and 3'UTR) of the mRNA of *CYP4C64* in susceptible and resistant strains of *B. tabaci* as determined by m⁶A RNA immunoprecipitation followed by qPCR ($n = 3$, mean \pm SE; $**P < 0.01$ and $*P < 0.05$, ANOVA with Tukey's HSD post hoc test). (D) Western blot analysis of the expression of *METTL3* and *METTL14* in susceptible and resistant strains of *B. tabaci*. Actin was used as a loading control. (E) Expression of *CYP4C64* protein after knockdown of *METTL3* or *METTL14* in the resistant strain ($R^{\#2}$) as assessed by Western blot. Actin was used as a loading control. (F) Sensitivity of susceptible and resistant strains of *B. tabaci* to 1.0 mM thiamethoxam after RNAi knockdown of *METTL3* or *METTL14*. Adults fed on dsEGFP were used as a negative control ($n = 3$, mean \pm SE; $**P < 0.01$ and $***P < 0.001$, ANOVA with Tukey's HSD post hoc test).

38%, $P = 1.41 \times 10^{-4}$; *CYP4C1*, 46%, $P = 5.47 \times 10^{-7}$; *CYP4G68*, 55%, $P = 2.44 \times 10^{-7}$; fig. S8; and *METTL14*: *CYP6DZ4*, 49%, $P = 1.64 \times 10^{-7}$; *CYP6DZ7*, 33%, $P = 1.62 \times 10^{-5}$; *CYP4C1*, 42%, $P = 4.35 \times 10^{-4}$; *CYP4G68*, 43%, $P = 4.26 \times 10^{-5}$; fig. S9). Consistent with this, the 5'UTR regions of each of these four genes were found to contain two or more predicted m⁶A consensus sequences (fig. S13). Furthermore, two of these genes, *CYP6DZ4* and *CYP6DZ7*, were also found to be constitutively overexpressed in both the $R^{\#2}$ and $R^{\#8}$ strains compared to the $S^{\#2}$ strain (fig. S10).

Further RNAi experiments were used to individually knock down the two m⁶A writer genes in the $S^{\#2}$, $R^{\#2}$, $R^{\#5}$, and $R^{\#8}$ strains, and the effect of this on sensitivity to thiamethoxam was examined. After 48 hours of feeding on a diet containing *METTL3* and *METTL14* dsRNA, the sensitivity of the resistant strains of whiteflies to 1.0 and 5.1 mM thiamethoxam was significantly increased relative to the control (fed EGFP dsRNA) (Fig. 3F; *METTL3*: $R^{\#2}$, $P = 1.32 \times 10^{-4}$; $R^{\#5}$, $P = 6.71 \times 10^{-3}$; $R^{\#8}$, $P = 1.86 \times 10^{-3}$; *METTL14*: $R^{\#2}$, $P = 2.30 \times 10^{-3}$; $R^{\#5}$, $P = 1.87 \times 10^{-2}$; $R^{\#8}$, $P = 1.39 \times 10^{-3}$) (fig. S3E; *METTL3*: $R^{\#2}$, $P = 1.52 \times 10^{-4}$; $R^{\#5}$, $P = 5.57 \times 10^{-4}$; $R^{\#8}$, $P = 1.29 \times 10^{-3}$; *METTL14*: $R^{\#2}$, $P = 1.02 \times 10^{-3}$; $R^{\#5}$, $P = 1.10 \times 10^{-3}$; $R^{\#8}$, $P = 2.19 \times 10^{-3}$).

DISCUSSION

Here, we uncover the posttranscriptional regulation of an insecticide resistance gene by the m⁶A pathway (Fig. 4). Our findings advance understanding of the epitranscriptomic regulation of the xenobiotic response in arthropods, provide new insight into the

functional roles of m⁶A in insects, and have applied implications for the control of a highly damaging global crop pest. We briefly discuss these topics below.

We show that the P450, *CYP4C64*, is overexpressed in field strains of *B. tabaci*, with the level of expression positively correlating with the resistance of these strains to thiamethoxam. Using a range of in vivo and in vitro approaches, we demonstrate that up-regulation of this P450 confers resistance. Previous monitoring of *B. tabaci* field populations revealed that overexpression of *CYP4C64* is also correlated with imidacloprid and cyantraniliprole resistance (38). Together, these results suggest that *CYP4C64* may be an important generalist P450 for the detoxification of insecticides in whiteflies. Thus, understanding the regulation of this gene in resistant strains of *B. tabaci* may have broad-scale relevance for insecticide resistance management programs.

