Epitranscriptomic regulation of insecticide resistance

Xin Yang1†, Xuegao Wei1†, Ying Yang1†, Tianhua Du1, Cheng Yin1, Buli Fu1, Mingjiao Huang1, Jinjin Liang1, Peipan Gong1, Shaonan Liu1, Wen Xie1, Zhaojiang Guo1, Shaoli Wang1, Qingsun Wu1, Ralf Nauen2, Xuguo Zhou3, Chris Bass4*, Youjun Zhang1‡

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 posttranscriptional 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 MEA1/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...
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 WxxxxR motif (positions 127 to 131), the heme-binding motif (AGxxT, 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*<sup>6</sup>), *WH-R* (*R*<sup>6</sup>), *TZ-R* (*R*<sup>7</sup>), *LF-R* (*R*<sup>9</sup>), and *NK-R* (*R*<sup>10</sup>)), and high resistance levels to thiamethoxam were observed in the *THR* (*R*<sup>11</sup>), *HZ-R* (*R*<sup>2</sup>), *JZ-R* (*R*<sup>4</sup>), *JY-R* (*R*<sup>5</sup>), and *CS-R* (*R*<sup>8</sup>) 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*<sup>2</sup>, *R*<sup>2</sup>, *R*<sup>5</sup>, and *R*<sup>8</sup> 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 = 1.11×10<sup>−4</sup>) (Fig. 1B and fig. S3D). Furthermore, when two resistant strains (*R*<sup>6</sup> and *R*<sup>8</sup>) and one susceptible strain (*S*<sup>2</sup>) 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.](http://advances.sciencemag.org/)

*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*<sup>2</sup>) and two resistance strains (*R*<sup>2</sup> and *R*<sup>8</sup>) 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*<sup>2</sup>) and two resistance strains (*R*<sup>2</sup> and *R*<sup>8</sup>) 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 ± 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 × U: UAS-CYP4C64/Tub-GAL4 line; n = 3, mean ± SE; **P < 0.01, ANOVA with Tukey’s HSD post hoc test). Yang et al., Sci. Adv. 2021; 7 : eabe5903 5 May 2021*
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 × 10⁻⁵; figs. S3A and S14A).

Knockdown of CYP4C64 significantly decreased the resistance of adult whiteflies of the R², R⁵, and R⁸ strains, relative to the control [fed enhanced green fluorescent protein (EGFP) dsRNA], when treated with 1.0 mM thiamethoxam (R²: P = 4.26 × 10⁻³; R⁵: P = 7.41 × 10⁻³; R⁸: P = 3.85 × 10⁻³; Fig. 1E and fig. S3B) and 5.1 mM thiamethoxam (R²: P = 3.99 × 10⁻⁴; R⁵: P = 8.41 × 10⁻⁴; R⁸: P = 2.56 × 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 enhanced resistance to thiamethoxam in vivo, we created a transgenic line expressing this P450. Flies expressing the CYP4C64 transgene displayed enhanced 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 CAGCA (Arrhenia ssp.) that closely resembles an N⁶-methyladenine (m⁶A) 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 enhancer element. E. coli cells were transformed with these constructs, and luciferase reporter assays were performed to determine the effect of this mutation on gene expression.

**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/ 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 ± SE; **P < 0.01 and ***P < 0.001, ANOVA with Tukey’s HSD post hoc test). (D) RNAi knockdown of two D. melanogaster m⁶A writer genes, METTL3 and METTL14, modulates CYP4C64 expression in S2 cells. The 5′UTR of CYP4C64 (pGL4.26-CYP4C64/5′UTR-R-WT) derived from resistant or susceptible (pGL4.26-CYP4C64/5′UTR-R-WT) whiteflies was transfected into S2 cells together with dsRNA for either METTL3 or METTL14 (n = 3; mean ± 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 ± 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 × 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 thiabendazole 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 thiabendazole-resistant whiteflies was changed from CGAC to CGGC (5'UTR-R-Mut). A second construct was generated where the consensus thiabendazole-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 × 10⁻⁸) 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 × 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 thiabendazole-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 × 10⁻⁵; METTL14: P = 8.52 × 10⁻⁵) 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 thiabendazole-resistant B. tabaci.

