Resistance mutation conserved between insects and mites unravels the benzoylurea insecticide mode of action on chitin biosynthesis

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Despite the major role of chitin biosynthesis inhibitors such as benzoylureas (BPUs) in the control of pests in agricultural and public health for almost four decades, their molecular mode of action (MoA) has in most cases remained elusive. BPUs interfere with chitin biosynthesis and were thought to interact with sulfonylurea receptors that mediate chitin vesicle transport. Here, we uncover a mutation (I1042M) in the chitin synthase 1 (*CHS1*) gene of BPU-resistant *Plutella xylostella* at the same position as the I1017F mutation reported in spider mites that confers etoxazole resistance. Using a genome-editing CRISPR/Cas9 approach coupled with homology-directed repair (HDR) in *Drosophila melanogaster*, we introduced both substitutions (I1056M/F) in the corresponding fly *CHS1* gene (*kkv*). Homozygous lines bearing either of these mutations were highly resistant to etoxazole and all tested BPUs, as well as buprofezin—an important hemipteran chitin biosynthesis inhibitor. This provides compelling evidence that BPUs, etoxazole, and buprofezin share in fact the same molecular MoA and directly interact with CHS. This finding has immediate effects on resistance management strategies of major agricultural pests but also on mosquito vectors of serious human diseases such as Dengue and Zika, as diflubenzuron, the standard BPU, is one of the few effective larvicides in use. The study elaborates on how genome editing can directly, rapidly, and convincingly elucidate the MoA of bioactive molecules, especially when target sites are complex and hard to reconstitute in vitro.

insecticide resistance | benzoylureas | CRISPR/Cas9 | resistance management | mosquito control

Insects pose tremendous threats to humans in two main areas. Pathogens causing diseases such as malaria, dengue fever, and more recent problems caused by the Zika virus, are vectored by mosquitos, such as the *Anopheles gambiae* and *Aedes aegypti*, and cause severe global health problems (1). Furthermore, the sustainability of agricultural yields, which need to meet predicted population growth (2), is seriously threatened by pest insects and mites. The diamondback moth *Plutella xylostella*, a global lepidopterous pest of brassicaceous vegetables, is one of the economically most important agricultural pests in the world, particularly due to it having developed resistance to almost all chemical classes of insecticides applied for its control under continuous insecticide pressure (3).

Protection of food sources and human health from invertebrate pests is critically reliant on insecticides (4, 5). Insecticides are classified according to mode of action (MoA) and chemistry into several groups through the IRAC (Insecticide Resistance Action Committee) insecticide grouping system, which is the basis for their rational use and resistance management strategies (4). The vast majority of current insecticides have neurotoxic and muscle action (>80%), whereas only a relatively small proportion interfere with growth and development (insect growth regulators, IGRs) and thus are highly selective to targeted arthropod pests as there are often no physiologically related processes or target sites present in vertebrates. IGRs are a group of chemically diverse compounds including the microbial-derived pyrimidine-nucleoside peptides, benzoylureas (BPUs), oxazolines, and thiadiazines (6) that all interfere with chitin biosynthesis or transport and deposition pathways. The MoA of the antifungal pyrimidine-nucleoside antibiotics is by their function as substrate analogs of UDP-*N*-acetylglucosamine at the catalytic site of chitin synthase (CHS) and are thus considered competitive inhibitors (7–9). BPUs (10), such as the major mosquito larvicide diflubenzuron and the agriculturally widely used insecticides triflumuron and lufenuron, represent a group of compounds (group 15 with regard to the IRAC grouping system; see also Fig. S1) that inhibit chitin biosynthesis by a unique yet elusive mechanism of action independent of the catalytic reaction of CHS itself (6, 10, 11). Although the sulfonylurea receptor (SUR) has been suggested as the

Significance

An old enigma in insect toxicology, the mode of action (MoA) of selective chitin biosynthesis inhibitors in arthropods, is resolved. Benzoylureas, buprofezin, and etoxazole share a MoA by directly interacting with chitin synthase 1. The finding that a single mutation confers striking levels of insecticide resistance against three putative different MoAs has important ramifications on resistance management strategies and rational use of insecticides against major agricultural pests and vectors of human diseases. Our results also show that CRISPR/Cas9-mediated gain-of-function mutations in single-copy genes of highly conserved target sites in arthropods provide opportunities for comprehensive insecticide resistance investigations across species boundaries and against several insecticide classes.

