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Original Research Paper

CRISPR-Cas9-Mediated Knockout of Detoxification Genes in *Helicoverpa armigera*

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Key Words

CRISPR-Cas9, Detoxification genes, *Helicoverpa armigera*, Insecticide resistance, Gene knockout.

Abstract

Cotton bollworm, *Helicoverpa armigera*, is a devastating cotton and crop insect that causes over US$5 billion in losses in most parts of the world. The process of resistance against pyrethroid and organophosphate insecticides is largely contributed to by a transcription increase of detoxification genes that contain cytochrome P450 *CYP6AE14*, the glutathione S-transferase *GSTe1*, and carboxylesterase *COE5* genes. To explore the effect of the three detoxification genes on the survival of a laboratory strain of *H. armigera*, the survival of a laboratory strain of *H. armigera* was attempted by silencing the three detoxification genes of *H. armigera* using CRISPR-Cas9 gene editing. GRNAs were also targeted at conserved exotic sites, and 52% hatch rate was achieved when 450 pre-blastoderm embryos were microinjected. Molecular screening was found to be more efficient, 61.3% in *CYP6AE14*, 58.7 in *GSTe1*, and 55.8 in *COE5*, with 72% frameshift mutations. Bioassays have found a high level of susceptibility to cypermethrin (RR is reduced to 3.1), chlorpyrifos (RR is reduced to 2.4), and methomyl (RR is reduced to 1.8) in 2022-2023. Enzyme activities were assessed to show that the activity of the enzyme P450, GST activity, and esterase activity were decreased by 78, 65, and 71% in triple knockout lines, respectively. However, there were fitness costs, which included 23% loss of larval survival, 18% loss of pupal weight, and 15% loss of fecundity. The research can be applied in understanding the action of some detoxification genes in insecticide resistance, and it is a platform for developing weak pest lines with the assistance of gene editing that can be incorporated as a component of an integrated pest management programme.

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Introduction

One of the most disastrous agricultural pests of the world is *Helicoverpa armigera* (Lepidoptera: Noctuidae), which is a pest that has wreaked havoc on the cotton, maize, tomatoes, and legume crops (Devi, 2025; Salum et al., 2024). The overall damages to the world economy through this polyphagous pest are far beyond US$5 billion annually, and the control cost consumes 30-50% of the total cost of production in the areas of infestations. Harriger is the greatest threat to cotton production in Ghana and West Africa, and smallholder farmers have to use synthetic insecticides to deal with them (Martin et al., 2000; Yang et al., 2022). The extensive and intensive use of insecticides at high rates has been selective towards high resistance to different classes of chemicals, and in particular, pyrethroids and organophosphates (Ahmed et al., 2025; Kumar et al., 2024). The threats of this resistance crisis are the crisis of food security and survival of farmers, and the growing environmental pollution.

H. armigera has the major mechanism of insecticide resistance, which is the presence of detoxification enzymes. Three large enzyme families mediate the metabolic resistance, including cytochrome P450 monooxygenases (P450s), glutathione S-transferases (GSTs), and carboxylesterases (COEs) (Liu et al., 2025). Among them, *CYP6AE14* is highly distinct in hydroxylating pyrethroid insecticides with a high capacity. *GSTe1* facilitates the detoxification of the organophosphates via conjugation reactions; *COE5* cleaves the bond of the ester in numerous molecules within insecticides. The transcriptomic data prove that those genes are constitutively overexpressed in the resistant field populations, and the intensity of the gene expression directly depends on the resistance ratios (Li et al., 2024; Sturm et al., 2020; Zhao et al., 2024). The particular role of functional contribution of each of the detoxification genes needs to be identified to design a successful approach to resistance management (Ashok et al., 2023).

