Genome Editing in Insects: CRISPR Technology and its Prospects

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Abstract: Insects, as one of the largest animal groups, play a crucial role due to their vast diversity, economic significance in agriculture and cottage industries, and their ecological functions as pollinators and vectors of various diseases. Significant advancements in genetics have provided extensive information on gene identity and sequences for many insect species. These genetic resources have facilitated genome editing studies aimed at developing improved genetic traits. One such strategy is the Sterile Insect Technique (SIT), which has been effectively employed against the screwworm in North America and continues to be used for managing insect pests. Gene silencing via RNA interference (RNAi), a fundamental genomic tool in model insect research, has also been applied in various biological studies. However, its variable efficiency among insect pests has limited its widespread use. Other gene-editing approaches include the induction of Double-Strand Breaks (DSBs) in DNA using Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), which stimulate non-homologous end joining or homology-directed repair at targeted sequences. More recently, the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) system has rapidly emerged as a transformative genome-editing approach across multiple fields, including agriculture, insect resistance management, environmental safety, human health, and industry. This article provides an overview of various genome-editing techniques employed in insects, with a specific focus on the application and future potential of the cutting-edge CRISPR/Cas system, which holds promise in surpassing other genome-editing approaches.

Keywords: Genome editing, Sterile Insect Technique (SIT), Ribonucleic acid interference (RNAi), Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR).

Background: Genome Editing in Insects

Recent advancements in insect genetics have provided a wealth of genetic tools that can be employed for genome editing to tackle agricultural insect pests, control vectors affecting human health, advance biological or medical research, and address environmental challenges. The introduction of engineered traits into wild populations of insect pests could address resistance development and issues with invasive species. Furthermore, genetic control strategies targeting vector-borne diseases have made gene editing a significant focus of current research (Xu et al., 2018). For example, suppressing sex-determination pathway genes that hinder vector competence, inducing lethal recessive mutations (mutations that only manifest

when two recessive alleles are present), or producing a biased sex ratio in targeted insect populations could reduce the burden of vector-borne diseases (Ranian et al., 2022; Zulhussnain et al., 2023).

The Sterile Insect Technique (SIT) is one of the earliest and most successful genetic control strategies used against insect pests. A major achievement in insect pest management was the eradication of *Cochliomyia hominivorax* (screwworm) from North and Central America. This pest endangered livestock, wildlife, and human health, resulting in billions of dollars in losses annually. These losses included reduced exports of cattle and sheep hides, decreased meat and milk production, and wastage of human resources (Scott et al., 2017). The SIT approach involves mass-rearing target insect species, sterilizing them through radiation, and releasing them to mate with wild-type populations. Sterile males produce no offspring, and a typical release ratio of 1:10 sterile to normal males significantly reduces the chances of normal males mating (Franz et al., 2021). However, the need for mass-rearing and frequent releases of sterile males remains a limiting factor in the widespread use of SIT (Schliekelman et al., 2005).

Several genetic manipulation techniques have since been developed in insects. For example, gene function characterization, economic trait improvement, and the production of recombinant proteins in silkworms have been achieved through various genome editing methods such as RNAi, Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and the CRISPR/Cas9 system (Isobe et al., 2004; Takasu et al., 2010; Xu et al., 2018; Chen et al., 2023b). In the 1990s, RNAi was first introduced in *Caenorhabditis elegans* to silence endogenous mRNA through the introduction of exogenous double-stranded RNA (Fire et al., 1998; Belles, 2010; Terenius et al., 2011).

RNAi has since been used to analyze gene function, typically by injecting double-stranded RNA (Jiang et al., 2021). However, RNAi-mediated gene silencing in silkworms often shows low efficiency, particularly for certain genes such as the bilin-binding protein and pheromone-binding protein, which are expressed in larval epidermis and pupal wing discs (Kobayashi et al., 2012). Similarly, RNAi systems have exhibited low efficiency and non-specificity in other Lepidoptera due to unknown mechanisms (Daimon et al., 2014; Kolliopoulou and Swevers, 2014). By contrast, CRISPR-based genome editing has demonstrated high and stable knockdown efficiency in silkworms. For instance, CRISPR/Cas13-mediated knockdown of the homeobox gene *Scr* resulted in stunted growth, abnormal sex comb and salivary gland development in larvae, and malformed head and pre-thoracic segments in adults. This suggests that CRISPR-RNAi editing systems may offer a more effective alternative to RNAi in both silkworms and other Lepidopteran species (Huynh et al., 2020; Mahas et al., 2019). Since the widespread use of RNAi in *Drosophila* in the 1990s, the CRISPR/Cas9 technique has rapidly emerged over the past decade and is now being successfully employed in various insects (Fig. 1).