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direct target of BPUs (12) by affecting chitin biosynthesis indirectly by altering vesicle trafficking, its role in chitin biosynthesis inhibition remains controversial (13, 14). Furthermore, it was recently shown that SUR is dispensable for cuticle formation and chitin biosynthesis in *Drosophila melanogaster* (15).

Buprofezin (group 16) and etoxazole (group 10B) are two other, chemically different compounds (Fig. S1) highly selective to sucking agricultural pests that have also been proposed to interfere with chitin biosynthesis or cuticle formation (16, 17). Etoxazole is an oxazoline acaricide widely used against pest mite species but with limited activity on insects (18). Genomic mapping of a recessive monogenic etoxazole resistance locus in the two-spotted spider mite *Tetranychus urticae*, together with additional genetic and biochemical evidence, suggests that a single mutation in *CHS1* is associated with etoxazole resistance; this mutation, I1017F (*T. urticae* numbering), is located in the C-terminal transmembrane domain. Therefore, it is likely that CHS1 is the molecular target of etoxazole as well as the chemically different acaricides clofentezine and hexythiazox (19, 20). Based on the similarity of symptoms for poisoning observed following exposure to both BPUs and etoxazole, as well as their inhibitory potential on chitin biosynthesis in isolated integuments of lepidopteran larvae, it has been hypothesized that they share the same MoA (18). The same direct MoA of BPU on CHS1, but not SUR, was later also postulated (19). However, no molecular evidence for such a possible association exists; there have been reports of BPU resistance in the diamondback moth in subtropical areas with intensive use of BPUs (21), but the molecular mechanism remains unknown. Furthermore, functional evidence of the involvement of the I1017F mutation in resistance could not be provided, given that in vitro approaches using recombinant protein expression are not feasible for large oligomeric integral protein complexes, especially when interactions are pre- or postcatalytic or involve the oligomerization of the complex (6, 19). As functional evidence is missing, the MoA through which chitin biosynthesis inhibitors exert their insecticidal activity remains uncertain.

Recent advances in genome modification technology, and especially the emergence of CRISPR/Cas9 (22), allow the application of "reverse" genetics approaches to provide in vivo evidence of the linkage between genotypes with phenotypes, including the study of insecticide MoA via generation of gain-of-function/loss-of-function mutations.

Here, we study and further select BPU resistance in *P. xylostella* and analyze the genetics of resistance as well as the possible association of identified point mutations in its *CHS1* gene with the phenotype. We use CRISPR to generate the corresponding single mutations associated with BPU (and etoxazole) resistance in *D. melanogaster*, a model organism that is equipped with an efficient genetic "toolbox" enabling the fast and reliable study of the contribution of individual mutations to resistance. Toxicity bioassays with genome-modified flies are used to reveal insensitivity to BPUs and buprofezin, thus attempting to provide compelling evidence for the functional interaction with CHS1 as the molecular target site.

Results

Selection and Characterization of BPU Resistance in *P. xylostella*. Low but significant resistance levels against diflubenzuron and triflumuron were detected in a *P. xylostella* strain (Sudlon) recently sampled in a Philippine cabbage field. The strain was maintained under laboratory conditions since 2011 to investigate target-site mutations in ryanodine receptors conferring resistance to diamide insecticides (23, 24). BPU insecticides have been used for diamondback moth control in Philippine cabbage in the past and were recently abandoned due to development of resistance. The Sudlon strain was reselected with triflumuron under laboratory conditions, resulting in the strain Sudlon-Tfm. Selection for 10 generations resulted in high BPU cross-resistance compared with the parental strain and reference strains BCS-S and Japan (Table 1). The selected strain Sudlon-Tfm was not only resistant to chemically diverse BPUs but also etoxazole (>178-fold), a chitin biosynthesis inhibitor of a different chemical class. Reciprocal crosses between Sudlon-Tmf and BCS and Sudlon revealed that the resistance was inherited autosomal recessive (Fig. 1) with a degree of dominance ranging from –0.73 to –0.88 in all reciprocal crosses (Table S1). Comparison of the postembryonic developmental time of strains Sudlon and Sudlon-Tfm showed that Sudlon-Tfm had a significantly longer larval (fourth instar) and pupal development time (Fig. S2), which could be indicative of possible fitness costs associated with the selected BPU resistance trait in *Plutella*.