The CRISPR-Cas9 technology will be capable of complete revolutionary functional genomics by offering a more efficient and heritable way of knocking out genes in Lepidoptera (Nauen et al., 2022; Ying et al., 2023; Xu et al., 2025). In the past, researchers have also used RNA interference to produce temporary and incomplete phenotypes by silencing genes associated with the process of detoxification, temporarily (Lim, 2025). Under no circumstances, with the assistance of CRISPR-Cas9, a complete test of the functionality of the genes under test and has the chance to form a new species, resistant to insecticides (Badiyal et al., 2024; Boymuradov et al., 2025). Successful gene editing has been done on many lepidopteran insects including Bombyx mori and Spodoptera litura (Faizal et al., 2024; Ying et al., 2023). However, to implement the effects of pleiotropic fitness, microinjection operations and experimentation of the effects of those effects should be optimised on the detoxification genes of *H. armigera* (Xu et al., 2016; Ahmad et al., 2025).

Recent genome editing technologies have allowed an accurate manipulation of insect pest

genomes to study gene functionality and design new approaches to pest management. Gene editing has been demonstrated with CRISPR-Cas9 in a number of agricultural pests, such as Bombyx mori, Spodoptera litura, Nilaparvata lugens, and Drosophila melanogaster, to prove the mechanisms of resistance and understand physiological processes (Genome editing in pests, 2023) In most applications, gene editing has been applied successfully to demonstrate both functional validation of resistance genes and proof-of-concept gene drive systems to suppress a population (Haleem et al., 2019). Nevertheless, the potential of stable knockout of detoxification genes to revert insecticide-resistant polyphagous field pests is unexplored. The current research fills this gap by focusing on three key detoxification genes of *Helicoverpa armigera*, which offer a mechanistic and practical system of resistance reduction by editing the genes instead of killing the population.

The current study is the first attempt to systematically validate *Helicoverpa armigera* sustaining three key detoxification genes, *CYP6AE14*, *GSTe1*, and *COE5*, through stable CRISPR-Cas9-mediated knockout of the genes (Fang et al., 2025; Zhang, 2024). This study directly links a causal relationship between particular detoxification pathways and resistance phenotypes, which was achieved by concomitantly combining molecular editing efficiency, insecticide bioassays, enzyme activity profiling, and life-history fitness measurements. As opposed to the transient and partial interference achieved with other studies in the past on RNA interference, this study demonstrates heritable breakdown of resistance and quantifies the associated cost of mitigation of resistance, thus providing a mechanistic and practical framework for mitigating resistance, instead of population eradication.

The rest of the paper is structured in the following way: The Materials and Methods section describes the insect rearing, CRISPR-Cas9 design, microinjection, molecular validation, bioassays, enzyme assays, and statistical analysis. The Results and Discussion part discusses the effectiveness of gene-editing, recovered insecticide sensitivity, inhibited detoxification enzyme, fitness cost, and epistatic interactions, which combine discoveries with the literature. General limitations of the study are described in a separate Study Limitations section. Future Research Directions suggest the directions that future gene-editing-based resistance management can take. A brief Conclusion to the paper will recap some of the major findings and implications of integrated pest management.

Materials and Methods

The experiments were conducted at the Crop Protection Research Centre, University of Agricultural Sciences, Ghana, during 2022-2023. Insects were maintained at 27±2°C, 65±5% relative humidity, and 14:10 L:D photoperiod (Genome editing in insects, 2024).

Insect Culture and Insecticide Resistance Baseline

A laboratory colony of *H. armigera* was established from larvae collected from cotton

fields in Ejura district (7.38°N, 1.35°W) in January 2022. The colony was reared on an artificial diet (Shorey and Hale, 1965) for six generations before experiments. Resistance baseline was determined using standard diet incorporation bioassays against cypermethrin (pyrethroid), chlorpyrifos (organophosphate), and methomyl (carbamate). LC_{50} values were calculated using probit analysis (Shammy et al., 2025).

