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Supplementary Information for

MAPK-directed activation of the whitefly transcription factor *CREB* leads to P450-mediated imidacloprid resistance

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SI Results

Lifespan of no-imidacloprid controls

The mean mortality observed at the conclusion of the experiment in all of the no-imidacloprid controls run in RNAi experiments was 13% and did not exceed 17% in any strain (Dataset S1). No significant difference was observed between the survival curves of no-imidacloprid controls treated with control *EGFP* dsRNA and those treated with whitefly gene-specific dsRNA (Dataset S1). No significant difference was observed in the no-imidacloprid survial curves obtained from the IMS and IMR strains (Dataset S1). A significant difference was observed between the no-imidacloprid survial curves obtained from the IMS strain and the three field strains, NK, YC, SY, however in all cases the mortality at the conclusion of the experiment was very similar for all four strains (Dataset S1).

SI Materials and Methods

Insect Strains. A field strain of *B. tabaci* MED was collected in 2011 from Hangzhou in Zhejiang Province and split into two cultures one of which was treated with 2.0 mM imidacloprid for more than 30 generations by soil drench application to form the IMR strain, and the other left unexposed to insecticides to form the IMS strain. The IMR and IMS strains were maintained, and experiments were conducted, at 25 ^{o}C with a 14 h: 10 h light: dark cycle and 70% humidity on pepper (cv. Zhongjiao #4, *Capsicum annuum*) plants. Four field strains of *B. tabaci* MED were collected in 2018 from northern China (*SI Appendix*, Table S1). A total of approximately 2000 adults were collected per location, 500 of which were snap frozen in liquid nitrogen and stored at -80^{o}C for protein extraction and western bolt analysis, the remaining adults were used immediately in insecticide bioassays and RNAi experiments.

Extraction of DNA and RNA and qRT-PCR. Genomic DNA was extracted from a pool of 50 adult whiteflies of the IMR strain using the MiniBEST universal genomic DNA extraction kit (Takara Biotech) following the manufacturer's instructions. Total RNA was isolated from 50 whole adult whiteflies using standard TRIZOL (Invitrogen) protocols; the extracted RNA was converted to cDNA using oligo (dT) primer and Superscript II reverse transcriptase with gDNA Eraser (Taraka Biotech). qRT-PCR was performed using Power SYBR Green PCR Master Mix (Tiangen) in quadruplicate on the 7500 system (Applied Biosystems). A total of 150 adults (three biological replicates, n = 50) from different populations were used for qRT-PCR analysis. Primers were designed to amplify a 90-200 bp fragment. The total reaction volume of 20-µl comprised 1 µl of diluted cDNA, 10 µl of SYBR® Green Real-time PCR Master Mix (Tiangen) and 0.3 µl of each primer. Samples were run on an ABI 7500 real-time system using the following temperature cycling conditions: 15 min of activation at 95°C followed by 40 cycles of 30 s at 95°C and 40 s at 60°C. A 2-fold dilution series of cDNA was used to construct a relative standard curve, and the PCR efficiency was determined (see Dataset S5 for amplification efficiencies). PCR efficiency was used to convert quantification cycle (Ct-values) into processed data (relative quantities). The

fold-changes in the expression of whitefly genes of interest, normalized to three reference genes (*Actin*, *EF1α* and *RPL29*), were calculated using the $2^{-\triangle\triangle Ct}$ method (1).

Cell Culture. *Drosophila* S2 cells were cultured in Hyclone SFX-insect medium (Thermo Scientific) at 27 °C. Human HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator containing 5% CO_2. Approximately 5×10^{-5} cells per well were added to 24-well plates 6 h before transfection. Plasmids were transfected into the S2 and HEK293T cells using Lipofectamine 2000 (Invitrogen).

Promoter Analysis of *CYP6CM1*. The promoter sequence of *CYP6CM1* was analyzed by PCR and sequencing. Primers were designed based on the genomic sequence of *B. tabaci* (2) and used to characterize the ~6-kb putative promoter region immediately upstream of the translation start codon of *CYP6CM1* of the IMR strain (listed in Dataset S2). PCR products were recovered from agarose gels, PCR amplified using LA Taq (TaKaRa) and direct-sequenced. For reporter gene assays, a 1.2 kb promoter sequence was amplified from genomic DNA extracted from the IMR strain using a high-fidelity taq (TaKaRa). These were ligated into pGL4.10-Basic (Promega) and transformed into Trans1-T1 (Transgen). Plasmids were extracted with the EndoFree Mini Plasmid Kit (TIANGEN), sequenced, and adjusted to 100 ng/μL for use in dual luciferase assays.

Dual-luciferase Reporter and Dominant Negative Assays. The 5'-flanking and first intron regions of *CYP6CM1* were amplified from *B. tabaci* genomic DNA by PCR and were subcloned into the pGL4.10 reporter plasmids (Promega) carrying the indicated promoter regions conjugated to firefly luciferase. Reporter plasmids carrying deleted and mutated 5'-flanking regions of *CYP6CM1* were constructed from the $pGL4.10^{-1196}$ plasmids. The primers used for the construction of reporter plasmids are listed in Dataset S5. The coding sequence of *CREB* and 15 other transcription factors (3-22) were cloned into the pAC5.1b/V5/His expression vector (Invitrogen) for expression in S2 cells and into the pcDNA3.1V5/His vector for expression in 293T cells. q-PCR was used to estimate the expression of each transcription factor in cells. Two mutated forms of CREB (DN1, deletion of amino acids 178-282; and DN2, deletion of amino acids 1-206) were also cloned into the pAC5.1b/V5/His expression vector. pGL4.10-$CYP6CM1^{-939\ to\ +1}$ (200 ng) and a reference reporter pGL4.73 plasmid (100 ng, containing the hRluc reporter gene and an SV40 early promoter) were then cotransfected with pAC5.1b-transcription factors (600 ng) into S2 cells (24-well plates) and were kept at 27 °C. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System and a GloMax 96 Microplate Luminometer (Promega) at 48 h post-transfection. Construct luciferase activity was normalized to Renilla luciferase activity.