Based on (*i*) the identical symptoms of poisoning observed following exposure to both BPUs and etoxazole, (*ii*) the inheritance of resistance in an autosomal and recessive way in line to the etoxazole resistance phenotype previously reported in spider mites (19), and (*iii*) the strong genetically based evidence that etoxazole likely acts on CHS1 but not SUR (19), we subsequently cloned and sequenced the full-length *CHS1* gene of *P. xylostella* strains BCS-S (GenBank accession no. KX420688), Sudlon (GenBank accession no. KX420689), and Sudlon-Tfm (GenBank accession no. KX420690) to compare the sequences between BPU-resistant and -susceptible strains. Compared with the *CHS1*, cDNA sequence of both susceptible strains BCS-S and Sudlon, a single nonsynonymous SNP resulting in a isoleucine (I)-to-methionine (M) amino acid change at position 1042 (*P. xylostella* numbering) in the C-terminal region of CHS1 of strain Sudlon-Tfm was found (Fig. 2). Genotyping of individual larvae by pyrosequencing of amplified *CHS1* fragments covering that region revealed that the I-to-M amino acid substitution at position 1042 (I1042M), which was completely absent in the BCS-S strain, was present at low frequency in the Sudlon strain and fixed (100%) in the resistant Sudlon-Tfm strain after selection with triflumuron (Table 2 and Fig. S3).

The frequency of the 1042M/1042M alleles was 7% in a population from Japan, whereas the frequency of the 1042M/1042M in survivors of BPU treatment (>100 ppm) of the same population

Table 1. Log-dose probit mortality for commercial BPU insecticides and etoxazole tested against third instar larvae of different strains of diamondback moth in leaf-dip bioassays (96 h)

Compound	Strain	*n*	LC_{50}, ppm	95% CL*	Slope	RR†
Diflubenzuron	BCS-S	300	36	21.0–60.3	1.3	
	Japan	300	45	24–85	1.2	1
	Sudlon	300	317	118–855	1.2	9
	Sudlon-Tfm	300	>1,000			>28
Triflumuron	BCS-S	420	5.3	4.2–6.9	1	
	Japan	420	11.6	7.8–17.3	0.89	2
	Sudlon	420	17.6	10.5–29.5	0.88	3
	Sudlon-Tfm	180	>1,000			>188
Lufenuron	BCS-S	450	1.8	0.96–3.5	1.3	
	Japan	420	1.2	0.28–4.7	0.47	1
	Sudlon	390	0.63	0.2591–1.510	0.86	1
	Sudlon-Tfm	330	354	57–2189	0.94	196
Flucycloxuron	BCS-S	240	0.16	0.15–0.18	1.3	
	Japan	240	0.36	0.21–0.63	1.6	2
	Sudlon	240	0.091	0.068–0.12	1.1	1
	Sudlon-Tfm	540	179	27–1183	0.50	1,119
Etoxazole	BCS-S	120	2.8	1.7–4.4	0.99	
	Sudlon	120	5.3	3.2–8.7	0.99	2
	Sudlon-Tfm	120	>500			>178

*95% confidence limits.
†Resistance ratio (based on strain BCS-S).

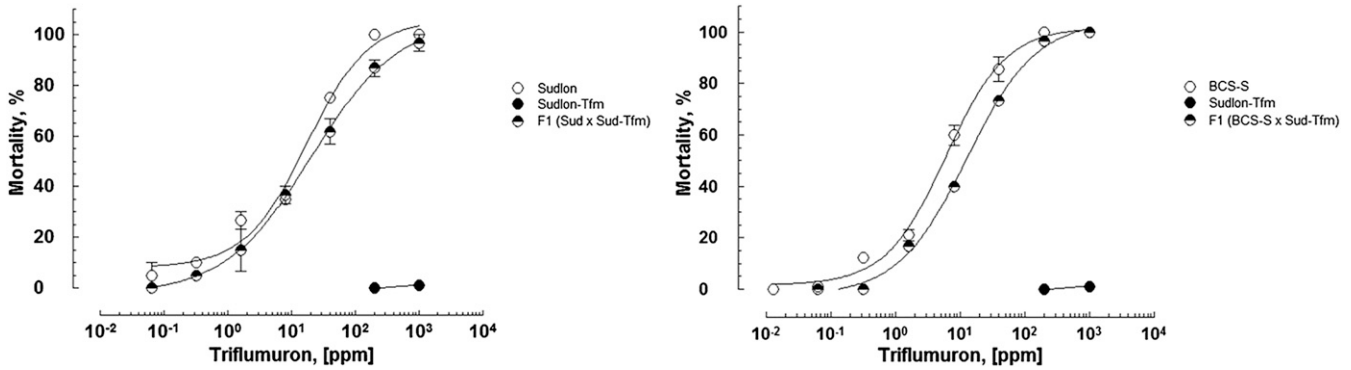

Fig. 1. Log-dose mortality data for triflumuron tested against third instar larvae of diamondback moth strains BCS-S, Sudlon, and Sudlon-Tfm as well as combined reciprocal crosses (F1). Error bars represent SEM.