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

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-CYP4C64UTR-R-WT and pGL4.10-CYP4C64UTR-S-WT. In reporter gene assays, cells expressing the 5'UTR from resistant whiteflies in combination with either whitely METTL3 or METTL14 exhibited significantly (METTL3: P = 1.82 × 10⁻⁵; 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 thiabendazole-susceptible and thiabendazole-resistant B. tabaci strains, we used dot blot and liquid chromatography–tandem mass spectrometry (LC-MS/MS) to compare m⁶A levels in the thiabendazole-susceptible strain (S⁶²) and two resistant strains (R⁶² and R⁶⁸), which exhibit high levels of thiabendazole resistance and CYP4C64 expression. Global m⁶A levels were significantly higher in both resistant strains (R⁶²: P = 0.0099; R⁶⁸: 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 S⁶² and R⁶² strains 24 and 48 hours after exposure to 1.0 mM thiabendazole 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 (R⁶²: P = 1.5 × 10⁻³; R⁶⁸: P = 2.1 × 10⁻²; R⁶⁸: P = 1.56 × 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 thiabendazole-resistant and thiabendazole-susceptible B. tabaci strains, homo antibodies were used in Western blots of the S⁶², R⁶², R⁶⁸, and R⁶⁸ 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 thiabendazole-resistant strains R⁶² and R⁶⁸ with the susceptible strain S⁶². This revealed that WTAP and KIAA1429 are significantly overexpressed in the resistant strains (WTAP: R⁶²: P = 0.003; R⁶⁸: P = 1.49 × 10⁻⁵; and KIAA1429: R⁶²: P = 0.001; R⁶⁸: 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 R⁶⁸. 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). Moreover, a corresponding significant decrease (METTL3: 34%, P = 0.03; METTL14: 51%, P = 6.64 × 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 whitely 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: CYP6D2A, 64%, P = 3.44 × 10⁻⁹; CYP6D2Z7,
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...
stress (47), and m^6^A in the 5′UTR guides mRNA alternative translation during the integrated stress response (48). As previous studies have shown that a single 5′UTR m^6^A 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 m^6^A levels and expression of this P450 gene. The finding that m^6^A is associated with up-regulation of CYP4C64 also supports recent work on the silkworm, B. mori (33), in suggesting that m^6^A positively regulates gene expression in insects. The biological functions of m^6^A 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 m^6^A methyltransferase complex, WTAP and KIAA1429, as significantly overexpressed in the resistant strains, suggesting that these accessory proteins may also play a role in enhancing m^6^A levels. Together, these findings reveal how an m^6^A site may act in concert with other components of the m^6^A 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 m^6^A 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 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, m^6^A has been shown to act as a dynamic and reversible modification, and stressful stimuli can alter both m^6^A 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\(^3\)), 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\(^3\)) 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 construct a relative standard curve, and the PCR efficiency was determined by the germ line of a strain carrying the attP40 docking plasmid. Using the PhiC31 system, clones were transformed into the genome of D. melanogaster via a feeding satchet. The feeding satchet consisted of a glass tube (20 mm in diameter × 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 μg/μl) 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 0.5 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 \(w^{1118}; P[w(w+Mc) = UAS-CYP4C64]attP40(25C6)/CyO\). The transgenic lines obtained were balanced, and the incorporation 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 strain \(w^{1118}; P[w(w+Mc) = act5C-gal4]attP2(68A4)/TM6B\) were crossed with males of the UAS-CYP4C64 strain (FunGene). Biosays 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). RNAsi of Drosophila S2 cells was conducted as described previously (30, 31), and dsMETTL3 and dsMETTL14 (3 μg) 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 pGL4.26BS 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/V5His expression vector (Invitrogen) for expression in S2 cells. pG4.10-CYP4C64BS (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 μg of total protein of each sample was then analyzed (CBio). 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 subjected 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 μg 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 μl of 1× 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 EF1α (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 μg 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 μl of nuclease S1 (180 U/μl; Sigma–Aldrich), and 1 μl of double-distilled water (ddH2O) were added, and samples were incubated at 37°C for 4 hours. Subsequently, 10 μl of buffer 2 [0.5 M tris–HCl and 10 mM MgCl2 (pH 9.0)], 5 μl of venom phosphodiesterase I (2 μl/μl; Sigma–Aldrich), and 1 μl of alkaline phosphatase (30 U/μl; Sigma–Aldrich) were added, and samples were incubated at 37°C for 2 hours. ddH2O was then added to 200 and 10 μl of the solution injected into LC-MS/MS. Nucleosides were separated by reverse-phase ultra-performance liquid chromatography on a T3 C18 column (2.1 × 100 mm, 1.7 μm, 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 ± 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.