RNAi Experiments. dsRNA was prepared using the T7 RiboMAX Expression RNAi system (Promega) following the manufacturer's instructions. *Enhanced green fluorescent protein* (*EGFP*) was used to generate control *EGFP* dsRNA. All primers used for producing dsRNA are listed in Dataset S4. dsRNAs were fed to adult whiteflies, within 2 days after emergence, using previously

described methodology (23). Briefly, dsRNA was fed to whitefly adults in a feeding chamber comprising 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 0.2 mL of diet solution (5% yeast extract and 30% sucrose, wt/vol) 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 sachet. 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% RH. The ends of the tubes with the Parafilm sachets were turned toward the light source, which was approximately 0.2 m away. Whiteflies were collected after 48 h of feeding and snap frozen in liquid nitrogen prior to molecular analyses.

Lifespan Measurements. The effect of the neonicotinoid insecticide imidacloprid (Sigma) on the lifespan of *B. tabaci* adults was determined in glass tubes as described by Yang et al (23). Two imidacloprid concentrations (0.4 and 2.0 mM) were dissolved in the diet solution, and introduced to adult whiteflies using the same satchet feeding system used for RNAi. An additional control comprised whiteflies fed on diet without imidacloprid.100–200 adult whiteflies were tested for each whitefly strain for each treatment/control with each glass tube containing 10 adult whiteflies. Mortality was recorded every 3 hours. The Kaplan-Meier method was used for survival curve analysis, and the log-rank (Mantel-Cox) test was used to determine the statistical significance of differences between survival curves using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA) and SPSS 19.0 (SPSS for Windows, Chicago, SPSS Inc.). Plant lifespan experiments used tomato plants (*Lycopersicon esculentum*, cv. Zhongza 9) treated with imidacloprid (both spray and soil drench) for 7 days. Inhibitors were then applied by spray application and 100 whiteflies placed on plants, with mortality recorded every day until all whiteflies had died.

Insecticide Bioassays. Leaf-dip bioassays were conducted with formulated imidacloprid (700 g kg-1 WG, Bayer Hangzhou Crop Science China Co. Ltd.) on adult whiteflies using the method described by Feng et al (24). Imidacloprid was dissolved and diluted with distilled water containing Triton X-100 (0.01%) to obtain serial concentrations of insecticide. Leaf discs (22 mm diameter) from cotton plants were dipped for 10 s in the insecticide solution or in distilled water (containing 0.01% triton X-100) as a control. After they were air dried, the leaf discs were placed with their adaxial surface downwards on agar (2 mL of 15 g/L) in a flat-bottomed glass tube (78 mm long). 15–30 adult whiteflies were added to each tube and kept in an incubator at 25°C with a 14:10 (L:D) photoperiod. Mortality was recorded after 48 h, and each combination of population and imidacloprid concentration was represented by four replicate tubes.

Western Blot. Total protein was extracted from 200 adult whiteflies per sample with the ProteinExt Mammalian Total Protein Extraction Kit (Transgen) following the manufacturer's

instructions. Rabbit polyclonal antibodies of CYP6CM1, total CREB, phosphorylated CREB, total ERK, and total p38 were raised against a synthetic peptide. The sequence of the peptide of CYP6CM1 was T-T-P-K-T-P-K-K-I-T-F-D-T-N (from tyrosine 486 to asparagine 500). The sequence of the peptide of total CREB was S-G-D-P-L-S-S-S-P-S-A-N-T-T (from serine 11 to tyrosine 25). The sequence of the peptide of phosphorylated CREB was D-I-L-T-R-R-P-pS-Y-R-K-I-L-N (from aspartic acid 103 to asparagine 117), in which Ser-111 was phosphorylated. Western blots were probed for phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (#4370), phosphor-p38 (Thr180/Tyr182) (#4631) (Cell Signaling Technologies). The sequence of the peptide of total ERK was L-E-Q-Y-Y-D-P-A-D-E-P-V-A-E (from serine 315 to tyrosine 328), and the sequence of the peptide of total p38 was D-P-E-D-E-P-T-S-P-P-Y-D-Q-S (from serine 309 to tyrosine 322), and β-actin antibody (#ab8227) (Abcam). The sequence of peptides of total CYP6CM1, CREB, ERK, and p38 were analyzed in the genome of *B tabaci* MED, they are present only in the target proteins. β-actin was used as a loading control in Western blot.

Inhibitor Assays. The following kinase inhibitors were purchased from Selleck: PD98059 (#S1102), SB203580 (#S1076), and SP600125 (#S1460). Dual luciferase reporter assays, in which pGL4.10-CYP6CM1$^{-939\ to\ +1}$ and pGL4.73 were co-transfected into S2 cells with either the ERK inhibitor PD98059 (75 μM), the p38 inhibitor SB203580 (53 μM), or the JNK inhibitor SP600125 (45 μM) were performed. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System and a GloMax 96 Microplate Luminometer (Promega) at 48 h post-transfection. Inhibitors were dissolved in DMSO as stock solutions with DMSO alone added to the cell culture as a control. An ERK inhibitor (PD98059 at 1.8mM) or a p38 inhibitor (SB203580 at 1.3 mM) were fed to adult whiteflies, within 2 days after emergence, using the RNAi system as previously described (23). The efficacy of these inhibitors was further explored on tomato plants in combination with imidacloprid (0.4 mM) with 100 *B. tabaci* adults released onto each plant. The lifespan of adult *B. tabaci* was analyzed on plants exposed to 0.4 mM imidacloprid treatment + PD98059 or SB203580 in comparison to plants exposed to imidacloprid without inhibitors.