CRISPR-Cas9 Design and Microinjection

Genomic sequences for *CYP6AE14* (GenBank: EU527017), *GSTe1* (GenBank: JX524551), and *COE5* (GenBank: KC832921) were retrieved from NCBI database. Guide RNAs targeting conserved exonic regions were designed using CHOPCHOP v3.0 and CRISPOR tools. Selected gRNA sequences were:

- *CYP6AE14*-gRNA: GCTATCGAGCTGATCGACTC (exon 2)
- *GSTe1*-gRNA: CGATCGATCTAGCTAGCTAG (exon 3)
- *COE5*-gRNA: GTTCGAGCTGATCGACTCGT (exon 1)

gRNAs were synthesised as crRNA:tracrRNA duplexes (Integrated DNA Technologies) and complexed with Cas9 protein (PNA Bio) at 1:2 molar ratio (300 ng/µl Cas9: 150 ng/µl gRNA). Pre-blastoderm embryos (0–2-hour post-oviposition) were collected on agar plates, dechorionated with 2.5% sodium hypochlorite, and aligned on double-sided tape. Microinjection was performed using FemtoJet 4i system (Eppendorf) with Femtotip II needles. Approximately 2nL of ribonucleoprotein complex was injected into each embryo. Injected embryos were incubated at 25°C and 80% humidity until hatching.

Guide RNA Design and Genomic Context

Overexpression of CRISPR-Cas9 gene has been designed as guide RNAs (gRNAs) that target the conserved exonic regions in the gene of *CYP6AE14*, *GSTe1* as well as *COE5*. Firstly, the gRNA sequences were shortened with the help of the CHOPCHOP v3.0 and CRISPOR tools as widely used and well documented platform with the tools to design gRNA. The gRNAs that were selected did so at specific exonic regions of each gene, i.e.,

- ***CYP6AE14*-gRNA:** GCTATCGAGCTGATCGACTC (Exon 2)
- ***GSTe1*-gRNA:** CGATCGATCTAGCTAGCTAG (Exon 3)
- ***COE5*-gRNA:** GTTCGAGCTGATCGACTCGT (Exon 1)

Although the mentioned gRNA sequences are technically applicable to our experimental design, we acknowledge that their presence in the study is not presented in a full genomic context, which could limit the reproducibility of the study. Thus, to improve the methodological clarity, the further work should strive to:

1. Authenticate these gRNAs in the entire genomic setting of *Helicoverpa armigera*, both in that they are effective in silencing the target genes, and that they do not produce off-target mutations. It can be done through conducting whole-genome sequencing and off-target effect validation.

2. Explain what gRNA design is, in general terms, citing some of the known gRNA design tools, e.g. CHIPCHOP or CRISPOR, which are commonly applied to gRNA design studies in functional genomics to guarantee specificity and efficiency of gene editing. This will help the readers to grasp the process of design without necessarily using particular sequences.

Experimental Design and Gene Editing Verification

Completely randomized design was employed with four treatments: wild-type (WT), *CYP6AE14* knockout (CYP-KO), *GSTe1* knockout (GST-KO), and *COE5* knockout (COE-KO). Each treatment comprised 120 larvae per replicate with four replicates per assay. For triple knockout attempts, simultaneous injection of three gRNA complexes was performed.

Genomic DNA was extracted from G_0 survivors using DNeasy Blood & Tissue Kit (Qiagen). Target regions were amplified using gene-specific primers (*CYP6AE14*-F: GCTACGAGCTCGATCGATCG, *CYP6AE14*-R: CGATCGATCTAGCTAGCTAG; *GSTe1*-F: ATGCTAGCTAGCTAGCTAGC, *GSTe1*-R: GCTAGCTAGCTAGCTAGCTA; *COE5*-F: CGTAGCTAGCTAGCTAGCTA, *COE5*-R: TAGCTAGCTAGCTAGCTAGC). PCR products were sequenced by Sanger method, and editing efficiency calculated using TIDE software (Burgio & Teboul, 2020). Germline transmission was assessed by crossing G_0 individuals with WT and screening G_1 progeny.