**REFERENCES AND NOTES**

1. X. Li, M. A. Schuler, M. R. Benesbaum, Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu. Rev. Entomol.* **52**, 231–253 (2007).

2. P. J. Daborn, J. L. Yen, M. R. Bogwitz, G. Loffe, E. Feil, S. Jeffers, N. Tiget, T. Perry, D. Heckel, P. Battenham, R. Feyereisen, T. G. Wilson, R. H. French-Constant, A single p450 allele associated with insecticide resistance in Drosophila. *Science* **297**, 2253–2256 (2002).

3. X. Li, J. Baudry, M. R. Benesbaum, M. A. Schuler, Structural and functional divergence of insect CYP6B proteins: From specialist to generalist cytochrome P450. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 2939–2944 (2004).
D. T. Timpte, Induction of sporulation in Saccharomyces cerevisiae leads to the formation of N\textsuperscript{6}-methyladenosine in m\textsuperscript{6} A: A potential mechanism for the activation of the IRE1A gene. *Nucleic Acids Res.* **30**, 4509–4518 (2002).

7. T. Lence, J. Akhtar, M. Bayer, K. Schmid, L. Spindler, C. H. Ho, N. Keim, M. A. Andrade-Navarro, B. Poeck, M. Helm, J.-Y. Roignant, m\textsuperscript{6} A modulates neuronal functions and sex determination in *Drosophila*. *Nature* **540**, 242–247 (2016).

8. I. U. Hausmann, Z. Bodi, E. Sanchez-Moran, N. P. Mongan, N. Archer, R. G. Fray, M. Soller, m\textsuperscript{6} A potentiates Si alternative pre-mRNA splicing for robust *Drosophila* sex determination. *Nature* **540**, 301–304 (2016).

9. X. Zhang, Y. Zhang, K. Dai, Z. Liang, M. Zhu, J. Pan, M. Zhang, B. Yan, H. Zhu, Y. Dai, M. Cao, R. Xue, G. Cao, X. Hu, C. Gong, N\textsuperscript{6}-methyladenosine level in silkworm midgut/Ovary cell line is associated with Bombyx mori nucleopolyhedrovirus infection. *Front. Microbiol.* **10**, 2988 (2020).

10. B. Li, X. Wang, Z. Li, C. Lu, Q. Zhang, L. Chang, W. Li, T. Cheng, Q. Xia, P. Zhao, Transcriptome-wide analysis of N\textsuperscript{6}-methyladenosine uncovers its regulatory role in gene expression in the lepidopteran *Bombyx mori*. *Insect Mol. Biol.* **28**, 703–719 (2019).

11. P. J. De Barro, S.-S. Liu, L. M. Boykin, A. B. Dinsdale, *Bemisia tabaci*: A statement of species status. *Annu. Rev. Entomol.* **56**, 1–19 (2011).

12. A. R. Horowitz, M. Ghanim, E. Roktidakis, R. Nauen, J. Ishaaya, Insecticide resistance and its management in *Bemisia tabaci* species. *J. Pest Sci.* **93**, 893–910 (2020).

