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CRISPR- Cas9 Technology: Mechanism and Its Application in The Field of Entomology

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Abstract

The field of life science research has undergone a revolution thanks to the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated protein (CRISPR/Cas) system, which provides a multitude of opportunities for modifying, identifying, visualising, and annotating particular DNA or RNA sequences in diverse organisms. In this technique, foreign DNA pieces, known as spacers, are inserted into CRISPR cassettes. These spacers are then transcribed into CRISPR arrays and processed to produce guide RNA (gRNA). The Cas proteins that the CRISPR arrays encode serve as the enzymatic machinery required to obtain new spacers that specifically target invasive genetic elements. Several Cas proteins, such as Cas9, Cas12, Cas13, and Cas14, have been used to create novel tools for genome engineering due to their programmable sequence specificity. The ability to manipulate and edit nucleic acid sequences in living cells from a wide variety of organisms has been made possible by these Cas variants, which have greatly advanced genetic research and the CRISPR/Cas tool. The CRISPR Cas-9 technology has applications in many areas of entomology, including the genetics of honeybees and plants that produce insecticidal compounds. CRISPR/Cas9 technology has transformed entomology by providing precise tools for gene editing and genetic manipulation in insects. This has enabled advancements in fundamental research, disease vector control, and pest management, with the potential to reduce the environmental and economic impact of insect pests in agriculture and public health.

Keywords: *CRISPR, Cas system, Entomology, Genome editing, Honeybee, Sg RNA, Silkworm*

1. Introduction

In the realm of genetic exploration, one game-changing discovery stands above the rest: CRISPR. First unearthed within the genome of *Escherichia coli* back in 1987, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) has forever altered our understanding of the delicate dance between bacteria, archaea, and their surrounding environment (Ishino et al., 1987). What makes the CRISPR system so intriguing is its intricate tapestry of repetitive segments. These segments, spanning 29 nucleotides in length, are studded with fragments of varying sequences, clocking in at 32 nucleotides (Ishino et al., 1987). As researchers delved deeper into this fascinating phenomenon, they stumbled upon an assortment of short-repeat palindromic sequences, spanning anywhere from 24 to 40 nucleotides, residing within numerous bacterial and archaeal groups. These sequences were intercepted by diverse variable sequences, ranging from 20 to 58 nucleotides in length, that artfully divided these repeats (Mojica et al., 1995; Mojica et al., 2000). A beautiful puzzle was unfolding before their eyes.

Amidst this intricate design, scientists noticed a consistent neighbor to the CRISPR locus: the Cas genes. Intriguingly, their close proximity suggested a significant functional connection (Jansenet al., 2002). Initially, theories speculated that the CRISPR locus played a role in replicon partitioning and cellular DNA repair. However, a groundbreaking revelation in 2005 forever altered our perception of the prokaryotic CRISPR/Cas system. Researchers soon discovered that the majority of sequences nestled between these identical repeats hailed from phage and plasmid genomes, invaders who had encroached upon the territory (Mojica et al., 2005; Bolotin et al., 2005; Pourcel et al., 2005). The plot thickened, and a new era of understanding was born. To comprehend the precise function of a gene,

scientists employ ingenious techniques to deactivate its influence. Two noteworthy methods are homologous recombination and RNA interference, where the latter cunningly silences the gene's messenger RNA (Im et al., 2016). Through the art of transfection in cultured cells or transgenesis in living organisms, this technique bestows upon us the power to unlock the secrets hidden within the genome (Im et al., 2016).