Electrophoretic Mobility Shift Assays. EMSA assays were performed using the Electrophoretic Mobility Shift Assay (EMSA) Kit (Invitrogen #E33075) according to the manufacturer's protocols. The assays were conducted using a synthetic DNA segment that contained putative *CREB* binding sites. The samples of CREB protein were expressed using the pEASY-Blunt E1 Expression Kit (Transgen) and purified with the CapturemTM His-tagged purification Kit (TaKaRa). The DNA amount was constant at 1.0 μg per reaction, and the protein/DNA ratios were 1:1, 1:2, 1:3, and 1:4. Samples were size-fractionated by gel electrophoresis using 6% TBE DNA retardation native gels at 150 V for 30 min. The DNA in the gel was stained using SYBR Green (provided in the same kit) and was visualized using the GE Typhoon LFA 9500 Imaging System (GE Healthcare LifeScience).

Statistical Analysis. The statistical significance of differences between samples was analyzed using Student's t-test and ANOVA with Tukey's HSD post hoc test (GraphPad 6.0, San Diego, CA). All quantitative data are reported as means ± SEMs from at least three independent experiments.

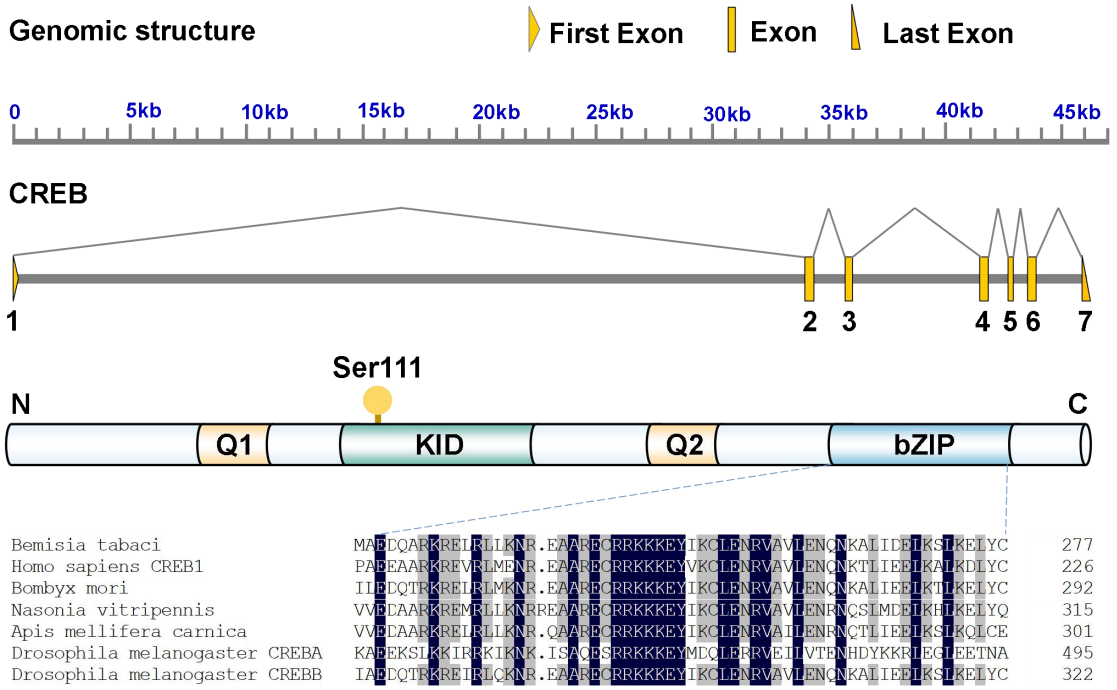

Fig. S1. Gene structure of *CREB* from the whitefly *B. tabaci*. The full-length gene that encodes *CREB* in *B. tabaci* contains an 846-bp ORF encoding 282 amino acid residues. Genomic structure analysis indicated that *CREB* contains 7 exons and 6 introns. *CREB* family members share similar modular organization: they all contain a carboxy-terminal basic Leu zipper (bZIP) DNA-binding and dimerization domain and an amino-terminal transactivation domain (TAD). The TAD contains a central kinase-inducible domain (KID) and two Gln-rich (Q1 and Q2) constitutive activation domains. A homo CREB1 Ser133-like site (RRP**S**YRK) is present in the *B. tabaci* KID domain (Ser 111). The deduced amino acid sequence includes important conserved domains common to other bZIP families, such as the leucine zipper and basic motif.