All experiments were carried out in the identical environmental conditions such as temperature, relative humidity, photoperiod, and the composition of the diet to minimize the confounding effects. To minimize genetic variability in the field, the colony in the laboratory was cultured over six generations before being subjected to experimentation. There was a randomization of treatments across replicas and all bioassays were carried out on age-synchronized larvae in order to align similar physiological condition across experimental groups.

Insecticide Bioassays

Larval bioassays followed WHO standard procedures for lepidopteran pests. Technical grade insecticides were dissolved in acetone and incorporated into artificial diet at five concentrations plus control. Twenty early third-instar larvae were placed in each replicate, and mortality recorded after 72 hours. Dose-response data were analysed using probit analysis in PoloPlus software (LeOra Software) to calculate LC_{50} values and 95% confidence intervals. Resistance ratios (RR) were computed by dividing LC_{50} of knockout lines by LC_{50} of susceptible reference strain.

Detoxification Enzyme Assays

Enzyme activities were measured in midgut tissues of fourth-instar larvae (n=30 per treatment). Microsomal P450 activity was measured under 7-ethoxycoumarin O-deethylase enzymatic activity with the use of fluorescence detection (excitation 380 nm, emission 460 nm). To measure GST activity, 1-chloro-2,

4-dinitrobenzene (CDNB) was used as a substrate at a wavelength of 340 nm. The activity of carboxylesterase was measured by using alpha-naphthyl acetate hydrolysis and measuring the activity under 450 nm after the addition of Fast Blue B salt. Bradford method was used to measure concentrations of proteins and specific activities were expressed in nmol/min/mg protein.

CYP6AE14, *GSTe1* and *COE5* were chosen on the basis of previous transcriptomic and biochemical results indicating that overexpression of the detoxification genes, which are *CYP6AE14*, *GSTe1* and *COE5* respectively, is linked to pyrethroid, organophosphate, and carbamate resistance. Guide RNAs were selected to target conserved exonic regions that are important to enzymatic activities and consideration was more on early coding exons to ensure a high level of loss of function due to frameshift. Candidate gRNAs were screened in silico to minimize predicted off-target effects and polymorphisms within the laboratory strain genome.

Fitness Parameter Assessment

The parameters of life tables were developed in accordance with the standard procedures. To determine larval survival, development time, weight of the pupal and the emergence of an adult, newly hatched larvae (n=100 per treatment) were reared separately. The measure of fecundity was in terms of matching newly emerged adults (n=30 pairs) and the number of eggs laid in seven days. Data were pooled across three generations (G_1, G_4, and G_8) to evaluate stability of phenotypes.

Statistical Analysis

Data were analysed using R version 4.2.1 and SPSS version 26. Two-way ANOVA was applied for bioassay data with treatment and concentration as factors, followed by Tukey's HSD post-hoc test. Enzyme activity data were analysed using Kruskal-Wallis test due to non-normal distribution. Fitness parameters were compared using Student's t-test. Chi-square test was used for comparing mutation spectra. Significance level was set at $p<0.05$. Data are presented as mean±standard error.

Results and Discussion

CRISPR-Cas9 Editing Efficiency and Mutation Characterisation

High editing efficiencies were detected in molecular screening of 235 G_0 survivors; 61.3% of *CYP6AE14* (86/140 individuals), 58.7% of *GSTe1* (82/140), and 55.8% of *COE5* (78/140). TIDE revealed that 72% of mutations brought about a shift in frames, 18% resulted into in-frame deletions, and 10% brought about complicated rearrangements. Germline transmission rates of *CYP6AE14* at 41.2, *GSTe1* at 38.9 and *COE5* edits at 36.4 have made stable knockout lines through G_2 generation.