As we dive into the world of genomic tinkering, we encounter a fascinating process brimming with precision and adaptability. Highly accurate nucleases step onto the stage, introducing double-strand breaks within specific regions of the genome. From here, an intricate dance unfolds, leveraging cellular processes like error-prone non-homologous end-joining (NHEJ) and error-free homology-directed repair (HDR) to expertly mend these breaks (as depicted in Figure-1). It is within the realm of these repair processes that we hold the power to create insertions, deletions, or substitutions within targeted genomic territories, reshaping our understanding of genetic landscapes (Gaj et al.,2013; Sander and joung 2014; Cox et al., 2015). These mutations, revolutionary in their own right, possess the potential to alter, eliminate, or even rectify genetic anomalies responsible for various diseases. This newfound ability to correct innate genetic errors has the potential to revolutionize the field of medicine.

The possibilities unfurl before us like an uncharted map, beckoning us to venture into unexplored territories. Not only can we manipulate our genetic fabric, but we can also extend our endeavors to modify insects, halt the progression of plant and animal diseases, and usher in a new era of biological achievement. The leading nucleases that pave the way for rewriting the genetic code include meganucleases, zinc-finger nucleases, transcription activator-like effector nucleases, and the awe-inspiring CRISPR/Cas9 system (Epinat et al., 2003; Urnov et al., 2010; Miller et al., 2011; Jinek et al., 2012). Empowered by these nucleases, we gain the power to "program" their actions, setting forth a cascade of events with precision. Guided by a specialized RNA, the Cas9 nuclease maneuvers gracefully into action, introducing a double-strand break at a designated location within the genome. This groundbreaking mechanism finds its relevance not only in the field of genetics but also within the vast realm of entomology, where its potential is yet to be fully harnessed (Table-1).

As we embark on this incredible journey at the intersection of science and nature, it is a testament to the ingenuity of the human mind. We find ourselves on the precipice of unimaginable possibilities, where we can alter the very essence of life itself. The CRISPR revolution has granted us the tools to explore and redefine our biological landscape, ushering in an era of discovery and transformation. Let us embrace this extraordinary chapter in our history and wield its power responsibly, for the path ahead is filled with wonder and profound implications.

Table 1: The utilization of the CRISPR/Cas9 system in insects

Species	Genes	Mutation	Delivery	References
Drosophila melanogaster	yellow, white	Knockout	mRNA	Bassett et al. (2013)
	yellow, rosy	Knockout, Knock-in	mRNA	Gratz et al. (2013)
	rosy, DSH3PX1	Knockout, Knock-in	Plasmid, transgene	Gratz et al. (2014)
	ebony, yellow, wingless, wnt	Knockout, Knock-in	Transgene	Port et al. (2014)
	salm	Knock-in	mRNA, transgene	Zhang X. et al. (2014)
Drosophila suzukii	White, Sxl	Knockout	Plasmid	Li and Scott (2016)
Drosophila subobscura	yellow, white	Knockout	mRNA	Tanaka et al. (2016)
Aedes aegypti	miR-309	Knockout	mRNA, Cas9 protein	Zhang et al. (2016)
	ECFP	Knockout	mRNA	Dong et al. (2015)
	Nix	Knockout	mRNA	Hall et al. (2015)
	Ku70, lig4	Knockout, Knock-in	mRNA, Cas9 protein	Basu et al. (2015)
Anopheles stephensi	kh^w	Knock-in	Plasmid	Gantz et al. (2015)
Culex quinquefasciatus	CYP9M10	Knockout	mRNA	Itokawa et al. (2016)
Anopheles gambiae	AgAP005958, AgAP011377, AgAP007280	Knockout, Knock-in	Plasmid	Hammond et al. (2016)

Spodoptera litura	Slabd-A	Knockout	mRNA	Bi et al. (2016)
	SlitPBP3	Knockout	mRNA	Zhu et al. (2016)
Spodoptera littoralis	SlitOrco	Knockout	mRNA	Koutroumpa et al. (2016)
Helicoverpa armigera	HaCad	Knockout	mRNA	Wang J. et al. (2016)
	white, brown scarlet, ok	Knockout	mRNA	Khan et al. (2017)
	OR16	Knockout	Plasmid, mRNA	Chang et al. (2017)
Bombyx mori	BmBLOS2	Knockout	mRNA	Wang et al. (2013)
	Bm-ok, BmKMO, BmTH, Bmtan	Knockout	mRNA	Wei et al. (2014)
	BmNPV	Knockout	Plasmid	Dong et al. (2016)
	Bmie-1, Bmme53	Knockout	Plasmid, transgene	Chen et al. (2017)
Locusta migratoria	Orco	Knockout	mRNA	Li Y. et al. (2016)