13. C. Bass, J. Denholm, M. S. Williamson, R. Nauen. The global status of insect resistance to neonicotinoid insecticides. *Pest. Biochem. Physiol.* **121**, 78–87 (2015).

14. X. Yang, W. Xie, S.-L. Wang, Q.-J. Wu, H.-P. Pan, R.-M. Li, N.-Y. B. M. Liu, B.-Y. Yu, X. Zhou, Y.-J. Zhang. Two cytoplasmic P450 genes are involved in imidacloprid resistance in field populations of the whitefly, *Bemisia tabaci*, in China. *Pest. Biochem. Physiol.* **107**, 343–350 (2015).

15. R. Wang, W. Che, J. Wang, C. Luo, Monitoring insecticide resistance and diagnostics of resistance mechanisms in *Bemisia tabaci* Mediterranean (Q biotype) in China. *Pest. Biochem. Physiol.* **163**, 117–122 (2020).

16. I. Kunaruk, J. Benting, B. Lueke, T. Ponge, R. Nauen, E. Roktidakis, J. Vontas, K. Gorman, J. Denholm, S. Morin. Over-expression of P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (hemiptera: Aleyrodidae). *Insect Biochem. Mol. Biol.* **38**, 634–644 (2008).

17. Y. T. Feng, Q. J. Wu, B. Y. Xu, S. L. Wang, X. L. Chang, W. Xie, J. Y. Zhang. Fitness costs and morphological change of laboratory-selected thiamethoxam resistance in the B-type *Bemisia tabaci* (hemiptera: Aleyrodidae). *J. Appl. Entomol.* **133**, 466–472 (2009).

18. W. Xie, Q.-S. Meng, Q.-J. Wu, S.-J. Wang, X. Yang, N.-Y. B. M. Liu, X.-G. Pan, J. P. H. Pan, B.-M. Liu, Q. Xu, B.-Y. Xu, S.-H. Li, X.-G. Zhou, Y.-J. Zhang. Pyrosequencing the *Bemisia tabaci* transcriptome reveals a highly diverse bacterial community and a robust system for insecticide resistance. *PLOS ONE* **7**, e35181 (2012).

19. W. Xie, C. Chen, Z. Yang, L. Guo, X. Yang, D. Wang, M. Chen, J. Huang, Y. Wen, Y. Zeng, Y. Liu, J. Xian, L. Hui, C. Q. G. B. S. Wang, B. Xu, X. Li, X. Tan, M. Ghanim, B. Qiu, H. Pan, D. Chu, H. Delatte, M. M. Marmuth, F. Ge, X. Zhou, X. Wang, F. Wan, Y. Yu, D. Luo, F. Yan, L. E. Preiser, X. Jiao, B. C. Q. Zhao, G. Qiao, J. X. Yin, Y. Liu, J. K. Brown, X. J. Zhou, Y. Zhang. Genome sequencing of the sweetpotato whitefly *Bemisia tabaci* MED/Q. *Gigascience* **6**, 1–7 (2017).

20. A. Ilas, J. Langel, D. E. Kappanidiali, E. Roktidakis, C. S. Tsigenopoulos, J. Vontas, A. Tsagkarakou, Transcription analysis of neonicotinoid resistance in *Mediterranean* (Q biotype) populations of *B. tabaci* reveal novel cytochrome P450s, but no N\textsuperscript{6}H-twist mutations associated with the phenotype. *BMC Genomics* **16**, 939 (2015).

21. X. Yang, C. He, W. Che, Y. Liu, J. Xian, Z. Yang, L. Guo, Y. Wen, S. Wang, Q. Wu, F. Yang, X. Zhou, Y. Zhang, Glutathione S-transferases are involved in thiamethoxam resistance in the field whitefly *Bemisia tabaci* Q (hemiptera: Aleyrodidae). *Pest. Biochem. Physiol.* **134**, 73–86 (2013).