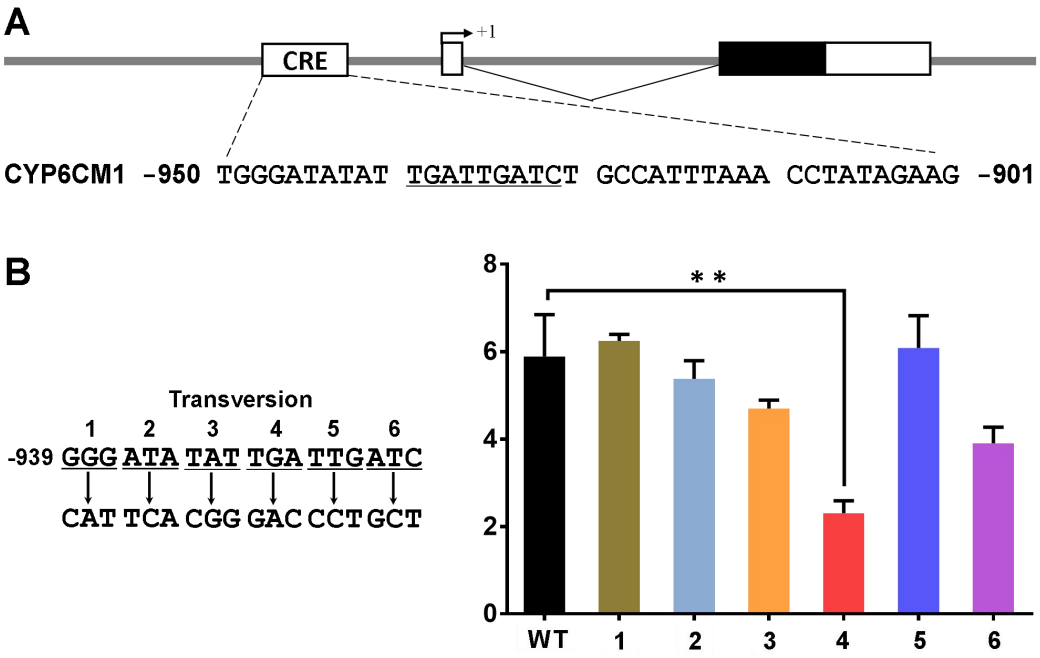

Fig. S2. The CRE-like site upstream of *CYP6CM1*. (**A**) Analysis of the CRE site in *CYP6CM1*. The upstream sequences of the *CYP6CM1* gene were analyzed in the MED/Q genomic sequences, and a half CRE-like site was identified at position –930 and -926bp. (**B**) The functionality of the CRE-like region was assayed with mutations causing a triplet transversion (each triplet has a transition) in the −939 to −920bp region of the upstream sequences of the *CYP6CM1* reporter (pGL4.10-*CYP6CM1*$^{-939\ to\ +1}$). Mutations of the core sequence of the CRE-like region (TGA) significantly decreased the expression of *CYP6CM1*. Values are means ± SEs (n=3). Data were analyzed using the Tukey-Kramer test (** $P < 0.01$).

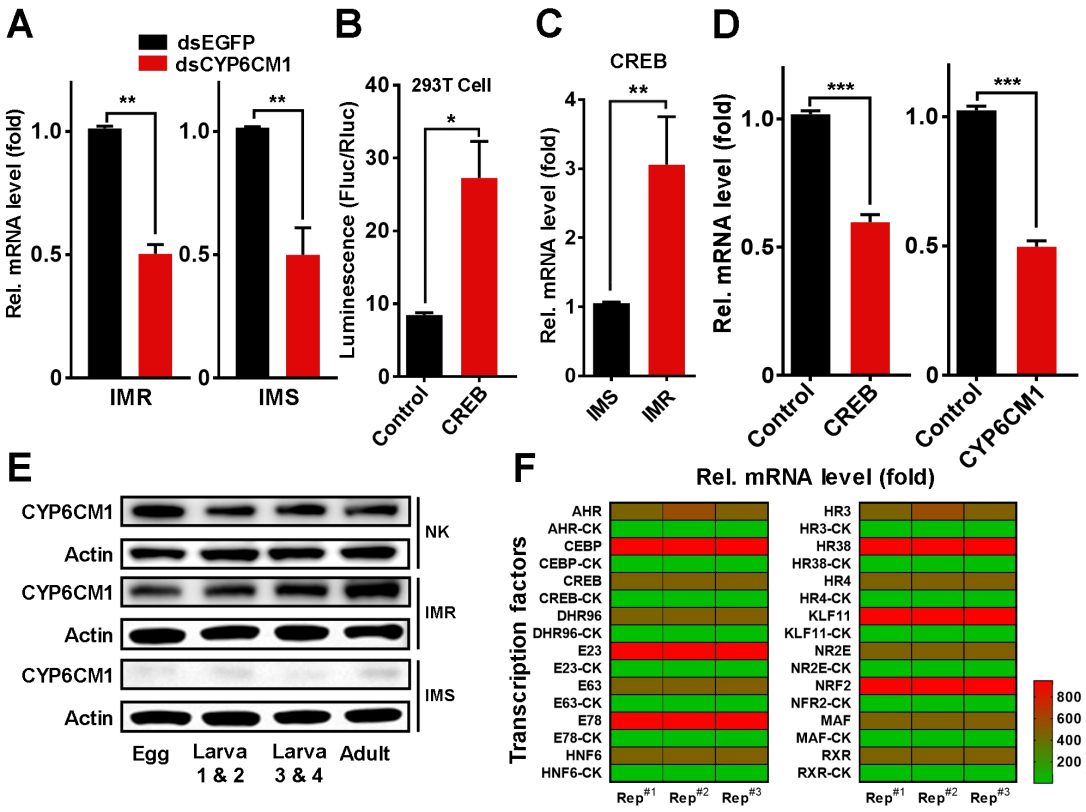

Fig. S3. (**A**) Efficiency of RNA knockdown of *CYP6CM1* as assessed by qRT-PCR. Relative mRNA abundance was measured in *B. tabaci* adults (IMR strain) that were fed ds*CYP6CM1*; adults that were fed ds*EGFP* served as the negative control. (**B**) Dual luciferase reporter assay, in which pGL4.10-*CYP6CM1*$^{-939\ \text{to}\ +1}$ and pGL4.73 plasmids were cotransfected into 293T cells with either the pcDNA3.1-CREB vector or the pcDNA3.1 empty vector (control). (**C**) Expression of *CREB* mRNA levels in the IMR and IMS strain as determined by qPCR. (**D**) The effect of feeding *CREB* dsRNA to *B. tabaci* for 48 h on the expression of *CREB* and *CYP6CM1*. mRNA levels were determined by qRT-PCR and are expressed as fold-change between control and treated whiteflies. Whiteflies fed on ds*EFGP* were used as a control. All values are means ± SEs (n=3). Data were analyzed using the Tukey-Kramer test (n = 3, mean ± SE; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, two-tailed Student's t-test). (**E**) Levels of CYP6CM1 protein in different developmental stages of *B.tabaci* in the IMR and IMS strains, and a field resistance NK strain. Actin was used as a loading control. (**F**) The expression of 16 whitefly transcription factors after transfection into S2 cells in pAC5.1b as assessed by qPCR. Empty pAC5.1b was used as a control.