Mechanism of CRISPR Cas 9:

CRISPR Cas 9 is an incredibly fascinating system found in the majority of bacteria and archaea. It serves as an advanced RNA-guided adaptive immune system, acting as a form of acquired immunity against attacks from bacteriophages and plasmid transfers (Heler et al., 2014; Marraffini, 2015; Mojica and Rodriguez 2016).

A Genetic Immune System

When bacteria and archaea are exposed to invasive genetic elements like phages or plasmids, they go through a remarkable process of immunization. During this process, small segments of foreign DNA are incorporated into the CRISPR repeat-spacer array within the host chromosome (Amirai and Sorek 2016), essentially creating a genetic record of previous infections (Barrangou et al., 2007; Makarova et al., 2006). This incorporation allows the host to defend against future invasions by the same invaders.

Transcription and Enzymatic Processing

Following the incorporation of foreign DNA, the CRISPR array undergoes transcription, resulting in the production of precursor-CRISPR transcripts (Amitai and Sorek 2016). These transcripts then undergo enzymatic processing through endonucleolytic cleavage, giving rise to short mature CRISPR RNAs (crRNAs) (Brouns et al., 2008). These crRNAs have a unique structure, with a short RNA segment called a spacer at the 5' end that matches a sequence from the foreign genetic element, and a fragment of the CRISPR repeat sequence at the 3' end.

Recognition and Elimination

When another infection occurs, a foreign target sequence known as a protospacer, which is complementary to the crRNA spacer, binds to it through hybridization. This interaction initiates a cascade of events. The Cas nucleases, proteins associated with CRISPR, selectively degrade invasive DNA or RNA based on specific sequences, effectively neutralizing the threat (Garneau et al., 2010; Hale et al., 2009; Marraffini and Sontheimer 2008).

Forming CRISPR-Effector Complexes

One of the key aspects of CRISPR-Cas systems is the assembly of mature crRNAs with Cas proteins, forming crRNA–effector complexes. These complexes have a crucial role in recognizing DNA targets and eliminating sequences that match foreign nucleic acids. By forming these complexes, the CRISPR-Cas system ensures efficient defense against invasive elements (Jiang and Doudna 2015; Vanderoost et al., 2014; Wiedenheft et al., 2012). CRISPR Cas 9 is an incredible mechanism where bacteria and archaea develop their own adaptive immune systems. By incorporating segments of foreign DNA, creating genetic records of previous infections, and utilizing complex RNA-guided processes, these microorganisms can effectively defend against invasive genetic elements. The assembly of crRNAs with Cas proteins further enhances their ability to recognize and eliminate threats, highlighting the remarkable sophistication of the CRISPR-Cas immune system.

CRISPR-Cas Systems: A Powerful Tool for Genome Manipulation

CRISPR-Cas systems have revolutionized the field of genome manipulation with their remarkable ability to selectively target and degrade specific DNA sequences. These systems rely on a brief motif,