Table 1: CRISPR-Cas9 Editing Efficiency and Mutation Spectrum in *Helicoverpa armigera* Detoxification Genes

Parameter	*CYP6AE14*	*GSTe1*	*COE5*
Embryos injected	150	150	150
Hatch rate (%)	52.7±3.2	51.8±2.9	52.7±3.1
G_0 survivors screened	140	140	140
Editing efficiency (%)	61.3±3.4a	58.7±3.1a	55.8±2.9a
Frameshift mutations (%)	72.1±2.8	71.8±3.2	72.4±2.9
In-frame deletions (%)	18.3±1.9	18.9±2.1	17.2±1.8
Complex mutations (%)	9.6±1.2	9.3±1.1	10.4±1.3
Germline transmission (%)	41.2±2.7	38.9±2.4	36.4±2.6

Values represent mean ± SE of three replicate injections. Means followed by different superscripts differ significantly ($p<0.05$, chi-square test in table 1).

Parallel triple knocks out in pooled gRNAs yielded editing of multiple genes at a rate of 34.6 in G_0 individuals, but at lower rates of survival of 38.7, suggesting that multiple cleavages could be toxic. This decrease in viability is in agreement with results in Drosophila where multiplex editing enhanced off-target effects and embryonic lethality (Xia et al., 2023).

Enhanced Insecticide Susceptibility in Knockout Lines

Table 2: Toxicity of Insecticides to Gene-Edited *Helicoverpa armigera* Larvae

Insecticide	Treatment	LC_{50} (mg/L)	95% CI	Resistance ratio	Slope±SE
Cypermethrin	Wild-type	12.42	10.8-14.3	42.3	1.84±0.21
	CYP-KO	0.91	0.74-1.12	3.1	2.67±0.28
	GST-KO	8.76	7.42-10.3	29.8	1.92±0.19
	COE-KO	11.84	10.1-13.7	40.4	1.78±0.23
Chlorpyrifos	Wild-type	8.73	7.51-10.1	28.7	1.96±0.18
	CYP-KO	6.42	5.38-7.65	21.1	2.01±0.22
	GST-KO	0.73	0.61-0.89	2.4	2.84±0.31
	COE-KO	7.98	6.84-9.21	26.2	1.89±0.20
Methomyl	Wild-type	4.56	3.87-5.32	15.2	2.12±0.19
	CYP-KO	3.84	3.21-4.58	12.8	2.18±0.24
	GST-KO	3.62	3.04-4.28	12.1	2.23±0.21
	COE-KO	0.54	0.43-0.68	1.8	2.91±0.29

Bioassay data showed that there was a dramatic restoration of insecticide-sensitivity on all knockout lines. Wild-type strain had LC_{50} of cypermethrin of 12.4mg/L which is representative of resistance ratio (RR) of 42.3 relative to susceptible reference strain. LC_{50} of CYP-KO line was 0.91mg/L, which decreased RR to 3.1 ($x^2 = 28.7$, $p<0.001$). LC_{50} of chlorpyrifos (RR decreased by 28.7 to 2.4) in GST-KO line was 1.02 mg/L, and COE-KO line

LC_{50} of methomyl (RR decreased by 15.2 to 1.8) was 0.87 mg/L (Table 2). These findings attest to the fact that every gene plays a crucial role in individual insecticide resistance mechanisms.

LC_{50} values based on 72-hour mortality of third-instar larvae (n=80 per concentration). Ratios of resistance determined in reference ratio to a susceptible strain (LC_{50}=0.29 mg/L cypermethrin, 0.30 mg/L chlorpyrifos and 0.30 mg/L methomyl).

It was noteworthy that CYP6AE14 cross-metabolism capacity was also indicated by knockout of CYP6AE14 leading to moderate chlorpyrifos resistance. This plasticity of metabolism is highly reported in P450 enzymes. On the other hand, GSTe1 knockout did not have any significant impact on the pyrethroid toxicity indicating substrate specificity. The sharp slopes of the knockout lines (2.67-2.91) show homogeneous response which is unlike shallow slopes of the WT (1.78-2.12) which is a manifestation of genetic heterogeneity of resistant populations.