22. Q. Wang, M.-W. Wang, Z.-Z. Jia, T. Ahamt, L.-J. Xie, W.-H. Jiang. Resistance to neonicotinoid insecticides and expression changes of eighteen cytochrome P450 genes in field populations of *Bemisia tabaci* from Xingjiang, China. *Entomol. Res.* **50**, 205–211 (2020).

23. K. D. Meyer, Y. Saletore, P. Zumbo, O. Elemento, C. E. Mason, S. R. Jaffrey. Comprehensive analysis of mRNA methylation reveals enrichment in 3'UTRs and near stop codons. *Cell* **169**, 1635–1646 (2018).

24. K. D. Meyer, D. P. Patil, J. Zhou, A. Zinoviev, M. A. Skabkin, O. Elemento, T. V. Pestova, S.-B. Qian, S. R. Jaffrey. 5' UTR m\textsuperscript{6} A promotes cap-independent translation. *Cell* **163**, 991–1010 (2015).

25. J. Zhou, J. Wang, X. Sun, Y. Mao, X.-M. Liu, X. Yuan, X. Zhang, M. E. Hess, J. C. Brüning, S.-B. Qian, N\textsuperscript{6}-methyladenosine guides mRNA alternative translation during integrated stress response. *Mol. Cell* **69**, 636–647 e7 (2018).

26. L. B. Miller, A. D. Devos, R. H. French-Constant, B. G. Forde. Changes in DNA methylation are associated with loss of insecticide resistance in the peach-potato aphid *Myzus persicae* (Sulz.). *FEBS Lett.* **243**, 323–327 (1989).
50. M. L. Field, L. A. Devonshire, Evidence that the E4 and E4 esterase genes responsible for insecticide resistance in the aphid Myzus persicae (Sulzer) are part of a gene family. Biochem. J. 330, 169–173 (1998).

51. C. Bass, A. M. Fuinean, C. T. Zimmer, I. Denholm, L. M. Field, S. P. Foster, O. Gutbrod, R. Nauen, R. Slater, M. S. Williamson. The evolution of insecticide resistance in the peach potato aphid, Myzus persicae. Insect Biochem. Mol. Biol. 51, 41–51 (2014).

52. S. Li, F. Hussain, G. C. Unnithan, S. Dong, Z. UlAbdin, S. Gu, L. G. Mathew, J. A. Fabrick, X. Ni, Y. Carrière, B. E. Tabashnik, X. Li, A long non-coding RNA regulates cadherin transcription and susceptibility to Bt toxin Cry1Ac in pink bollworm, Pectinophora gossypiella. Pest. Biochem. Physiol. 158, 54–60 (2019).

53. L. Tian, T. Song, R. He, Y. Zeng, W. Xie, Q. Wu, S. Wang, X. Zhou, Y. Zhang. Genome-wide analysis of ATP-binding cassette (ABC) transporters in the sweetpotato whitefly, Bemisia tabaci. BMC Genomics 18, 330 (2017).

54. R. Hodge. Preparation of RNA dot-blots. Methods Mol. Biol. 86, 73–75 (1998).

55. K. Thüring, K. Schmid, P. Keller, M. Helm. LC-MS Analysis of Methylated RNA/RNA Methylation (Humana Press, New York, 2017), pp. 3–18.

56. G. Jia, Y. Fu, X. Zhao, Q. Dai, G. Zheng, Y. Yang, C. Yi, T. Lindahl, T. Pan, Y.-G. Yang, C. He. N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. Nat. Chem. Biol. 7, 885–887 (2011).
Epitranscriptomic regulation of insecticide resistance

Xin Yang, Xuegao Wei, Jing Yang, Tianhua Du, Cheng Yin, Buli Fu, Mingjiao Huang, Jinjin Liang, Peipan Gong, Shaonan Liu, Wen Xie, Zhaojiang Guo, Shaoli Wang, Qingjun Wu, Ralf Nauen, Xuguo Zhou, Chris Bass and Youjun Zhang

Sci Adv 7 (19), eabe5903.
DOI: 10.1126/sciadv.abe5903