Our data demonstrate that a point mutation (A-206T) in the 5'UTR of *CYP4C64* is observed at high frequency in thiamethoxam-resistant *B. tabaci* strains. This mutation introduces a predicted m⁶A site, and we provide several lines of evidence that mRNA methylation of the adenine at this position acts to increase expression of *CYP4C64* and resistance. m⁶A has not been previously implicated in the xenobiotic response of insects. The biological roles of this epitranscriptomic mark in most insect species remain largely unknown. Outside of insects, m⁶A has been shown to be particularly enriched in 3'UTR regions, and as a result, the function of m⁶A in 5'UTRs remains comparatively less well understood (46). However, recent reports have revealed that mRNA methylation in the form of m⁶A can promote cap-independent translation under conditions of

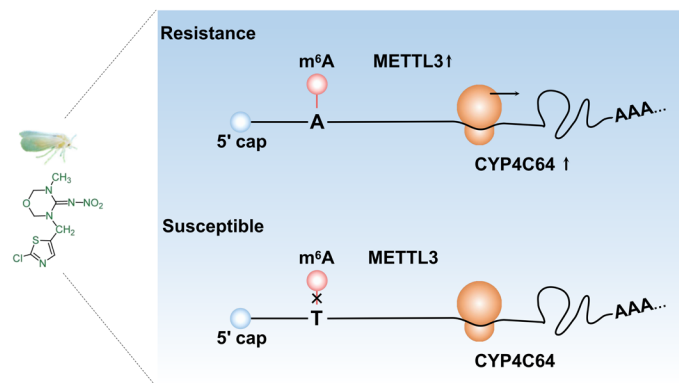


Fig. 4. A schematic of m⁶A-mediated regulation of *CYP4C64* expression in *B. tabaci*. A mutation (T-206A) in the 5'UTR of *CYP4C64* is observed at high frequency in thiamethoxam-resistant *B. tabaci*. The mutation introduces an m⁶A recognition site, and methylation of this site enhances the expression of *CYP4C64* in resistant strains. Overexpression of the methyltransferase METTL in resistant strains acts in concert with the mutation to increase levels of *CYP4C64* resulting in thiamethoxam resistance.

stress (47), and m⁶A in the 5'UTR guides mRNA alternative translation during the integrated stress response (48). As previous studies have shown that a single 5'UTR m⁶A is sufficient to mediate cap-independent translation (47), it is feasible that methylation of adenine -206 in the 5'UTR of resistant *B. tabaci* enhances *CYP4C64* expression by this mechanism. Furthermore, we show that insecticide exposure increases both global m⁶A levels and expression of this P450 gene. The finding that m⁶A is associated with up-regulation of *CYP4C64* also supports recent work on the silkworm, *B. mori* (33), in suggesting that m⁶A positively regulates gene expression in insects. The biological functions of m⁶A are mediated by specific binding proteins, namely, methyltransferases (“writers”), demethylases (“erasers”), and effectors (“readers”), that recognize this modification. We demonstrate that knockdown of the methyltransferases *METTL3* and *METTL14*, which methylate mRNA as a complex, leads to reduced expression of *CYP4C64* in resistant *B. tabaci* and increased sensitivity to thiamethoxam. We further show that *METTL3* is overexpressed in resistant strains of *B. tabaci*. Last, we identify two other components of the m⁶A methyltransferase complex, *WTAP* and *KIAA1429*, as significantly overexpressed in the resistant strains, suggesting that these accessory proteins may also play a role in enhancing m⁶A levels. Together, these findings reveal how an m⁶A site may act in concert with other components of the m⁶A regulatory pathway to regulate an insecticide resistance gene. Intriguingly, we also show that *METTL3* and *METTL14* knockdown results in the down-regulation of four other P450 genes (*CYP6DZ4*, *CYP6DZ7*, *CYP4C1*, and *CYP4G68*) previously implicated in neonicotinoid resistance in *B. tabaci*, all of which contain predicted m⁶A consensus sequences in their 5'UTRs. This suggests that *METTL3* and *METTL14* may be involved in the regulation of multiple P450 genes in *B. tabaci*. Thus, further research to examine the extent to which these methyltransferases act as posttranscriptional regulators of genes involved in the xenobiotic response of insects is warranted. The role, if any, of *CYP6DZ4*, *CYP6DZ7*, *CYP4C1*, and *CYP4G68* in neonicotinoid resistance in *B. tabaci* also requires further functional validation. In this regard, our finding that *CYP6DZ4* and *CYP6DZ7* are constitutively overexpressed in thiamethoxam-resistant *B. tabaci* strains suggests that they should be prioritized for further analyses.