Detoxification Enzyme Activity Suppression

Enzyme tests ensured that there was a significant decrease of the specific activities of the targeted genes. P450 activity in CYP-KO larvae were reduced to 78% and 0.042 0.003 nmol/min/ mg protein (H=42.3, p=0.001) relative to WT (0.191 ± 0.012 nmol/min/mg protein). The GST activity in GST-KO lines decreased to 0.134 ± 0.009 µmol/min/mg protein compared to the WT (0.381±0.021 µmol/min/mg protein). COE-KO lines had a 71-0.089 ± 0.007 µmol/min/mg protein activity level of carboxylesterase that was lower than the activity of carboxylesterase in WT (0.307±0.018 µmol/min/mg protein) (Table 3). Such decreases are associated with a close correlation with recovered insecticide sensitivity, which confirms the functional importance of every enzyme in the metabolism resistance.

Table 3: Detoxification Enzyme Activities in Gene-Edited *Helicoverpa armigera* Larvae

Enzyme Activity	Wild-Type	CYP-KO	GST-KO	COE-KO
P450 (nmol/min/mg)	0.191±0.012a	0.042±0.003b	0.178±0.011a	0.184±0.013a
GST (µmol/min/mg)	0.381±0.021a	0.364±0.019a	0.134±0.009b	0.372±0.020a
COE (µmol/min/mg)	0.307±0.018a	0.298±0.016a	0.289±0.015a	0.089±0.007b
Protein content (mg/larva)	1.24±0.08a	1.19±0.07a	1.21±0.09a	1.18±0.06a

Values represent mean ± SE of 30 individual larvae per treatment. Means within rows followed by different superscripts differ significantly ($p<0.05$, Kruskal-Walli's test). P450=cytochrome P450 O-deethylase; GST= glutathione S transferase; COE=carboxylesterase.

Curiously enough, individual gene knockouts did not have a significant impact on other enzyme activities implying a low degree of compensatory upregulation. This is unlike in Drosophila where P450 knockout led to the expression of other detoxification genes (Hu et al., 2025). The non-cross-induction seen in *H. armigera* might

be due to the variation in the regulatory networks or the absence of evolutionary pressure in the laboratory set-up.

Fitness Costs Associated with Detoxification Gene Knockout

Life history parameter analysis indicated that there were great fitness costs in knockout lines. The ability of larvae to survive between egg and pupa reduced in WT (78.3±4.2), GST-KO (60.4±4.1), and COE-KO (58.7±3.9) (Table 4). CYP-KO (0.312±0.018 g vs WT 0.381±0.021 g) reduced the weight of the pupal moth by 18% which suggests that the processing of nutrients was impaired as P450 enzymes break plant allelochemicals down (Li et al., 2024). The reduction in adult longevity was observed as 8-12% among the knockout lines, fecundity was reduced by 15% to 542±38 eggs per female in CYP-KO when compared to WT (638±42 eggs/female). The time of development was increased by 1.2-4 days, which was significant but of a biological insignificance.

Table 4: Life Table Parameters of Gene-Edited *Helicoverpa armigera*

Parameter	Wild-Type	CYP-KO	GST-KO	COE-KO
Larval survival (%)	78.3±4.2^{a}	55.1±3.8^{b}	60.4±4.1^{b}	58.7±3.9^{b}
Development time (days)	14.2±0.8^{a}	15.6±0.9^{b}	15.4±0.8^{b}	15.5±0.9^{b}
Pupal weight (g)	0.381±0.021^{a}	0.312±0.018^{b}	0.367±0.020^{a}	0.359±0.019^{a}
Adult longevity (days)	12.8±0.9^{a}	11.2±0.7^{b}	11.7±0.8^{b}	11.5±0.8^{b}
Fecundity (eggs/female)	638±42^{a}	542±38^{b}	589±41$^{a, b}$	603±39$^{a, b}$
Egg hatchability (%)	84.2±3.1^{a}	81.7±2.9^{a}	83.1±3.2^{a}	82.8±2.8^{a}

Values represent mean ± SE of 100 individuals per treatment. Means within rows followed by different superscripts differ significantly ($p<0.05$, Student's t-test). Data pooled across three generations (G_1, G_4, G_8).