was 100% (Table 2). The correlation between mutation and resistance is significant ($R^2 = 0.9779$, $P = 0.0002$). The I1042M mutation was also present at relatively high frequencies in field populations of *P. xylostella* sampled from cabbage fields in China and India with known BPU control failures (Table 2). Furthermore, genotyping of amplified *CHS1* fragments of individual larvae of the Chinese field strain revealed another mutation, I1042F, which has been associated (19) with etoxazole resistance in *T. urticae* (Fig. 2, corresponding position I1017F).

***Drosophila* Flies Bearing the Mutations Corresponding to I1042M and I1017F Are Resistant to BPUs and Other Chitin Biosynthesis Inhibitors.** We identified the ortholog CHS gene in *Drosophila* (*krotzkopf verkehrt* or *kkv*; *SI Results* and Fig. S4), and to generate in *kkv* the I1056F/M mutations corresponding to I1017F in *T. urticae* and I1042M in *P. xylostella*, respectively (Fig. 2), we injected strain y^1 M{nos-Cas9.P}ZH-2A w* (referred to as nos.Cas9 below) embryos with the appropriate gRNAs/donor plasmid mixes (*SI Materials and Methods* and Fig. S5) and screened progeny for genome-modified alleles. For the I1056F mutation, there were indications for the presence of homology-directed repair (HDR)-derived alleles within the sample at 16 different lines—that is, ~20% of the total number (i.e., 77) of lines that gave G_1 progeny. G_1 individuals from each of three different original (G_0) lines were crossed to balancer flies and screened to identify positive heterozygotes (Fig. S6). Several independent lines were established, and at least one became readily homozygous after balancing (line Et15); this line was verified by sequencing the relevant genomic region and shown to be genome-modified as expected, carrying the I1056F mutation at the *kkv* gene. Similarly, for the I1056M mutation, HDR-derived alleles were found (Fig. S6) in pools from 16 lines out of the 48 screened (~33%), and individuals from three lines were crossed to balancers and screened. Several lines were sequence-verified as homozygous; line Px39 was selected for conducting toxicity bioassays.

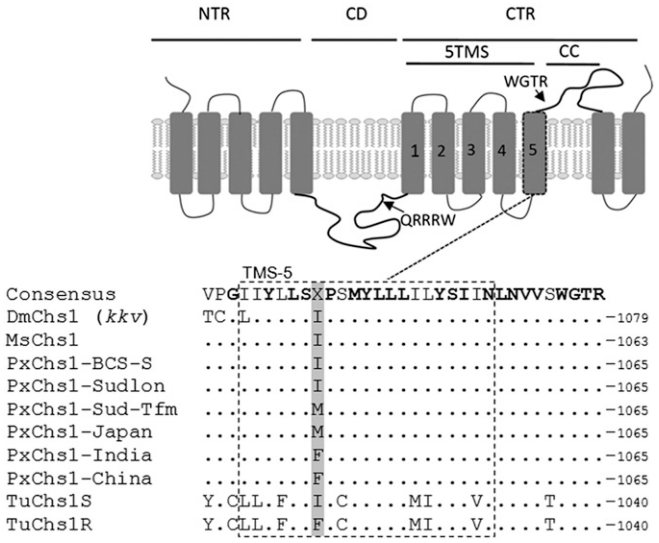

Fig. 2. Location of the two mutations conferring resistance. (*Top*) Schematic representation of domain architecture of CHS1, redrafted from ref. 19. 5TMS, cluster of five transmembrane segments; CC, coiled-coil motif; CD, catalytic domain; CTR, C-terminal region; NTR, N-terminal region. Rectangular boxes represent transmembrane domains. Arrows point to signature sequences QRRRW (catalytic domain) and WGTR (N-terminal region). (*Bottom*) Aligned amino acid sequences of helix 5 in the 5TMS clusters of CHS1 of *D. melanogaster* (Dm), *M. sexta* (Ms), six strains of *P. xylostella* (Px), and *T. urticae* (Tu; S, etoxazole susceptible; R, etoxazole resistant). Conserved residues are shown in bold. The position of the I1042M/F substitution in resistant *P. xylostella* (I1017F in etoxazole-resistant mites) is indicated in gray.