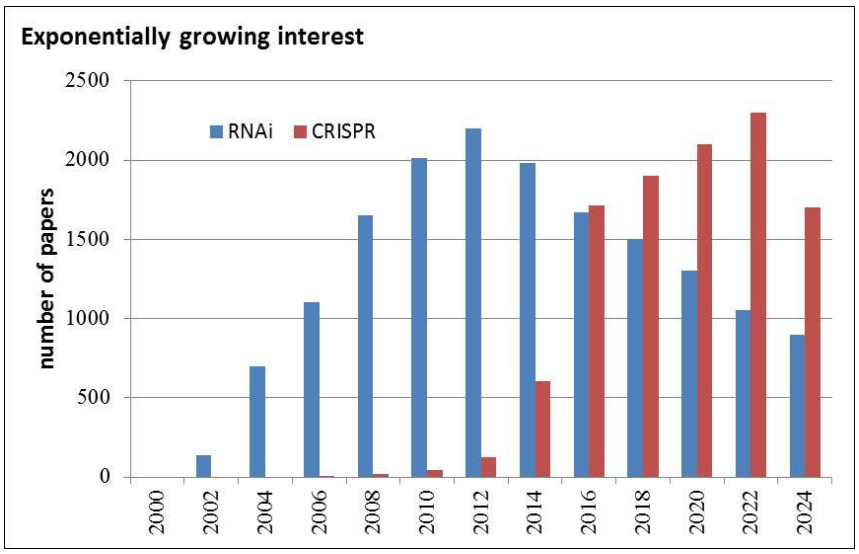

Figure 1: *Comparison of number of publications of RNAi and CRISPR*

Mosquitoes are well-documented vectors of numerous microorganisms responsible for diseases such as dengue, malaria, Zika, filariasis, and chikungunya (Zahoor et al., 2019; Reegan et al., 2016). For decades, synthetic pesticides have been the primary method used to control insect pests and vector mosquitoes worldwide. However, the extensive use of these pesticides has led to significant environmental damage. Their indiscriminate application affects non-target organisms, including humans, and has contributed to the development of pesticide resistance in many species (Bayen, 2012). This highlights the urgent need for safer, more environmentally friendly control strategies. In this context, genetic control methods are increasingly seen as a more sustainable alternative.

In addition to the successful use of the Sterile Insect Technique (SIT) in agricultural fields, which has shown significant results in Northern America, RNA interference (RNAi) has also garnered substantial scientific attention. With further advancements in genetic tools, techniques such as Transcription Activator-Like Effector Nucleases (TALENs) and Zinc-Finger Nucleases (ZFNs) have been introduced and successfully used to target genes of interest in various insect species, including crickets and mosquitoes (Watanabe et al., 2016; Awata et al., 2015; Aryan et al., 2013; Smidler et al., 2013; Watanabe et al., 2012). ZFN and TALENs have been employed to produce gene knockouts in hemimetabolous insects such as *Gryllus bimaculatus*. Site-specific mutations have been created using Surveyor (Cel-I) nuclease through microinjection of ZFNs and TALENs to generate homozygous knockout crickets. TALENs are artificial nucleases that induce double-strand breaks (DSB) at specific DNA loci, and this knockout strategy has been suggested for use in non-transgenic insect control (Watanabe et al., 2012). TALENs have also been used for knock-in genome editing in *Gryllus bimaculatus* (Watanabe et al., 2016). Similarly, the silkworm (*Bombyx mori*) has been widely employed for gene function characterization, improving economically important traits, and producing recombinant proteins using genome editing techniques such as RNAi, ZFNs, TALENs, and CRISPR-Cas9 (Chen et al., 2023).

The CRISPR-Cas system has emerged as a powerful tool for genetic manipulation, allowing precise edits to specific DNA sequences. This tool has been instrumental in exploring biological functions, dissecting signaling pathways, generating mutants for biological research, preventing disease, studying ecological interactions, and controlling agricultural pests. The CRISPR-Cas9 system, where the Cas9 protein is guided by RNA to target specific DNA sequences, is widely used for genome editing in various insects. These include flies such as fruit flies, mosquitoes (e.g., *Anopheles*, *Culex*, and *Aedes* species), bees (e.g., honeybees and bumblebees), beetles (e.g., lantern beetles and stored grain beetles), butterflies, moths, silkworms, crickets, and grasshoppers. The application of CRISPR-Cas9 has revolutionized functional genomics in insects, advancing research in pest control and resistance management (Rosli et al., 2024; Zulhussnain et al., 2023; Ranian et al., 2022; Zahoor et al., 2021; Martin et al., 2020; Tong et al., 2018; Taning et al., 2017; Chen et al., 2016; Reid and O'Brochta, 2016; Ma et al., 2017).

CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS (CRISPR)

Over the past decade, the clustered regularly interspaced short palindromic repeats (CRISPR) gene-editing technique has emerged as a highly successful genetic tool for inducing mutations and creating genetically edited insects. CRISPR has become a key technique employed across diverse fields, including biological sciences, agriculture, environmental conservation, health sciences, and industry (Rosli et al., 2024; Cannon and Kiem, 2021; Knott and Doudna, 2018; Hsu et al., 2014).

Initially discovered in bacteria and archaea, CRISPR functions as a defense mechanism, providing adaptive immunity against invading phages and foreign nucleic acids. This system consists of two main components: a CRISPR-associated (Cas) nuclease, which cleaves the target DNA sequence and generates precise double-stranded breaks (DSBs), and a single guide RNA (sgRNA), which directs the nuclease to the target DNA site (Wiedenheft et al., 2012). The sgRNA is formed by combining two RNA molecules—CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA)—that are expressed separately. In bacterial cells, the Cas proteins process these RNA molecules to produce mature guide RNA (gRNA), which then forms a complex with Cas9 to recognize and cleave DNA sequences near a proto-spacer adjacent motif

(PAM). This cleavage results in DSBs at the specific target site, which are subsequently repaired via two primary pathways: non-homologous end-joining (NHEJ) or homology-directed repair (HDR) (Jinek et al., 2012; Hsu et al., 2014; Sorek et al., 2013; Shen et al., 2017; Wang and Doudna, 2023; Wang et al., 2022; Sander and Joung, 2014; Salsman and Dellaire, 2017).