An emerging body of work is beginning to characterize how the genes involved in insecticide resistance are regulated (1, 7). While a range of cis- and trans-acting factors has been shown to modulate detoxification gene expression in resistant insects by influencing transcription (3–12), unexpectedly few examples of posttranscriptional regulation have been described. This is especially true for epigenetic mechanisms, where just a handful of studies have implicated DNA methylation or noncoding RNA in the regulation of resistance genes (49–52). Our study demonstrates that methylation of mRNA can provide an alternative epigenetic mechanism of resistance gene regulation. One of the few previous examples of epigenetic regulation of an insecticide resistance gene was the demonstration that the highly duplicated *E4* esterase gene that is overexpressed in organophosphate-resistant clones of the aphid, *Myzus persicae*, can be silenced by DNA methylation (50). This allows the costly production of this esterase enzyme to be “switched off” in the absence of insecticide selection pressure. Related to this, m⁶A has been shown to be a dynamic and reversible modification, and stressful stimuli can alter both m⁶A levels and its regulatory network (16–20). Thus, this may allow the regulation of resistance gene expression regulated by this epitranscriptomic mark to be fine-tuned depending on insecticide exposure. Further work is required to investigate how readily *CYP4C64* expression can be silenced by demethylation of A-206 in the absence of insecticide selection and then reactivated following resumption of insecticide use. Such knowledge is of relevance for resistance management strategies, which commonly rely on the rotation of several insecticides with different modes of action. This strategy is based on the premise that individuals carrying mechanisms that confer resistance to a specific insecticide exhibit fitness costs in the absence of this compound, which means they are outcompeted by susceptible individuals. However, if resistance gene expression can be readily switched off, these fitness costs can be avoided, making this resistance management strategy less effective.

A key component of effective resistance management is monitoring target pest populations for the mechanisms that confer resistance. However, in the case of metabolic resistance, this is frequently time-consuming and costly as identification of changes in detoxification gene expression often requires the extraction of RNA from samples. This also precludes the use of many of the approaches commonly used to store specimens before molecular analyses (i.e., in ethanol or on silica). In this study, we identify a genetic alteration (T-206A) that is linked to the regulation of a resistance gene by an epitranscriptomic mechanism. This provides a marker for the development of DNA-based diagnostics that can be used to monitor the frequency and distribution of resistance in field-collected individuals stored in a variety of cost-effective ways. Such information will inform the development and deployment of strategies to control *B. tabaci* while reducing ineffective insecticide application. Related to this, the identification of the key mechanisms involved in the regulation of *CYP4C64* also highlights potential new targets for control interventions against this global crop pest. The demonstration in this study that knockdown of *METTL3* and *METTL14* reduces *CYP4C64* expression and increases the sensitivity of resistant strains of *B. tabaci* to thiamethoxam illustrates the promise of this approach.

MATERIALS AND METHODS

Insect strains

B. tabaci MEAM1 (Middle East-Asia Minor 1) adults were collected from a greenhouse in the Haidian district in the municipality of

Beijing in 2000 and used to establish the susceptible strain *TH-S* ($S^{#1}$), which has been maintained on cabbage (*Brassica oleracea* L. var. capitata) for more than 18 years without exposure to any pesticide (table S1) (40). The TH-S strain was selected with thiamethoxam each generation (approximately 30 days) to form a resistance strain *TH-R* ($R^{#1}$) that exhibits a level of resistance 79-fold that of the THS strain. In 2011, another susceptible *B. tabaci* MED strain (HZ-S) was collected from tomato in Hangzhou, Zhejiang province, and maintained on cotton plants (*Gossypium herbaceum* L. cv. Zhongmian 49). The sensitivity of this strain to thiamethoxam is similar to that of the TH-S strain. The HZ-S strain was selected with thiamethoxam to form the HZ-R ($R^{#2}$)-resistant strain. The resistance level of this strain to thiamethoxam is 151-fold that of the TH-S strain. Last, eight *B. tabaci* MED field strains ($R^{#3}$ to $R^{#10}$) were collected in different regions of China from 2011 to 2018, and further details and the resistance levels of these strains to thiamethoxam are shown in tables S1 and S2. All strains were maintained in a glasshouse at 25°C.