Toxicity assays with *Drosophila* larvae of strains nos.Cas9 and *yw* (both of which contribute to the genetic background of genome-modified flies) indicated that the strains carrying the wild-type *kkv* allele were sensitive to etoxazole at concentrations around 10 mg/L, without any significant differences observed between the two strains. Larvae did not manage to pupate or even grow to third instar. On the contrary, larvae from the genome-modified strains Et15 and Px39 bearing either the I1056F or I1056M homozygous mutation managed to grow and undergo molting without any visible problem, virtually all pupated, and adults eclosed normally when exposed to etoxazole concentrations as high as 10,000 mg/L, although at the highest concentrations (>1,000 mg/L) adults were dying just after eclosion. The LC_{50} values (with their corresponding 95% fiducial limits) for the susceptible (nos.Cas9) and resistant (Et15, Px39) lines and the associated resistance ratios are shown in Table 3.

Bioassay screens indicated a gross difference in the toxicity between both Px39 (I1056M) and Et15 (I1056F) *Drosophila* lines for diflubenzuron (LC_{50} nos.Cas9, 0.322 mg/L vs. LC_{50} Et15 and LC_{50} Px39, >5,000 mg/L), lufenuron (LC_{50} nos.Cas9, 0.148 mg/L vs. LC_{50} Et15, 16.659 mg/L and LC_{50} Px39, >20 mg/L), and buprofezin (LC_{50} nos.Cas9, 53.2 mg/L vs. LC_{50} Et15, >1,000mg/L and LC_{50} Px39, 1,276.654 mg/L). Such levels of at least partial cross-resistance support a common MoA between etoxazole, BPUs, and buprofezin. However, cyromazine toxicity is not affected either by the I1056M or the I1056F mutation, indicating either a different binding mode or another MoA.

The genome-modified fly lines used for bioassays were examined for certain life table parameters (*SI Materials and Methods* and Fig. S7), but no significant difference was observed in the flies

Table 2. Genotyping (individual larvae) by pyrosequencing for a CHS1 target-site mutation (I1042M) in different strains of diamondback moth

Strain	*N*	Frequency of genotype, %					
		SS I1042, ATT/ATT	RS I/M1042, ATT/ATG	RR M1042, ATG/ATG	RR L1042, TTG	RR M/L1042, ATG/TTG	RR M/F1042, ATG/TTT
BCS-S	30	100	0	0			
Sudlon	30	97	0	3			
Sudlon-Tfm	40	0	0	100			
Japan	30	50	43	7			
Japan*	6	0	0	100			
Px-China	59	27	20	25	3	2	22
Px-India	23	52	30	17			
Reciprocal crosses							
F1-A (BCS-S × Sudlon-Tfm)	40	0	100	0			
F1-B (Sudlon × Sudlon-Tfm)	63	0	89	11			

*Survivors of BPU treatment (>100 ppm).

bearing the I1042M mutation, contrary to the result from *Plutella* (Fig. S2).

Discussion

Resistance against the major chitin biosynthesis inhibitor class of insecticide chemistry (i.e., BPUs) was detected and subsequently selected in a recently collected Philippine field population of the diamondback moth *P. xylostella*, one of the most important agricultural pests in brassicaceous crops worldwide. The presence and frequency of the amino acid substitution I1042M was highly correlated with cross-resistance against several BPUs such as diflubenzuron, triflumuron, lufenuron, and flucycloxuron. Surprisingly, the *P. xylostella* mutation in *CHS1* gene lies at the same location of a previously documented mutation (I1017F) conferring etoxazole resistance (19). Introduction of either mutation in *D. melanogaster* by a CRISPR/Cas9 coupled with HDR genome modification approach showed a similar resistance phenotype across different chemical classes of IGRs, such as BPUs, etoxazole, and buprofezin, but not cyromazine. This is compelling evidence that BPUs, buprofezin, and etoxazole share the same MoA and directly interact with CHS1.