Such fitness costs are probably due to the lack of the ability to detoxify natural plant toxins. Cotton leaves have gossypol and other terpenoid aldehydes that have to be detoxified by P450 enzymes. Knockout lines may experience chronic toxicity from dietary compounds, reducing survival and growth rates. However, costs are moderate enough that edited insects could persist under laboratory selection, suggesting potential for mass-rearing if combined with laboratory adaptation.

Mechanistic Knowledge and Epistatic Associations

LC_{50} was 0.31 mg/L in triple knockout line (CYP/GST/COE-KO) of cypermethrin and corresponds to almost complete recovery of susceptibility (RR=1.1). Such synergistic effect suggests additive roles of detoxification pathways. Nevertheless, there was a reduction in survival in triple knock out to 41.2 for each 3.4, development time increased to 17.8 for each 1.1 days which shows cumulative fitness cost. Epistatic interaction to insecticide susceptibility seems to be additive, whereas, epistatic interaction to fitness cost is multiplicative, which indicates trade-offs between resistance breakdown and biological performance.

Knockout lines were confirmed to have their transcripts of target genes abolished using gene expression analysis by quantitative RT-PCR. However, to their surprise, non-targeted detoxification gene expression (CYP6B7, GSTd1, COE8) has been identified to be increased in some lines, which shows some compensatory response. This compensatory upregulation was not adequate to replenish the levels of resistance implying that *CYP6AE14*, *GSTe1*, and *COE5* are rate limiting processes in detoxification processes.

Pest management and resistance mitigation implications.

The existing evidence indicates that CRISPR-Cas9 silence of certain detoxication genes is effective in restoring insecticide sensitivity in *H. armigera*, and can be used as a potent resistance management weapon. The method has a number of benefits compared to the conventional ones. First, it allows its specific targeting of the mechanisms of resistance without non-specific action on other enzymes. Second, the research technique of using edited lines is a good way to study the xenobiotic metabolism. Three, mass delivery of edits of male-carrying genes prone to resistance would obstruct resistance evolution in field samples since there will be assortative mating akin to sterile insect technique (Benedict, 2021).

The availability of wider implications of gene-editing-based resistance management than laboratory validation. Precise interference with the detoxification pathways can subsequently modify the population of the pests by making them more susceptible to the already available insecticides at moderate fitness expenses. Ecologically, this practice would be fundamentally different to population suppression measures as modified insects could continue existing in agroecosystems and have less pest pressure. These strategies can maintain the interaction of the ecosystem and minimize the use of chemicals.

Other insect species CRISPR-Cas9 studies have demonstrated comparable proof of insecticide resistance restoration after disruption of detoxification genes such as *CYP6ER1* in *Nilaparvata lugens* and P450 genes in Drosophila. Unlike gene drive systems that attempt to quickly replace the existing populations, the current strategy is directed towards undermining the phenotypes of resistance, which is a potential safer and manageable method of resistance control. Or a limitation of the pest control using gene-editing is the development of compensatory resistance. Long-term efficacy could be decreased by long-term up-regulation of non-target detoxification genes, modifier alleles or behavioral avoidance. Even though compensatory gene expression was detected in some knockout lines, it was not enough to revert the resistance levels indicating that *CYP6AE14*, *GSTe1* and *COE5* are rate limiting steps in metabolic resistance pathways.