Insecticides and whitefly bioassays

Formulated thiamethoxam, WG (250 g kg⁻¹; Syngenta China Investment Co. Ltd.), was used for all insecticide bioassays. These were conducted on adult *B. tabaci* using the leaf dipping method described by Feng *et al.* (40). Thiamethoxam was dissolved and diluted with distilled water containing Triton X-100 (0.1%). Leaf discs (22 mm in diameter) from cotton plants were dipped for 10 s in insecticide solution. After air drying, leaf discs were placed with their adaxial surface downward onto a bed of agar (2 ml of 20 g/liter) in a flat-bottomed glass tube (78-mm length). Adult whiteflies were collected by inverting the tubes so that adults (mixed sex) would fly into the tube. The open end of the tube was sealed with a cotton plug. Control groups were treated with distilled water (containing 0.1% Triton X-100) applied to leaf discs in the same manner as the treatment groups. Each tube contained 15 to 30 adults and was placed in an incubator at 25°C and a 14:10 (Light: Dark) photoperiod. Mortality was recorded after 48 hours, and each combination of population and thiamethoxam concentration was represented by four replicates.

Extraction of RNA and RACE

Total RNA was isolated from 60 whole adult *B. tabaci* using standard TRIzol (Invitrogen) protocols; the extracted RNA was converted to cDNA using oligo(dT) primer and SuperScript II reverse transcriptase with gDNA Eraser (TaKaRa Biotech). The complete cDNA sequence of *CYP4C64* was obtained by 3' and 5' RACE using the SMARTer RACE cDNA Amplification Kit (Clontech).

Whitefly qRT-PCR

A total of 150 adults (three biological replicates, $n = 50$) from different strains were used for the quantitative reverse transcription PCR (qRT-PCR) analysis. Primers were designed to amplify a 90- to 200-bp fragment, and amplification efficiencies are listed in data file S5. The total reaction volume of 20 μ l comprised 1 μ l of diluted cDNA, 10 μ l of SYBR Green Real-time PCR Master Mix (Tiangen), and 0.3 μ l of each primer. Samples were run on an ABI Q3 real-time system (Applied Biosystems) using the following temperature cycling conditions: 15 min of activation at 95°C, followed by 40 cycles of 30 s at 95°C, 40 s at 60°C. A threefold dilution series of cDNA was used to construct a relative standard curve, and the PCR efficiency was determined and used to convert quantification cycle [cycle threshold (Ct) values] into processed data (relative quantities). The fold changes

in the expression of whitefly genes of interest, normalized to two reference genes (*EF1 α* and *RPL29*) (12), were calculated using the $2^{-\Delta\Delta C_t}$ method on the basis of three biological replicates. Nine cytochrome P450 genes [*CYP6CM1*, *CYP6CX1*, *CYP6CX4*, and *CYP6DZ7* (37); *CYP6CX3* and *CYP6CX5* (43); and *CYP6DZ4*, *CYP4C1*, and *CYP4G68* (45)], two GST genes [*GSTd7* (38) and *GST14* (44)], and one ATP-binding cassette subfamily G member 3 gene [*ABCG3* (38)] previously implicated in neonicotinoid resistance in whitefly were selected for qRT-PCR analysis.