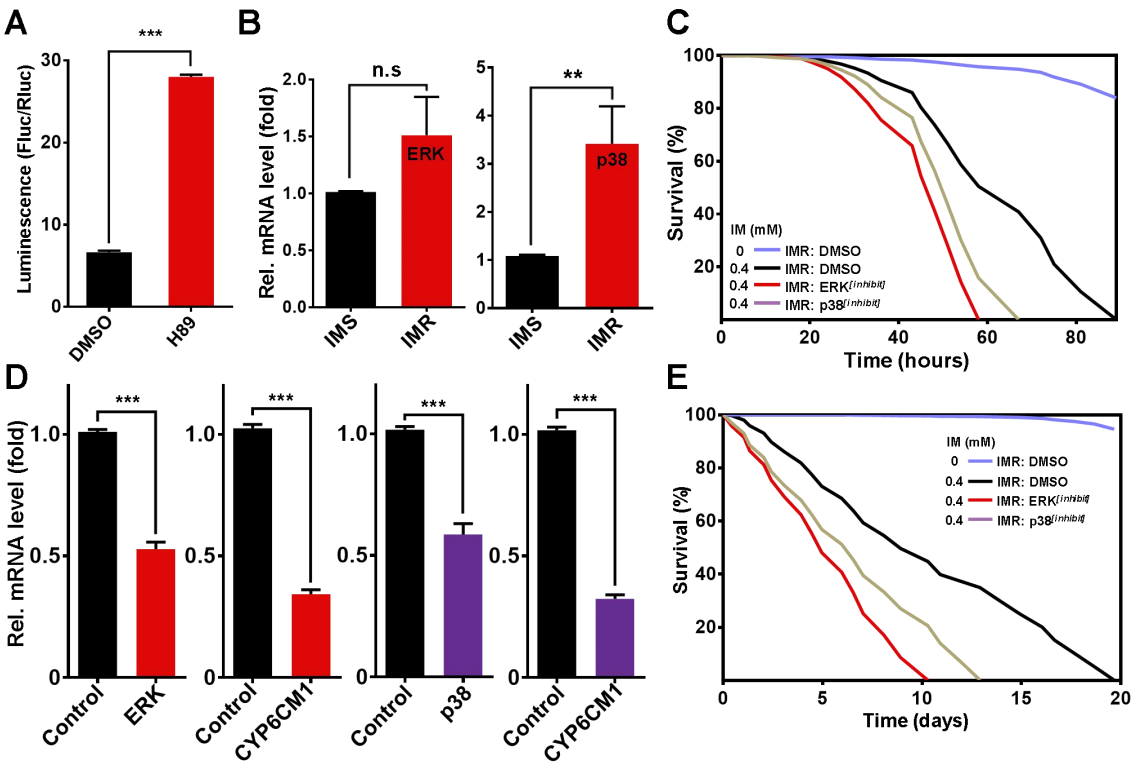

Fig. S4. (**A**) Dual luciferase reporter assays, in which pGL4.10-*CYP6CM1*$^{-939\ to\ +1}$ and pGL4.73 plasmids were cotransfected into S2 cells with either the PKA inhibitor H89 or DMSO alone as the control. Fluc/Rluc represents the ratio of firefly to Renilla luciferase activity. (**B**) Real-time qPCR analysis of the mRNA levels of *ERK* and *p38* in the imidacloprid resistant IMR strain and the susceptible strain IMS. mRNA levels are shown as fold-change between the susceptible and resistant strain. (**C**) The lifespan of adult whiteflies of the IMR strain after feeding on the inhibitors PD98059 (1.8 mM) and SB203580 (1.3 mM) for 24 hours on exposure to 0.4 mM imidacloprid. Adults fed on equivalent concentrations of DMSO were used as a negative control. (**D**) Expression of *CYP6CM1* mRNA after feeding adult whiteflies dsRNA of *ERK* (red) or *p38* (purple) for 48 h. For reference the expression of *ERK* and *p38* after dsRNA feeding is also shown. Adults fed on ds*EFGP* were used as a control. mRNA levels were determined by qRT-PCR. (**E**) Inhibition of *ERK* and *p38* enhances the control of *B. tabaci* (IMR adults) on tomato using imidacloprid. Lifespan of adult *B. tabaci* was significantly decreased when adults were exposed to 0.4 mM imidacloprid and to an *ERK* inhibitor (PD98059 at 1.8mM) or a *p38* inhibitor (SB203580 at 1.3 mM). For the control, adults were exposed to 0.4 mM imidacloprid without inhibitor. All values are means ± SEs (n=3). Data were analyzed using the Tukey-Kramer test (n = 3, mean ± SE; ** $P < 0.01$, *** $P < 0.001$, two-tailed Student's t-test).