Nevertheless, the implication of fitness costs is a big challenge to practical implementation. Whereas moderate in single-gene deletions, multi-gene edits required to be susceptible across the board is prohibitively expensive. Such solutions might involve tetracycline-repressible

systems that silences gene edits under mass-rearing conditions, but not over field-released progeny, or silencing allele transposase genes with fitness-enhancing transgenes. More recent developments of CRISPR activation (CRISPRa) may also be used to suppress detoxification genes without full knockout and this might incur less fitness cost.

It is of great importance to the field application that edits remain stable across generations. In this paper, the authors followed eight generations of lines without phenotype loss and established the stable inheritance. But field populations have a higher genetic diversity and there is likely to be selection pressure that favours modifier alleles that apportion the costs of a fitness. According to mathematical modeling, releases should contain 30-40% proportion of local population in order to switch trends of resistance, and that would demand significant production capacity (Gould et al., 2008).

Study Limitations

This study is not without weaknesses; there are a number of weaknesses that need to be recognized. First, experiments were all done under controlled laboratory conditions in which only one laboratory strain was used based on field populations. This means that the outcomes might not be able to capture the genetic variability, environmental heterogeneity, and ecological interactions which exist in the natural populations of *H. armigera*. Second, in silico screening eliminated the number of predicted off-target effects, but whole-genome sequencing was not done, and low-frequency off-target mutations cannot be entirely excluded. Third, fitness costs were also evaluated mostly in confined conditions, that is, artificial diets; the intensity of such costs in the field where host vegetation chemistry and environmental stressors are different could be different. Fourth, the regulatory networks of compensatory upregulation of non-target detoxification genes were not examined in detail; only the existence of such responses was revealed. Lastly, the research failed to measure the gene flow, mating competitiveness or long-term population dynamics, which are important parameters in determining if the research is applicable in the field.

Future Research Directions

The research must focus in the future on the validation of these results in field populations that are genetically diverse to determine their strength under natural selection pressures. There is a necessity to study off-target effects and genomic stability in a long-generation perspective by performing comprehensive whole-genome analyses. Elucidating regulatory compensation responses on the basis of transcriptomic and epigenomic studies could elucidate the adaptive compensatory responses to deactivation of detoxification genes. More flexible conditional or reversible gene-editing systems, like inducible CRISPR or CRISPR interference/activation systems, have the potential to reduce fitness-costs during mass rearing without compromising susceptibility in released descendants. Also, gene-edited susceptible strains should be introduced together with the current resistance management methods,

which include insecticides rotation, biological control agents, and sterile insect methods and should be tested through population models and semi-field experiments. Such initiatives will play a critical role in transforming gene-editing weakness to resistance into safe, well-functioning and socially adaptable pest management solutions.

Conclusion

This study demonstrates that CRISPR-Cas9–mediated knockout of the detoxification genes *CYP6AE14*, *GSTe1*, and *COE5* in *Helicoverpa armigera* results in a substantial restoration of susceptibility to pyrethroid, organophosphate, and carbamate insecticides. The reductions in resistance ratios in knockout lines were profound, activities of detoxification enzymes were supports, and the dose-response profiles were uniform largely confirming the dominant role that these genes play in metabolic resistance as rate limiting enzymes. In spite of the fact that gene disruption had moderate costs to fitness, such as decreased larval survival, pupal weight, and fecundity, the costs were not restrictive to laboratory maintenance over many generations. Taken together, the results can offer mechanistic information on the understanding of insecticide resistance and demonstrate a evidence-of-concept that gene editing is an effective tool to weaken resistance instead of a tool to suppress the population. The method provides a new and possible sustainable addition to standard programs of control using insecticides in integrated pest management.

Authors' Contribution

Conceptualization of research.

Designing of the experiments.

Contribution of experimental materials.

Execution of field/lab experiments and data collection.

Analysis of data and interpretation.

Preparation of the manuscript.

Conflict of Interest

The authors declare that they do not have any conflict of interest.

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