The NHEJ pathway typically introduces random insertions or deletions (InDels) at the DSB site, often leading to gene knockouts. These InDels disrupt the reading frame of the target gene, resulting in truncated or non-functional proteins. In contrast, HDR allows for precise gene editing by introducing specific mutations, resulting in gene knock-ins. Both NHEJ and HDR pathways are widely utilized to generate mutants, contributing to advancements in functional genomics research across numerous fields (Sun et al., 2017).

Moreover, the availability of genetic tools has expanded considerably, thanks in part to the Addgene repository, which offers a large number of gRNA and Cas9 plasmid constructs that facilitate gene editing in a wide range of insect species (https://www.addgene.org/) (Rosli et al., 2024; Zahoor et al., 2021; Wang et al., 2022; Ranian et al., 2022; Wang and Doudna, 2023; Zulhussnain et al., 2023).

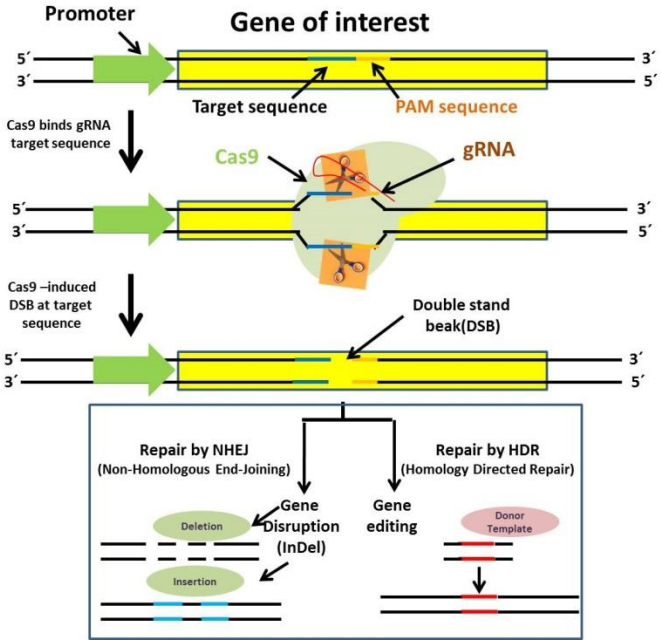

Figure 2: *Mechanism of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based genome editing (Source: www.addgene.org/crispr/guide/).*

The development of comprehensive genome databases has significantly advanced the field of genetic research, with vast amounts of data now available through resources such as NCBI BioProjects, i5K, InsectBase, and IAS1000. Additionally, transcriptome data for many insect species can be accessed, which is essential for accelerating research in genetics and genome editing. This wealth of information has enabled researchers to delve into various areas such as functional genetics, genetic screening, and the identification of genes involved in reproduction, sex determination, and insecticide resistance. Furthermore, these resources support studies on critical signaling pathways that regulate metabolic functions, the genetic mechanisms behind gene drives, and gene silencing techniques used for genetic control (Qian and Wan, 2018; Li et al., 2019).

The availability of these databases has been a boon for the scientific community, providing novel molecular tools to enhance gene editing research. Researchers can now efficiently explore and manipulate genes, leading to innovations in the management of insect resistance and the broader understanding of biological systems.

APPLICATION OF CRISPR/CAS IN INSECTS

The CRISPR system is regarded as one of the most advanced and powerful genome editing tools available today. It has been successfully applied to approximately 40 insect species across seven different insect orders, contributing significantly to a broad range of biological research areas (Chen et al., 2024). The versatility and precision of the CRISPR system make it an essential tool in various fields, including genetics, agriculture, and pest management. Below, the diverse applications of CRISPR are discussed in different categories (Fig. 3).

1. AGRICULTURE AND ENVIRONMENT

With the increasing global population, the demand for food continues to rise. However, agricultural production is severely impacted by both biotic and abiotic factors. It is estimated that insect pests alone contribute to approximately 20% of annual crop yield loss worldwide. This has led to the indiscriminate use of pesticides, which poses significant risks to human health and severely degrades the environment. Pesticide resistance is becoming a critical threat to agricultural productivity, further complicating the control of vector-borne diseases.

Recent research has focused on the potential of gene editing, particularly using CRISPR/Cas9 technology, to address insecticide resistance. Notably, studies on *Drosophila melanogaster* have provided key insights. The role of ATP-binding cassette (ABC) transporters in pesticide resistance has been identified, highlighting their significance in the development of resistance mechanisms (Douris et al., 2020). Both RNAi and CRISPR/Cas9 have made substantial contributions to our understanding of pesticide toxicity and resistance in insects, offering promising avenues for the development of more sustainable pest control strategies (Amezian et al., 2024).