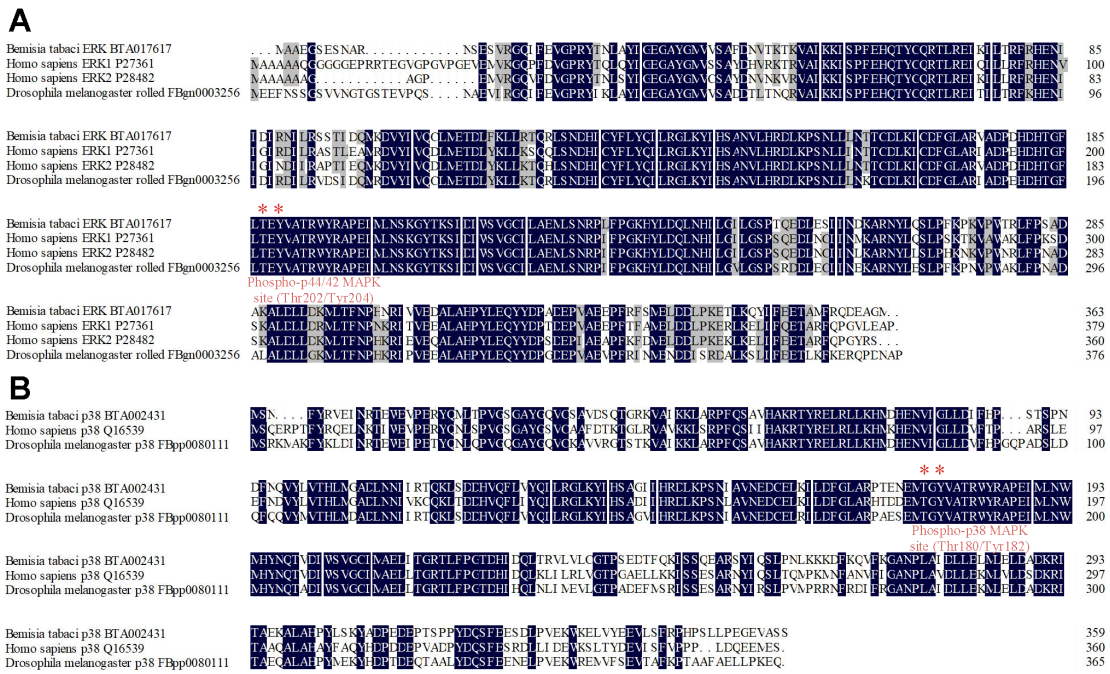

Fig. S5. Analysis of phosphorylation sites of the MAPKs *ERK* and *p38* in *B. tabaci* in comparison with the orthologs from *Homo sapiens* and *Drosophila melanogaster*. Asterisks indicate the phosphorylation sites of *Homo sapiens*.

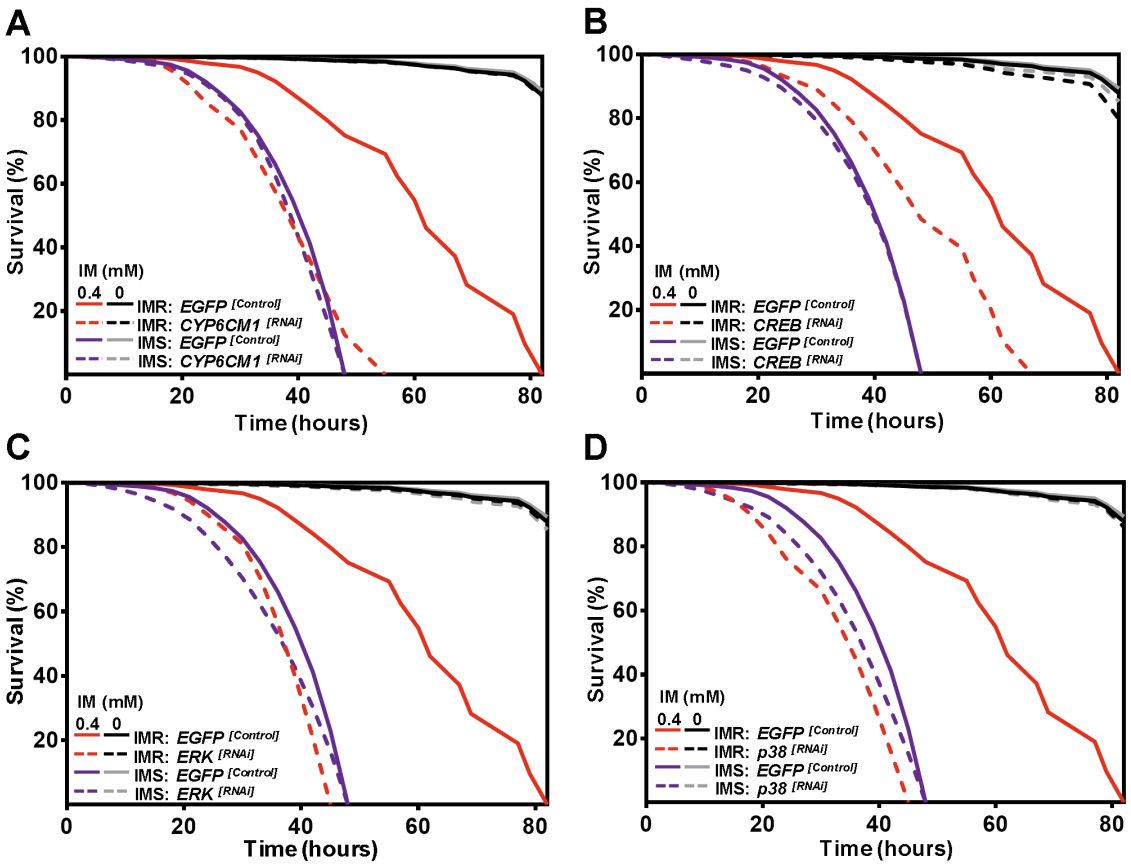

Fig. S6. Lifespan of IMR and IMS strains adults exposed to 0.4 mM imidacloprid (0.4) or no imidacloprid (0) after RNAi knockdown of *CYP6CM1* (**A**), *CREB* (**B**), *ERK* (**C**), and *p38* (**D**). Adults fed on ds*EFGP* were used as a negative control.