Our chosen genetic validation approach is further supported by a contemporary study showing that the introduction of a single point mutation in an *alpha6* subunit of the nicotinic acetylcholine receptor of *Drosophila* by CRISPR/Cas9 genome editing copying a mutation associated with spinosad resistance in thrips resulted in a spinosad-resistant phenotype in genome-modified flies (25). Our results show that a reverse genetics strategy is exceptionally suitable for the elucidation of the MoA of insecticides and/or functional validation of mutations associated with insecticide resistance in a wide array of targets that are otherwise difficult to study. CRISPR/Cas9 has already been used in *Drosophila* for resistance research before (25, 26). However, in this study, we have generated lines bearing homozygous recessive gain-of-function mutations in a single-copy gene, thus enabling comprehensive investigation—that is, comparative bioassays for these particular mutations against several insecticide classes. The fact that most target sites between arthropods are highly conserved allows screening of different mutations across species boundaries. This strategy has several potential valuable ramifications, as it can be used in a large number of molecular targets and a wide array of chemical classes of insecticides.

Procedures toward the investigation of insecticide MoA and resistance mechanisms typically involve in vitro screening systems (27), electrophysiology (28), direct ligand/receptor–insecticide interactions either in vivo (24) or in silico (29), functional expression of enzymes (30, 31), or genetic mapping linkage analysis (19, 32). However, there are cases where in vitro screening is not applicable because the native proteins or protein complexes cannot be reconstituted or recombinantly expressed. One such example is CHS1 because of its structure as a large oligomeric integral membrane protein that catalyzes both polymerization of sugars and translocation of the nascent chitin fiber across the plasma membrane. No structural information is available on CHS1 complexes, and even the quaternary structure is not known (although trimeric complexes have been purified from *Manduca sexta*, they could be building blocks of higher order complexes) (33), thus rendering impossible any effort to model interactions. Attempts in recombinant expression have failed to generate active complexes. In this and other cases, the interaction between target site and insecticides can be more complex than simply inhibiting natural substrate or ligand binding, making it even harder to develop a functional screening assay.

The elucidation of the MoA of the chitin biosynthesis inhibitor classes BPU and buprofezin (i.e., IGR insecticides) that have been used against major agricultural pests and disease vectors for many years, directly acting on CHS, as well as the identification of BPU target-site resistance mutations has important implications and impact for the rational use of insecticides and insecticide resistance management. It will directly affect the IRAC classification (4) of those molecules, which are currently assigned to a MoA group (MoA group 15) different from etoxazole (MoA group 10) and buprofezin (MoA 16). Our study provides compelling evidence that both classes affect the same target protein, CHS1, thus justifying their subgrouping in a single MoA class. The finding that a single mutation confers high levels of insecticide resistance against three putative different MoAs has important effects on resistance management strategies, which are largely based on rotation of insecticide MoA groups, to avoid selection for target-site resistance by repeatedly applying chemistries addressing the same binding site.

The presence of the *CHS1* resistance mutation in diamondback moth populations from different countries, in particular, is an important consideration for rational use and management of insecticides against this major pest. The slightly but significantly extended development time of fourth instar larvae and pupae in strain Sudlon-Tfm indicated a putative fitness cost in *Plutella*, possibly associated with this mutation. However, this was not confirmed in *Drosophila* lines, where the mutation was isolated in an isogenic background. It is possible that unrelated genetic loci in the multiresistant Sudlon-Tfm laboratory strain (24) might have contributed to the fitness cost observed.

The developed pyrosequencing diagnostic as well as possible additional field-applicable technologies to detect the presence of

Table 3. Bioassay results (LC_{50} values and associated resistance ratios) of genome-modified flies (Et15, Px39) versus relevant unmodified controls (nos.Cas9) for five different insecticides

Insecticides	Strains	LC_{50}, ppm (95% CL)	Resistance ratio
Etoxazole	Et15 (I1017F)	>10,000	>1,077
	Px39 (I1042M)	>10,000	>1,077
	nos.Cas9	9.28 (0.73–14.00)	1
Diflubenzuron	Et15 (I1017F)	>5,000	>15,625
	Px39 (I1042M)	>5,000	>15,625
	nos.Cas9	0.32 (0.24–0.42)	1
Lufenuron	Et15 (I1017F)	16.66 (8.70–66.47)	111.06
	Px39 (I1042M)	>20	>133
	nos.Cas9	0.15 (0.11–0.18)	1
Cyromazine	Et15 (I1017F)	0.23 (0.21–0.25)	0.74
	Px39 (I1042M)	0.30 (0.21–0.41)	1
	nos.Cas9	0.31 (0.25–0.34)	1
Buprofezin	Et15 (I1017F)	>1,000	>18.79
	Px39 (I1042M)	1,276.65 (1,110.36–1,554.15)	24.07
	nos.Cas9	53.20 (41.24–65.72)	1

CHS1 target-site mutations provides a tool allowing us to screen rapidly for the presence of resistant genotypes to adjust resistance management strategies based on MoA rotation accordingly.