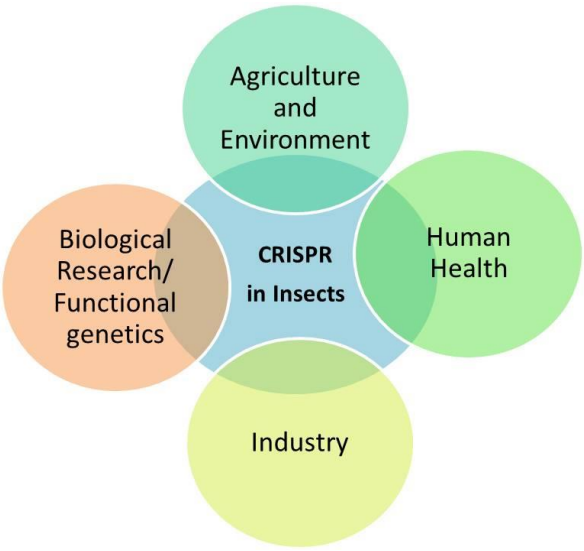

Figure 3: *A brief summary of applications of CRISPR*

RNAi targeting the BtACTB gene was induced through the expression of double-stranded RNA (dsRNA) in the whitefly, *Bemisia tabaci*, leading to the development of both nuclear transgenic and transplastomic tobacco plants. The nuclear transgenic plants were found to be more effective in controlling the whitefly *Bemisia tabaci* (Rosli et al., 2024; Dong et al., 2020), a pest that also affects major crops like cotton. In addition, a plastid-mediated RNAi (PM-RNAi) approach has been reported to genetically control *Bemisia tabaci* (Li et al., 2023), highlighting the potential for genetic control strategies to help eradicate other sap-sucking insect pests in the future (Dong et al., 2020).

The cutworm *Spodoptera litura*, a polyphagous and highly destructive pest of key crops such as cotton, fruit trees, tobacco, pulses, potatoes, sweet potatoes, and various vegetables, poses a significant agricultural threat (Chandel et al., 2022). CRISPR-Cas9 technology has been applied to *Spodoptera litura*, where the Slabd-A gene was targeted, resulting in abnormal body segmentation and pigmentation (Bi et al., 2016). Similarly, the *Orco* gene, which regulates sex pheromone production and

interaction with plant odors in *Spodoptera littoralis*, was targeted using CRISPR-Cas9 to control the pest (Koutroumpa et al., 2022; Sun et al., 2023).

Furthermore, CRISPR-Cas9 has been employed in other species, such as the silkworm *Bombyx mori*, targeting the *Orco* gene (Liu et al., 2017), the hawkmoth *Manduca sexta* (Fandino et al., 2019), and the locust *Locusta migratoria*, where homozygous and heterozygous mutant lines were produced to help genetically control locusts and other orthopteran species (Li et al., 2016). CRISPR-Cas9 has also been used in the fruit fly *Bactrocera dorsalis* (Xu et al., 2024) and the American bollworm *Helicoverpa armigera* (Fan et al., 2022). Moreover, CRISPR-Cas9 has been applied to genetically control *Spodoptera frugiperda*, commonly known as the fall armyworm, which is currently emerging as a significant agricultural pest (Gouda et al., 2024).

Insect Pest Control

The CRISPR-Cas9 technique has emerged as a highly effective tool for controlling insect pests in agriculture (Fig. 4). By employing various strategies within the CRISPR system, researchers can modify specific target DNA sequences to manage insecticide resistance or introduce traits that restore susceptibility to pests. One such strategy involves the release of gene-edited insects into the wild, which carry genes that can reduce the population of resistant individuals. This method has shown significant potential in the global fight against insect pests. Additionally, gene drive systems are being used to accelerate the spread of these genetic traits through populations (Ying et al., 2023). Insect pest control strategies using CRISPR often complement other methods, enhancing overall effectiveness (Zahoor et al., 2021; Ying et al., 2023).

CRISPR-Based Gene Drive

Gene drives, designed to spread genetic material rapidly through natural populations, have been instrumental in pest control efforts worldwide (Champer et al., 2016). By introducing beneficial genetic traits into pest populations, CRISPR-based gene drives can significantly modify reproductive patterns and increase susceptibility to pesticides. These selfish genetic elements use the natural sexual reproduction process to transmit DNA sequences or genes across generations, operating more swiftly than traditional Mendelian inheritance (Bier, 2022). Gene drives typically rely on transposable elements—naturally occurring sequences in insect genomes that can be harnessed for genetic modification (Sinkins and Gould, 2006; Wang et al., 2022a). Homing gene drives, in particular, utilize homing endonuclease genes, which recognize specific DNA sequences and induce targeted genome changes (Burt, 2003; Hillary et al., 2020).

Gene drives that exploit CRISPR-Cas9 operate through both the non-homologous end joining (NHEJ) and homology-directed repair (HDR) pathways. These systems introduce effector genes that disrupt critical biological processes, such as fertility or sex ratios, effectively reducing pest populations over time (Gantz and Bier, 2015; Galizi et al., 2016). The primary goal of gene drives is to modify populations by spreading genetic variants that eliminate harmful traits without destroying the species entirely (Champer et al., 2016; Raban et al., 2020, 2022; Devos et al., 2022a). For instance, gene-edited insects with traits like reduced fertility are bred in laboratories and then released into the wild. The introduction of these traits can lead to lower fertility rates, skewed sex ratios, or other traits that destabilize pest populations (Deredec et al., 2008; Oye et al., 2014). CRISPR-based gene drives have been successfully reported in various species, including *Drosophila*, beetles, moths, grasshoppers, and, most notably, vector-borne mosquitoes (Scott et al., 2014; Shukla and Palli, 2013).