Whitefly RNAi experiments

dsRNA was prepared using the T7 RiboMAX Expression RNAi system (Promega) following the manufacturer's instructions. EGFP was used to generate control EGFP dsRNA. All primers used for producing dsRNA are listed in data file S5. dsRNAs were fed to adult whiteflies, within 2 days after emergence, using the RNAi system as previously described (12). Whiteflies were collected after 48 hours of feeding and snap frozen in liquid nitrogen before molecular analyses. The efficiency of RNAi knockdown of *CYP4C64*, *METTL3*, and *METTL14* is shown in figs. S3A and S7 (C and D). dsRNA was introduced to adult *B. tabaci* via a feeding satchet. The feeding satchet consisted of a glass tube (20 mm in diameter \times 50 mm long, open at both ends), which was covered at the top by one layer of Parafilm membrane stretched as thinly as possible. A total of 0.1 ml of diet solution (5% yeast extract and 30% sucrose, w/v) was pipetted onto the outer surface of the stretched Parafilm. Insecticide or dsRNA (0.5 μ g/ μ l) was dissolved in the diet solution. A second layer of Parafilm was stretched on top of the first membrane to form a feeding satchet. Fifty adult whiteflies (mixed sexes) were transferred into each tube, and the remaining opening was sealed with a black cotton plug and covered with a shade cloth. The tubes were placed in an environmental chamber (Panasonic MLR-352H, Gunma, Japan) at 25°C and with a photoperiod of L14:D10 and 80% relative humidity. The ends of the tubes with the Parafilm sachets were turned toward the light source, which was approximately 0.2 m away.

Transgenic expression of *CYP4C64* gene in *D. melanogaster*

Wild-type *CYP4C64* was synthesized and provided in the pUASTattB40 plasmid. Using the PhiC31 system, clones were transformed into the germ line of a *D. melanogaster* strain carrying the attP40 docking site on chromosome 2 [w^{1118} ; $P\{w(+mC) = UAS-CYP4C64\} attP40(25C6)/CyO$]. The transgenic lines obtained were balanced, and the integration of *CYP4C64* was confirmed by PCR and sequencing using the LA DNA polymerase (TaKaRa) as detailed above with the primers detailed in data file S5. Virgin females of the Tub-GAL4 [w^{1118} ; $P\{w(+mC) = act5C-gal4\} attP2(68A4)/TM6B$] strain were crossed with males of the UAS-*CYP4C64* strain (FunGene). Bioassays were used to assess the susceptibility of adult F1 flies of this cross to thiamethoxam. Several concentrations of thiamethoxam were overlaid onto 2% agar containing 1% sucrose in standard *Drosophila* vials and allowed to dry overnight at room temperature. Ten to 20 adult flies (2 to 5 days after eclosion) were then added to each vial, and mortality was assessed after 48 hours (4). Four replicates were carried out for each concentration. Control mortality was assessed using vials containing agar/sucrose minus insecticide.

Cell culture and cell RNAi assay

Drosophila S2 cells were cultured in HyClone SFX-insect medium (Thermo Fisher Scientific) at 27°C. Approximately 5×10^{-5} cells per

well were added to 24-well plates 2 hours before transfection. Plasmids were transfected into the S2 cells using Lipofectamine 2000 (Invitrogen). RNAi of *Drosophila* S2 cells was conducted as described previously (30, 31), and ds*DMETTL3* and ds*DMETTL14* (3 μ g) were transfected into the S2 cells in combination with dual-luciferase reporter plasmids.

Dual-luciferase reporter assays

Wild-type 5'UTR-*CYP4C64*-R and 5'UTR-*CYP4C64*-S were amplified from cDNA of resistant and susceptible whiteflies and then cloned into the pGL4.26 reporter plasmid (Promega), which contains a mini-promoter, carrying the indicated promoter regions conjugated to firefly luciferase. Reporter plasmids carrying mutated 5'UTR regions of *CYP4C64* were constructed from the pGL4.26^{R/S} plasmids. The primers used for the construction of the reporter plasmids are listed in data file S5. The coding sequence of *METTL3* and *METTL14* were cloned into the pAC5.1b/V5/His expression vector (Invitrogen) for expression in S2 cells. pGL4.10-*CYP4C64*^{R/S} (600 ng) and a reference reporter pGL4.73 plasmid (200 ng, containing the *hRluc* reporter gene and an SV40 early promoter) were then cotransfected with pAC5.1b-*METTL3*/*METTL14* (600 ng) into S2 cells (24-well plates) and kept at 27°C. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System and a GloMax 96 Microplate Luminometer (Promega) 48 hours after transfection (12). Construct luciferase activity was normalized to *Renilla* luciferase activity.