The findings may also have implications for public health insecticide-based vector control interventions. The larvicide diflubenzuron is one of the most important insecticides that have been used against mosquitoes, particularly in regions such as Europe, where neurotoxic insecticides are banned from use in mosquito breeding sites. Screening of *A. aegypti* and *Aedes albopictus* populations, the major vectors of arbovirus including Dengue and Zika, from several geographical regions for possible resistant *CHS1* alleles will guide appropriate resistance management strategies to ensure the sustainability of control interventions. This discovery will also potentially have a bearing on the choice of insecticide for new human pathogen vector control, such as against the malaria mosquito *A. gambiae s.s* (34).

Materials and Methods

Chemicals. Insecticides (diflubenzuron, triflumuron, lufenuron, and flucycloxuron) used for *P. xylostella* bioassays were of technical grade (purity >98%) and provided in-house (Bayer CropScience). Commercial insecticide formulations were used for *Drosophila* bioassays, namely Borneo [11% (wt/vol) etoxazole; Hellapharm], Dimilin [48% (wt/vol) diflubenzuron; Syngenta], Match [50% (wt/vol) lufenuron; Syngenta], Trigard [75% (wt/vol) cyromazine; Syngenta], and Applaud [25% (wt/vol) buprofezin; Syngenta]. All other chemicals were purchased from Sigma-Aldrich.

Insects. The susceptible reference strain (BCS-S) of *P. xylostella* L. (Lepidoptera: Plutellidae) has been maintained under laboratory conditions for more than 20 y without exposure to insecticides. Strain Sudlon was collected in a cabbage field located in Sudlon, Cebu Island, in the Philippines in 2011 as described elsewhere (24). The BPU-resistant strain Sudlon-Tfm was obtained by selecting strain Sudlon for 10 generations with triflumuron by incrementally increasing its concentration to 1,000 $mg{\cdot}L^{-1}$. The Japan strain was collected in Mizobe, Japan in 2010. Finally, the strains from China and India were collected from cabbage in 2014. All strains were maintained on cabbage plants (*Brassica oleracea*) as recently described (24). Strain Sudlon-Tfm was maintained on triflumuron- (1,000 $mg{\cdot}L^{-1}$) treated cabbage plants. The *Drosophila* strain y^1 M{nos-Cas9.P}ZH-2A w* (nos.Cas9; stock no. 54591 at Bloomington Stock Center) (35) as well as yw strain and the strain yw; TM3 *Sb e*/TM6B *Tb Hu e* (containing third chromosome balancers, provided by Christos Delidakis, Institute of Molecular Biology and Biotechnology/ Foundation for Research and Technology Hellas and University of Crete, Heraklion, Crete, Greece) were used in this study. *Drosophila* strains were typically cultured at 25 °C temperature, 60–70% humidity, and 12/12-h photoperiod on standard fly diet.

Bioassays. Leaf dip bioassays with third instar diamondback moth larvae were conducted after IRAC method no. 7 (www.irac-online.org) as described recently (24). Control mortality was less than 10%. LC_{50} values and their corresponding 95% fiducial limits were calculated using Prism 5.03 (GraphPad Software, Inc.). For *Drosophila* bioassays, second instar larvae were collected and transferred in batches of 20 into new vials containing fly food supplemented with different insecticide concentrations. Larval development, molting, pupal eclosion, and adult survival were monitored for a period of 10–12 d. Five to six insecticide concentrations that cause 5–95% mortality (when applicable) were tested in triplicate, together with relevant negative (no insecticide) controls, in genome-modified flies and wild-type (nos.Cas9 and/or yw) controls. Dose-dependent molting and/or mortality curves were constructed from dose–response data, and LC_{50} values were calculated with PoloPlus (LeOra Software). A χ^2 test was used to assess how well the individual LC_{50} values agreed with the calculated linear regression lines.