Sex Distortion

Sex ratio distortion is another promising CRISPR-based strategy, especially in mosquito populations. Studies on *Drosophila melanogaster* have demonstrated two mechanisms of CRISPR-induced sex ratio distortion: X-shredding and X-poisoning. X-shredding operates during the meiotic phase of spermatogenesis, targeting the X chromosome to induce male-biased sex ratios, though it is counteracted by NHEJ repair. X-poisoning targets the RpS6 gene, leading to reduced reproductive output (Fasulo et al., 2020). These sex distortion approaches hold potential for genetic control of insect populations in agriculture and public health, offering a means to suppress populations of harmful pests through genetic manipulation. Further research in this area could yield significant advances in pest control and disease prevention.

Insecticide Resistance

Diamide resistance has been identified in numerous lepidopteran pests, which function as activators of Ryanodine Receptors (RyRs). Research on three diamides - chlorantraniliprole, flubendiamide,

and cyantraniliprole—revealed that mutant flies exhibited moderate resistance to flubendiamide but significantly lower resistance to chlorantraniliprole and cyantraniliprole. This established a link between RyR mutations and diamide resistance (Douris et al., 2017). Similarly, studies on the diamondback moth, *Plutella xylostella*, a major pest of Brassica plants, utilized CRISPR/Cas9 gene editing through the HDR pathway, confirming that resistance to diamide insecticides is associated with mutations in RyRs (Wang et al., 2020). Comparable findings have been observed in *Drosophila* and the beet armyworm, *Spodoptera exigua*, using CRISPR/Cas9, further confirming the role of RyRs in resistance (Zuo et al., 2017).

In addition to diamide resistance, spinosad resistance has been linked to mutations in the Alpha6 subunit of nicotinic acetylcholine receptors (nAChRs). A CRISPR/Cas9-edited *Drosophila* strain, with a mutation at position P146S in the DmAlpha6 gene, was found to confer resistance to spinosad (Somers et al., 2015). Similar CRISPR/Cas9-based research on mosquitoes has demonstrated diflubenzuron resistance in *Culex pipiens* via mutations in the chitin synthase (CHS) gene, with *D. melanogaster* used as a model organism (Fotakis et al., 2020). Resistance to pyrethroids, due to enzymes involved in detoxification mechanisms, has also been reported in *Culex quinquefasciatus* (Itokawa et al., 2016).

Recent advancements include engineering chloroplasts with double-stranded RNA (dsRNA) to combat the western flower thrips, *Frankliniella occidentalis*. CRISPR/Cas9 technology has been applied to control this pest as a potential strategy (Wu et al., 2022; Bulle et al., 2023).

Research on cadherin gene mutations in various cotton, maize, and vegetable pests has advanced through CRISPR/Cas9 techniques (Ahmed et al., 2021; Wang et al., 2016). Silencing of the *HaTSPAN1* gene has increased insect susceptibility to Bt cotton. This strategy was employed to convert resistant strains of the American bollworm, *Helicoverpa armigera*, into susceptible strains (Cheema et al., 2022; Wang et al., 2016). The cadherin gene has been found to interfere with Bt cotton feeding by bollworms, although resistance to Bt cotton has become widespread globally. In the case of the pink bollworm, *Pectinophora gossypiella*, CRISPR/Cas9 was used to modify *Cry1Ac* and *Cry2Ab* genes, with gene editing aimed at restoring susceptibility and inhibiting feeding in cotton pests, thereby reducing crop losses in the future (Zahoor et al., 2021).

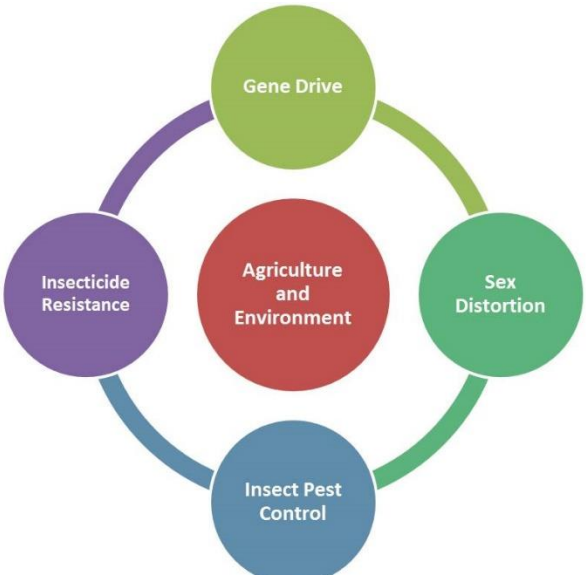

Figure 4: *Summary of applications of CRISPR in Agriculture and Environment*

The invasive whitefly, *Bemisia tabaci*, has caused significant crop yield losses globally. Despite various control strategies, insecticides remain the primary tool for managing this pest. Insecticides are often seen as a quick fix for pest control but come with considerable environmental and health risks (Bonner and Alavanja, 2017; Rani et al., 2021). Moreover, the overuse of insecticides has led to resistance in many pest species, including *Bemisia tabaci*. The indiscriminate application of chemicals

wastes valuable resources every year on a global scale. Therefore, there is a pressing need for alternative control measures that are environmentally safe, human health-conscious, and economically viable (Jurat-Fuentes et al., 2021; Bier, 2022; Ying et al., 2023). The CRISPR/Cas9 system, a powerful genetic tool, holds great promise for addressing insecticide resistance (Douris et al., 2020).