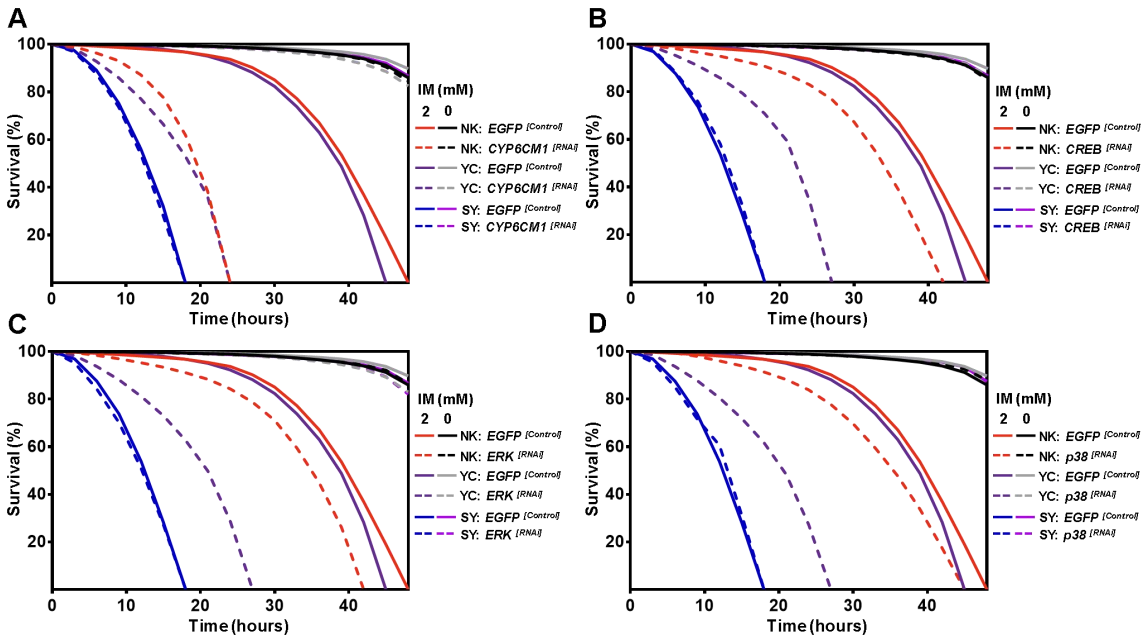

Fig. S7. Lifespan of three field resistance strains (NK, YC and SY) adults exposed to 2.0 mM imidacloprid (2) or no imidacloprid (0) after RNAi knockdown of *CYP6CM1* (**A**), *CREB* (**B**), *ERK* (**C**), and *p38* (**D**). Adults fed on ds*EFGP* were used as a negative control.

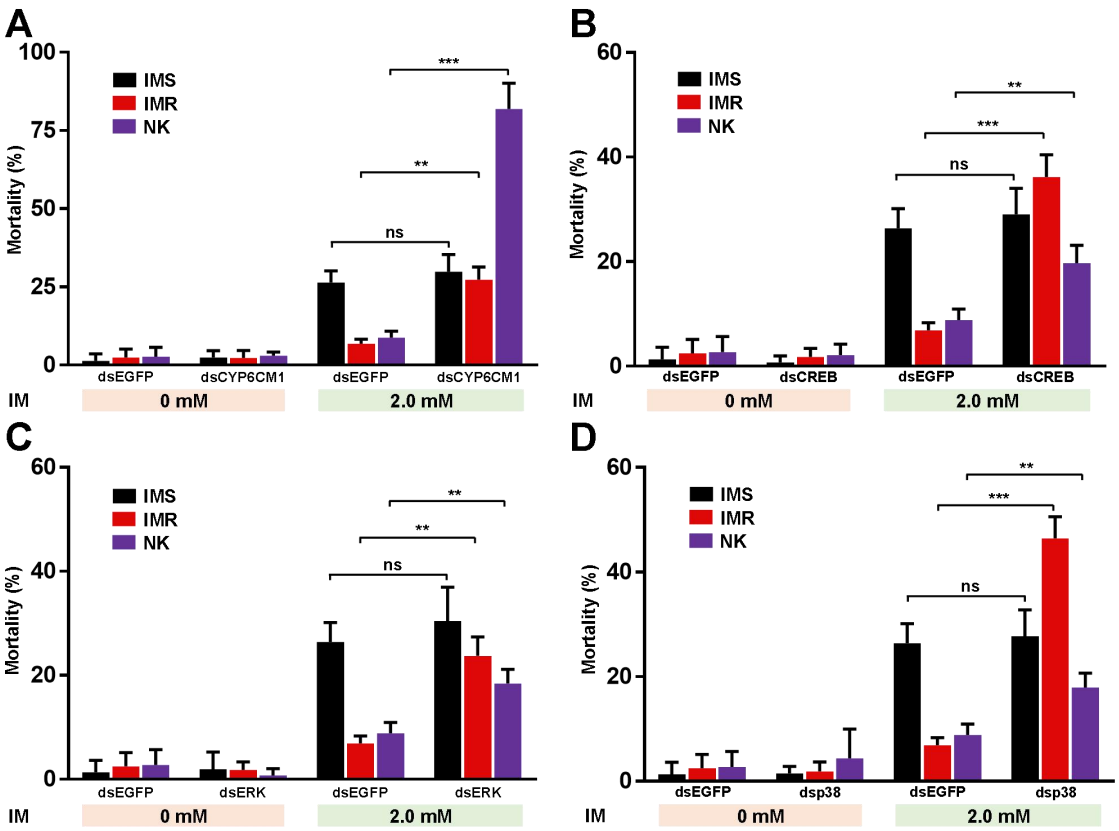

Fig. S8. Bioassays of one susceptible strain (IMS) and two resistance strains (IMR and NK) adults exposed to 2.0 mM imidacloprid (2.0) or no imidacloprid (0) for 24 hours after RNAi knockdown of *CYP6CM1* (**A**), *CREB* (**B**), *ERK* (**C**), and *p38* (**D**). Adults whitefly fed on ds*EFGP* were used as a negative control. (bioassays: n = 3, mean ± SE; ** $P < 0.05$,*** $P < 0.001$, two-tailed Student's t-test).