Crossing Experiments. Pupae of strains BCS-S, Sudlon, and Sudlon-Tfm were collected and kept in Petri dishes individually until they hatched. After sex determination, 50 virgin females of Sudlon-Tfm were crossed with 50 males of Sudlon strain or BCS-S strain and vice versa. Because there was no difference obtained between the two reciprocal crosses, the F1 generation was pooled for further studies. The F1 generation was backcrossed with the respective parental strains. The backcross was conducted following the same approach as the reciprocal crosses; there was no difference obtained among the offspring, so samples were pooled. Third instar larvae were used for leaf dip bioassays to obtain the individual LC_{50} values for triflumuron. The degree of dominance (D) was calculated using Stone's equation. (36). Larvae of the different strains were preserved in RNA*later* (Ambion) and analyzed for the I1042M/F mutation by pyrosequencing.

Pyrosequencing. Individual *P. xylostella* larvae were ground in lysis buffer, and total genomic DNA (approximately 400 ng per larvae) was extracted using DNAdvance Tissue Kit (Agencourt) according to the to the supplier's recommended protocol. A gene fragment of 210 bp was amplified by PCR from 50-ng aliquots of gDNA using the primer pair PxCHS1-forward and PxCHS1-reverse (Table S2), designed with Assay Design Software (PSQ-Biotage AB, now Qiagen). The primer pair is based on a ClustalW aligned consensus sequence of *CHS1* of diamondback moth found in GenBank (accession number AB271784) as well as internally sequenced *CHS1* of strains BCS-S, Japan, and Sudlon. The pyrosequencing protocol comprised 35 PCR cycles with 0.5 μM forward and biotinylated reverse primer in 30 μL reaction mixtures containing 1× Taq enzyme reaction mix (RedTaq Jumpstart Master Mix, Sigma-Aldrich) and cycling conditions of 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and a final elongation step at 72 °C for 5 min. The single-strand DNA required for pyrosequencing was prepared as described in ref. 23. The pyrosequencing reactions were carried out according to the manufacturer's instructions using the PSQ 96 Gold Reagent Kit (Qiagen), and the sequence-PxCHS1-seq (Table S2) for genotyping. The pyrograms were analyzed using the SNP Software (Qiagen).

Genomic Engineering Strategy. An ad hoc CRISPR/Cas9 genomic engineering strategy was devised to generate the I1056M/F mutations (equivalent to the I1042M and I1017F mutation in *P. xylostella* and *T. urticae CHS1*,

respectively; *SI Materials and Methods*, Fig. 2, and Fig. S5) at the *kkv* gene in *D. melanogaster*. Potential CRISPR targets in the region of interest were identified using the online tool Optimal Target Finder (37) (tools.flycrispr.molbio.wisc.edu/targetFinder/), and two targets with no predicted off-target hits were selected to generate RNA expressing plasmids gRNA444 and gRNA658, respectively, targeting the relevant genomic regions (*SI Materials and Methods* and Fig. S5). We constructed de novo (Genscript) two donor plasmids for HDR, encompassing genomic region 3R:5380538:5383542 but with certain modifications compared with the wild-type genomic sequence (Fig. S5).

***Drosophila* DNA Purification and Amplification.** DNA was purified from *Drosophila* tissues by DNAzol (MRC) according to the manufacturer's instructions. PCR amplification with relevant primer pairs (Table S2) was typically performed with Kapa Taq DNA Polymerase (Kapa Biosystems). The conditions used were 95 °C for 2 min, followed by 30–35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min followed by a final extension step for 2 min.

Generation and Selection of Genome-Modified Flies. We used transgenic flies with the genotype y^1 M{nos-Cas9.P}ZH-2A w* that carry a transgene expressing Cas9 protein during oogenesis under control of *nanos* regulatory sequences (35) and injected embryos as described in *SI Materials and Methods*. Screening was performed by isolating DNA from sets of ∼30 individuals per vial (mostly pupae, but also adults and third instar larvae, depending on availability). In case the presence of genome-modified alleles was indicated in the pool (Fig. S6), several individual G_1 flies from the same original cross were first crossed again with nos.Cas9 flies to generate G_2 progeny and then individually screened to positively identify which of these G_1 flies indeed carried genome-modified alleles. Lines originating from positive G_1 flies were established, and individual G_2 flies (expected to be heterozygous for the mutant allele at a 50% ratio) were balanced against a strain containing third chromosome balancers (yw; TM3 *Sb e*/TM6B *Tb Hu e*). Flies potentially containing modified alleles were screened as before after being back-crossed to the original balancer stock (Fig. S6); the progeny of positives (bearing the modified allele opposite to one of the balancer chromosomes) was used to generate homozygous lines by crossing between siblings and selecting against the marker phenotype (*Sb* or *Tb Hu*) for the relevant balancer. All lines used were sequence-verified.

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