The continuous and excessive use of insecticides has driven the development of resistance in *Bemisia tabaci*, regulated at the molecular level by specific genes. OMICS approaches, such as genomics, transcriptomics, proteomics, and metabolomics, have significantly contributed to understanding the mechanisms behind this resistance. Rosli et al. identified several detoxification genes, including Cytochrome P450 (CYP), Glutathione S-transferases (GST), Carboxylesterases (COE), UDP-glucuronosyltransferases (UGT), and ATP-binding cassette (ABC) transporters, that play a crucial role in regulating insecticide resistance in *Bemisia tabaci*. The study also suggested that the knockdown of these detoxifying genes through RNA interference (RNAi) and CRISPR/Cas9 technology could reduce infestation and help manage insecticide resistance in the future (Rosli et al., 2024).

2. Biological Research/Functional genetics

Drosophila genetics has significantly advanced biological research, serving as a foundation for studies ranging from basic functional analysis to more complex areas like development, evolution, metabolism, signaling pathways, and gene function. Drosophila is particularly valued for its role in studying diseases, RNA, proteins, and cellular processes, with thanks to the available genetic tools, making it a model organism of unparalleled importance (Fig. 5).

The first mutation in *Drosophila melanogaster* using the CRISPR/Cas9 system targeted two sgRNAs, successfully deleting a 4.6 kb sequence at the *yellow* locus gene (Gratz et al., 2013). Later, a site-specific mutation in the *sex lethal (Sxl)* gene led to reproductively abnormal flies (Li and Scott, 2016). CRISPR/Cas9 technology also played a key role in understanding spinosad resistance by inducing a site-specific mutation at the nicotinic acetylcholine receptor (Zimmer et al., 2016).

The link between drug resistance and ABC transporters was further explored when mutants for three ABC transporter genes—*Mdr49*, *Mdr50*, and *Mdr65* (homologous to mammalian *ABCB1*, P-glycoprotein)—were generated in *Drosophila melanogaster*. Among these, *Mdr65* knockouts were more susceptible to tested insecticides, while *Mdr49* and *Mdr50* mutants showed resistance. When combined with the calcium channel blocker verapamil, the *Mdr65* mutants retained more insecticides in their system, confirming that *Mdr65* is involved in insecticide transport (Denecke et al., 2017).

In other species, such as the silkworm *Bombyx mori*, CRISPR/Cas9 has been used to induce a transclucency mutation at the *BmBLOS2* gene (Wang et al., 2013). Targeting the *BmWnt1* gene, which regulates the Wnt1 signaling pathway crucial for development, resulted in abnormal pigmentation and body segmentation (Zhang et al., 2015). Recent advancements in CRISPR/Cas9 technology have broadened its applications in biological research, highlighting its powerful contributions to the field (Gratz et al., 2024).

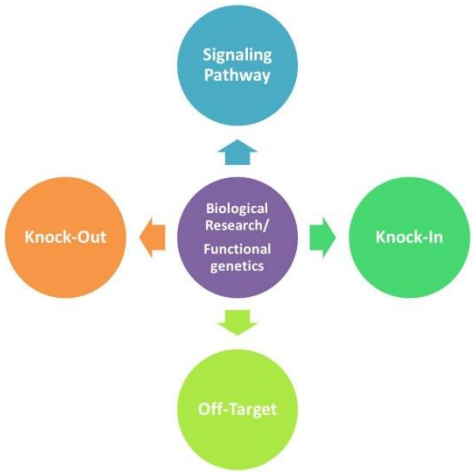

Figure 5: *Summary of applications of CRISPR in Biological Research/Functional Genetics*

A review of the scientific literature reveals that gene editing studies have predominantly focused on holometabolous insects, such as *Drosophila melanogaster*, *Bombyx mori*, *Aedes aegypti*, and butterflies like *Vanessa cardui* (Martin et al., 2020; Tong et al., 2018; Taning et al., 2017; Chen et al., 2016; Reid and O'Brochta, 2016). However, functional genetic tools for hemimetabolous insects, including economically and agriculturally important groups like Orthoptera, remain underdeveloped. This presents a significant barrier to the functional genetics and biological study of these insects.

In recent years, advancements in transcriptome data for crickets have enabled the application of CRISPR-Cas9 technology to orthopteran insects. Gene knock-out and knock-in strains of crickets and other orthopterans have been developed for potential use in agricultural pest control. For example, *Gryllus bimaculatus* has been used extensively for functional genomic studies, with gene knock-out and knock-in models developed to explore various biological processes (Watanabe et al., 2017). Additionally, the CRISPR-Cas9 system has been reported for use in exploiting crickets as a sustainable source of food and feed (Nakamura et al., 2022).

Research into *Gryllus bimaculatus* has also shed light on important biological processes such as behavior, neurology, physiology, and functional genetics. Notably, it has been shown that in crickets, octopamine and dopamine neurons mediate reward and punishment behavior. Subsequent studies in *Drosophila* confirmed similar findings through the type 1 dopamine receptor, *Dop1*. CRISPR/Cas9-generated knock-out crickets demonstrated the roles of dopamine and octopamine neurons in aversive and appetitive reinforcement, suggesting that CRISPR-based genome editing could provide deeper insights into learning and memory behaviors in insects (Awata et al., 2015).