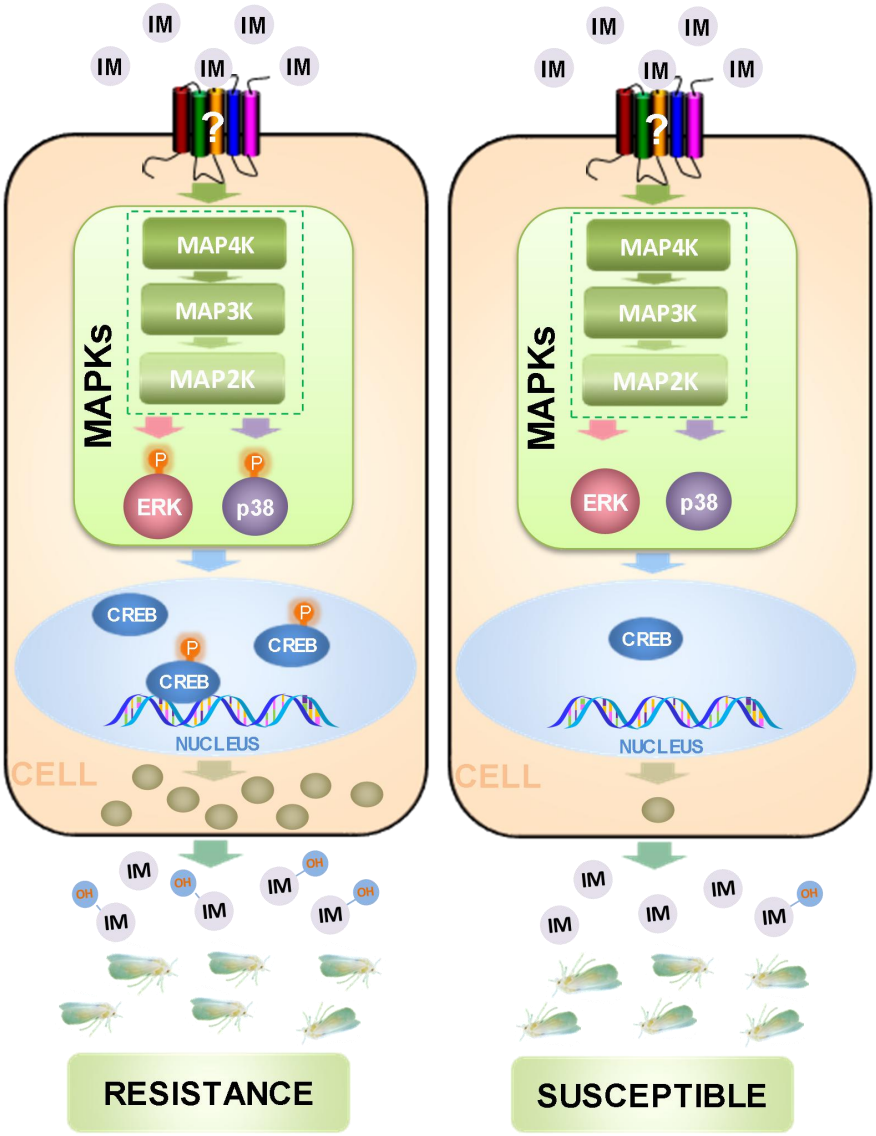

Fig. S9. A model of the regulation of *CYP6CM1* in *B. tabaci*. In this model, mitogen-activated protein kinases of the MAPK pathway phosphorylate ERK and p38. These in turn phosphorylate CREB, which then activates the expression of *CYP6CM1*. Activation and over-expression of *CREB* in the resistant strain increases the production of CYP6CM1 protein, which reduces the toxicity of imidacloprid.

Table S1. Origin of the *Bemisia tabaci* strains used in this study.

Population code	Population name	Sampling location	Sampling date	Host plant	*B. tabaci* composition
1	IMS/IMR	Hangzhou, Zhejiang (30°20′ N, 12°38′ E)	2011.10	Melon	MED/Q
2	NK	Nankou, Beijing (40°13′ N, 116°08′ E)	2018.09	Tomato	MED/Q
3	LF	Langfang, Hebei (39°36′ N, 116°35′ E)	2018.09	Pepper	MED/Q
4	YC	Yuncheng, Shanxi (35°26′ N, 110°58′ E)	2018.10	Cotton	MED/Q
5	SY	Shunyi, Beijin (40°11′ N, 116°43′ E)	2018.11	Tomato	MED/Q

Table S2: Log-dose probit-mortality data for *B. tabaci* strains in response to imidacloprid.

Strain	N[a]	Slope (±SE)	LC_{50} (mg L^{-1})	95% FL[b]	df[c]	χ^2	RR[d]
IMS	486	2.16 (± 0.18)	123	98-154	4	5.47	1
IMR	424	1.56 (± 0.19)	2790	638-3125	4	4.68	23
NK	639	1.72 (± 0.12)	1674	894-6869	3	7.81	14
YC	444	1.83 (± 0.24)	2146	1437-4731	4	5.36	17
LF	852	1.26 (± 0.10)	505	337-801	5	16.69	4
SY	353	1.17 (± 0.16)	35	7-71	3	3.86	0.3

[a]N = Number of *B.tabaci* used in each bioassay.

[b]df = Degrees of freedom.

[c]FL = Fiducial limit.

[d]RR (Resistance Ratio) = LC_{50} of the sample strains/LC_{50} of strain IMS.

Other Supplementary Material for this manuscript includes the following:

Dataset S1. Results and analysis of lifespan bioassays conducted on the IMR, IMS, SY, NK and YC strains.

Dataset S2. Upstream sequence analysis of *CYP6CM1* and *CREB* in the IMR and IMS strains.

Dataset S3. Information on the proteins chracterised in this study.

Dataset S4. Analysis of the sequences of eight nAChR α subunits and one β subunit in the IMR and IMS strains.

Dataset S5. Sequences of the oligonucleotide primers used in this study.

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