Western blot

Total protein was extracted from 200 adult whiteflies per sample with the cell lysis buffer for Western and IP Kit (Beyotime) following the manufacturer's instructions. Protein was quantified using the BCA Protein Assay Kit (Beyotime), and 20 μ g of total protein of each sample was then analyzed (CWBio). Rabbit polyclonal antibody of *CYP4C64* was raised against a synthetic peptide (Jiaxuan Biotech). The sequence of the peptide of *CYP4C64* was N-K-R-I-Q-L-V-R-T-M-N (from sites 25 to 35). Antibody specificity was confirmed by BLAST search of the peptide sequence against the genome of *B. tabaci* and transcriptome datasets of different development stages (53). Western blots were probed for *METTL3* (Abcam), *METTL14* (Cell Signaling Technology), and β -actin antibody (Abcam). β -Actin was used as a loading control in Western blot.

m⁶A dot blot

m⁶A dot blots were conducted as previously described with some modifications (54). Ploy(A)⁺ mRNA samples were denatured at 50°C for 15 min in three sample volumes of RNA incubation buffer. An equal volume of chilled 20x SSC buffer (Sigma-Aldrich) was then added before samples were spotted on a polyvinylidene difluoride (PVDF) membrane (Millipore) and fixed at 80°C for 40 min. The membrane was blocked with 5% nonfat milk and incubated with anti-m⁶A antibody (1:10,000; Synaptic Systems) overnight at 4°C. Then, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Beyotime) was added to the blots for 1 hour at room temperature, and the membrane was developed with Amersham ECL Prime Western Blotting Detection Reagent (Millipore).

Gene-specific m⁶A qPCR

Thiamethoxam-resistant and thiamethoxam-susceptible mRNAs were subject to methylated RNA immunoprecipitation (MeRIP)

analysis using the Magna MeRIP m⁶A kit (17-10499, Millipore) to profile m⁶A RNA methylation sites (55). *CYP4C64*-specific m⁶A qPCR was used to determine m⁶A abundance across transcripts of this gene. Briefly, 30 μ g of total RNA was sheared to about 100 nucleotides in length by metal ion-induced fragmentation and then purified and incubated with anti-m⁶A antibody-conjugated beads in 100 μ l of 1 \times immunoprecipitation buffer supplemented with ribonuclease inhibitors at 4°C for 2 hours. Methylated RNA was immunoprecipitated with beads, eluted by competition with free m⁶A, recovered with elution buffer, and converted to cDNA (Taraka Biotech), further analyzed by qPCR along with MeRIPed RNA (TIANGEN). The relative enrichment of m⁶A in each sample was calculated by normalizing to a reference gene *EF1 α* (*RPL29* cannot determine in the MeRIPed RNA). Each group was repeated in triplicate.

Quantitative analysis of whitefly m⁶A level using LC-MS/MS

LC-MS/MS for determination of m⁶A/A ratio was performed as previously described (56, 57). Briefly, 1 μ g of total RNA of whitefly adults was incubated at 95°C for 5 min and then on ice for 2 min. Two microliters of buffer [300 mM CH₃COONa, 2.8 M NaCl, and 10 Mm ZnSO₄ (pH 4.6)], 1 μ l of nuclease S1 (180 U/ μ l; Sigma-Aldrich), and 1 μ l of double-distilled water (ddH₂O) were added, and samples were incubated at 37°C for 4 hours. Subsequently, 10 μ l of buffer 2 [0.5 M tris-HCl and 10 mM MgCl₂ (pH 9.0)], 5 μ l of venom phosphodiesterase I (2 mU/ μ l; Sigma-Aldrich), and 1 μ l of alkaline phosphatase (30 U/ μ l; Sigma-Aldrich) were added, and samples were incubated at 37°C for 2 hours. ddH₂O was then added to 200 and 10 μ l of the solution injected into LC-MS/MS. Nucleosides were separated by reverse-phase ultraperformance liquid chromatography on a T3 C18 column (2.1 \times 100 mm, 1.7 μ m, Waters), with online mass spectrometry detection using a Sciex TripleTOF 6600 UPLC mass spectrometer in positive electrospray ionization mode. The nucleosides were quantified using the nucleoside to base ion mass transitions of 282 to 150 (m⁶A) and 268 to 136 (A). Quantification was performed by comparison with the standard curve obtained from pure nucleoside standards running as part of the same batch of samples. The ratio of m⁶A to A was calculated on the basis of the calculated concentrations.

Statistical analysis

The statistical significance of differences between samples was analyzed by Student's *t* test and analysis of variance (ANOVA) with Tukey's post hoc test (GraphPad 7.0). All quantitative data are reported as means \pm SEM from at least three independent experiments.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/7/19/eabe5903/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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