Furthermore, the biosynthesis of melanin and catecholamine in crickets has been linked to tyrosine hydroxylase (TH) and yellow protein, which play crucial roles in growth, development, and melanin biogenesis. Recent studies using the CRISPR/Cas9 system in *Gryllus bimaculatus* have elucidated the physiological roles of these proteins in dopamine synthesis and the pigmentation process, providing a foundation for further research into behavior and biosynthetic pathways (Bai et al., 2023).

Similarly, honey bees, particularly the European honey bee (*Apis mellifera*), are widely used in studies of learning, memory, and cognitive behavior. Many candidate genes have been identified in the brains and mushroom bodies of honey bees, linking them to sensory systems, learning, and memory. Forward genetics and CRISPR/Cas9 techniques are now being developed to study these genes, offering deeper insight into the molecular and neural bases of social behaviors in honey bees (Kohno and Kubo, 2019).

CRISPR-Knockout

Knockouts (KO) are created by inactivating genes or specific gene segments, resulting in a loss of gene function. This process occurs when random insertions and/or deletions are introduced at a specific site via the non-homologous end joining (NHEJ) DNA repair mechanism. NHEJ is activated after a double-stranded break (DSB) is made by CRISPR machinery (Fig. 2 & 5). The resulting gene knockouts are random disruptions and are often used to study gene functions, gene-gene interactions, explore signaling pathways, and perform genetic screens in model organisms like *Drosophila* and other insects (Hsu et al., 2014; Sorek et al., 2013; Shen et al., 2017; Wang & Doudna, 2023; Wang et al., 2022; Sander & Joung, 2014; Salsman & Dellaire, 2017).

CRISPR-Knock-in

Knock-ins (KI) occur when specific DNA bases are inserted into the genome to replace an existing genomic sequence. This precise editing is accomplished via homology-directed repair (HDR), a DNA repair method that ensures the integration of a designed sequence at the desired location (Fig. 2 & 5). Knock-ins are often used to introduce specific mutations, especially in genes linked to human diseases, and are a key tool in clinical trials involving model organisms (Shen et al., 2017; Sun et al., 2017; Wang & Doudna, 2023; Wang et al., 2022).

CRISPR-Off-Target

While CRISPR technology is known for its high precision, there remains a possibility of off-target effects—where unintended sequences, which closely resemble the target site, are also edited. These off-target genes may be unintentionally modulated during the editing process. Various strategies, including the use of a multiplexed nickase approach, have been developed to minimize off-target effects (Guo et al., 2023). Additionally, several tools and software are employed today to reduce such risks, including CHOPCHOP, CRISPR Design, and CRISPRdirect (Ranian et al., 2022). The selection of specific Cas enzymes and protospacer adjacent motifs (PAM) sequences can further help minimize off-target gene or sequence effects (Ran et al., 2013; Guo et al., 2023).

3. CRISPR and Industry

Insect larvae as Living Biofactory for the production of human growth factors

Growth factors (GFs) are naturally occurring proteins essential in signaling pathways that regulate cell proliferation and wound healing. Along with cytokines and other compounds, GFs form a complex network crucial for tissue repair (Velnar & Gradisnik, 2018). Due to their therapeutic properties, GFs are widely researched for their role in tissue repair and facial skin rejuvenation, helping combat aging (Dudognon et al., 2014). Both GFs and cytokines have garnered attention in diagnostic research and the pharmaceutical industry for their potential applications (Dudognon et al., 2014; Lee et al., 2021; Testa et al., 2022).

The fruit fly, *Drosophila melanogaster*, is often regarded as the "Swiss-army knife" of genetics. CRISPR-Cas9 has been used to advance research on growth factors in this model organism (Sustar et al., 2023). While growth factor expression in insect cell systems has been previously reported (Lee et al., 2006), there is still a need for large-scale and cost-effective GF production. Recently, insect larvae have emerged as promising biofactories for producing growth factors as an alternative to traditional fermentation technologies (Dudognon et al., 2014). The EntoEngine™ platform has successfully employed *Drosophila melanogaster* to produce growth factors on a large scale for industries like agriculture and pharmaceuticals in Canada (Bunnak et al., 2016; Future Fields, n.d.).

Nutraceuticals, food components with therapeutic properties, are also produced through microbial cell factories—a sustainable and promising method. CRISPR-Cas9 technology is proving to be highly adaptable for precise gene editing in these biofactories, aiding in the large-scale production of nutraceuticals (Hussain et al., 2023). Furthermore, the CRISPR-Cas9 system is being used to optimize host cell lines and improve recombinant protein yields. Targeting intrinsic genes in therapeutic studies and glycosylation pathways through CRISPR-Cas9 has also been reported (Khan et al., 2020).

One notable example of using insect larvae in recombinant protein production is the cabbage looper, *Trichoplusia ni*, in combination with baculovirus, a system known as the Improved Baculovirus

Expression System (IBES). The use of insect larvae as biofactories offers several advantages over conventional cell culture systems, including lower costs, faster production timelines, and simplified infrastructure without the need for complex fermentation systems (Katsuma et al., 2006).

By leveraging insect larvae for these purposes, researchers and industries can benefit from an efficient and scalable approach to producing growth factors, proteins, and other bio-products with potential applications in medicine, agriculture, and beyond.

4. CRISPR and Human Health

The powerful genetics of *Drosophila melanogaster* has made it a cornerstone of biological research for decades. This model organism shares 60% of its genome with humans, making it a valuable tool for studying genetic diseases. Importantly, approximately 75% of disease-causing genes in humans have homologs in *Drosophila*, and out of 300 human diseases, around 200 have been modeled in *Drosophila* (Ugur et al., 2016; Oriel et al., 2018; Yamaguchi, 2018; Verheyen, 2022). The first successful CRISPR/Cas9 gene editing in insects was carried out in *Drosophila*, followed by studies in *Bombyx mori* and other non-model organisms, including flies, mosquitoes, beetles, bees, moths, butterflies, whiteflies, and crickets (Bassett & Liu, 2014).

The signaling pathways that regulate fundamental biological processes are conserved in *Drosophila*. For instance, the growth regulation mechanisms have been thoroughly reviewed by Mirzoyan et al., who also explored the use of *Drosophila* as a cancer model (Mirzoyan et al., 2019). Similarly, Zahoor et al. (2019) conducted an RNAi screen in *Drosophila* and identified negative growth regulators interacting through dS6K. They further reported that the *archipelago* gene, which regulates *CycE* and *Myc*—oncoproteins linked to ovarian and breast cancer in humans—controls growth in *Drosophila* via S6K (Zahoor et al., 2019).

In mosquitoes, gene editing has been employed to control disease vectors. CRISPR-based gene drive systems have been used to control *Anopheles* mosquitoes, the primary vector for malaria, by modifying their immune responses and introducing anti-parasite effector molecules (Kistler et al., 2015; Naidoo & Oliver, 2024). Antiviral effectors and gene drive strategies have also been developed to suppress populations of *Aedes aegypti*, which transmit diseases such as dengue, chikungunya, and Zika (Williams et al., 2020). Additionally, efforts to make malarial mosquitoes refractory to *Plasmodium* using CRISPR/Cas9-based gene drives have been explored (Nola, 2021). In sub-Saharan Africa, genetically modified mosquitoes and transgenic schistosome-resistant snail vectors have been released to combat diseases like schistosomiasis (Famakinde, 2020).

Culex mosquitoes, vectors for diseases such as West Nile virus and lymphatic filariasis, pose a significant public health threat. A CRISPR-based homing gene drive for *Culex quinquefasciatus* has been tested, and similar approaches could be applied to control *Aedes* and *Anopheles* mosquitoes (Harvey-Samuel et al., 2023).

In *Aedes aegypti*, which transmits several viral diseases, the sex-determining *M* factor plays a crucial role. Only female mosquitoes, equipped with piercing-sucking mouthparts, feed on blood for egg development. CRISPR/Cas9 has been used to target the *Nix* gene, successfully converting females into non-biting males (Hall et al., 2015). The sex-determination pathway is regulated by upstream signals that affect the splicing of downstream genes, such as *transformer*, *doublesex*, and *fruitless* (Siddall et al., 2022). In particular, *doublesex* (*Aedsx*) regulates sex differentiation via alternative splicing, producing male and female-specific transcripts (*AedsxM* and *AedsxF*). Targeting *dsxF* in our lab led to abnormal female phenotypes, including reduced wing size, shortened proboscises, and underdeveloped ovaries before and after blood meals. These results suggest that CRISPR-Cas9 could be used as a genetic control strategy by distorting the sex ratio in *Aedes aegypti* populations (Ranian et al., 2022).

Recently, our lab has also made significant progress in suppressing *Aedes aegypti* populations by generating knockouts of *Aaedsx* and *AaeSxl* genes using CRISPR-Cas9. Further investigations into *AaeSxl* as an upstream regulator of *Aaedsx* are proposed for sex transformation studies in *Aedes aegypti* (Zulhussnain et al., 2023). Field trials of CRISPR-Cas9-based gene drives targeting *doublesex* (*dsx*) in *Aedes aegypti* have demonstrated substantial population suppression across large areas of West Africa, using a simulation modeling approach (North et al., 2020). In *Anopheles gambiae*, three genes responsible

for female sterility were targeted via CRISPR-Cas9, further highlighting the potential for population control (Hammond et al., 2016). However, the biosafety risks associated with releasing genetically modified mosquitoes into the environment must be carefully considered (Taning et al., 2017).

Future Perspectives:

Although significant progress has been made in genome editing across various biological fields using CRISPR, there is still room for improvement in data repositories, the availability of genetic tools, and accessibility to plasmid constructs. Platforms like Addgene (https://www.addgene.org/) provide essential resources by offering an extensive collection of plasmid constructs, facilitating access to genetic tools at affordable prices for researchers worldwide. However, with the rapid advancement of CRISPR technology, it is essential to enhance genetic networks, foster research collaboration, and improve resource availability on a broader scale to keep pace with developments.

In addition to technological and collaborative progress, biosafety and bioethical considerations must be prioritized before the release of CRISPR-edited organisms into natural environments. These measures are crucial for ensuring that genome-edited organisms do not negatively impact ecosystems or human health, and that regulatory frameworks are in place to manage potential risks associated with gene editing technologies.

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