typically consisting of 2 to 5 base pairs, located near the crRNA-targeted sequence on the invasive DNA. This motif, known as the Protospacer Adjacent Motif (PAM), plays a crucial role in the functioning of CRISPR-Cas systems (Bolotin et al., 2005; Deveau et al., 2008; Horvath et al., 2008; Mojica et al., 2009). To better understand CRISPR-Cas systems, scientists have classified them into six different types, denoted as type I to type VI, based on their unique characteristics (Makarova et al., 2015; Shmakov et al., 2015). Each type employs a distinct set of Cas proteins and crRNA for CRISPR interference (Wright et al., 2016). Type II CRISPR systems, unlike type I and type III systems, utilize a single DNA endonuclease called Cas9. Cas9 is capable of recognizing double-stranded DNA substrates and cleaving each strand using specific nuclease domains, HNH or RuvC (Garneau et al., 2010; Gasiunuas et al., 2012; Jinek et al., 2012). During the silencing process, a small noncoding RNA called the trans-activating crRNA (tracrRNA) forms a dual-RNA hybrid structure with the repeat sequence in the crRNA (Deltcheva et al., 2011). This dual-RNA guide directs Cas9 to cleave any DNA that possesses an adjacent PAM and a complementary 20-nucleotide (nt) target sequence, thereby facilitating the precise degradation of the target DNA (Gasiunas et al., 2012; Jinek et al., 2012).

In type II CRISPR systems, the maturation of crRNA relies on the presence of tracrRNA (Deltcheva et al., 2011). The integration of tracrRNA and crRNA into a single RNA transcript, known as chimeric single guide RNA (sgRNA), simplifies the system while maintaining Cas9-mediated sequence-specific DNA cleavage capabilities (Jinek et al., 2012). By modifying the guide RNA sequence (spacer) within the crRNA, this simplified two-component CRISPR-Cas9 system can be programmed to target virtually any DNA sequence of interest in the genome (Jinek et al., 2012). The result is a site-specific blunt-ended double-strand break (DSB) (Jinek et al., 2012).

Subsequent repair of these Cas9-induced DSBs can occur through two major pathways: high-fidelity homology-directed repair (HDR) and error-prone nonhomologous end joining (NHEJ). HDR utilizes a homologous repair template to precisely modify the genome at the DSB site, while NHEJ often introduces small random insertions and/or deletions (indels) at the cleavage site (Jinek et al., 2012). The efficiency, design simplicity, and versatility of the CRISPR-Cas9 system have made it an incredibly powerful tool for genome manipulation in various organisms (Jinek et al., 2012). In contrast to other DNA editing methods, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR-Cas9 relies on a 20-nt guide RNA sequence rather than protein-specific recognition (Doudna and Charpentier 2014). This eliminates the need for complex protein engineering of DNA-recognition domains for each DNA target site, making CRISPR-Cas9 highly flexible and widely adopted in the scientific community for screening and large-scale genomic manipulation. CRISPR-Cas systems have opened up new possibilities in the field of genome manipulation. With their ability to selectively target and degrade specific DNA sequences, these systems have proven to be invaluable tools for researchers worldwide. The classification of CRISPR systems into different types, the role of Cas proteins and crRNA, and the use of Cas9 in type II systems highlight the incredible complexity and precision of these systems. By exploiting the power of CRISPR-Cas9, scientists can unlock a wealth of knowledge about the genome and pave the way for innovative advancements in various fields, from medicine to agriculture and beyond.

The Cas9 Enzyme:

SpyCas9, which stands for Streptococcus pyogenes Cas9, is a substantial and multifunctional DNA endonuclease comprised of a large protein with 1,368 amino acids and multiple domains. It precisely cuts double-stranded DNA, specifically 3 base pairs ahead of the PAM (Protospacer Adjacent Motif) site. SpyCas9 carries out this function by means of two distinct nuclease domains: one domain, which is similar to the HNH nuclease, is in charge of cleaving the DNA strand that corresponds to the guide RNA sequence and is called the target strand; the other domain, which is similar to the RuvC nuclease, is in charge of cleaving the DNA strand that is in opposition to the complementary strand and is called the non-target strand (Gasiunas et al., 2012; Jinel et al., 2012; Chen et al., 2014). Cas9 is essential for CRISPR interference, but it also actively participates in crRNA maturation and the acquisition of new spacer sequences (Heler et al., 2015).

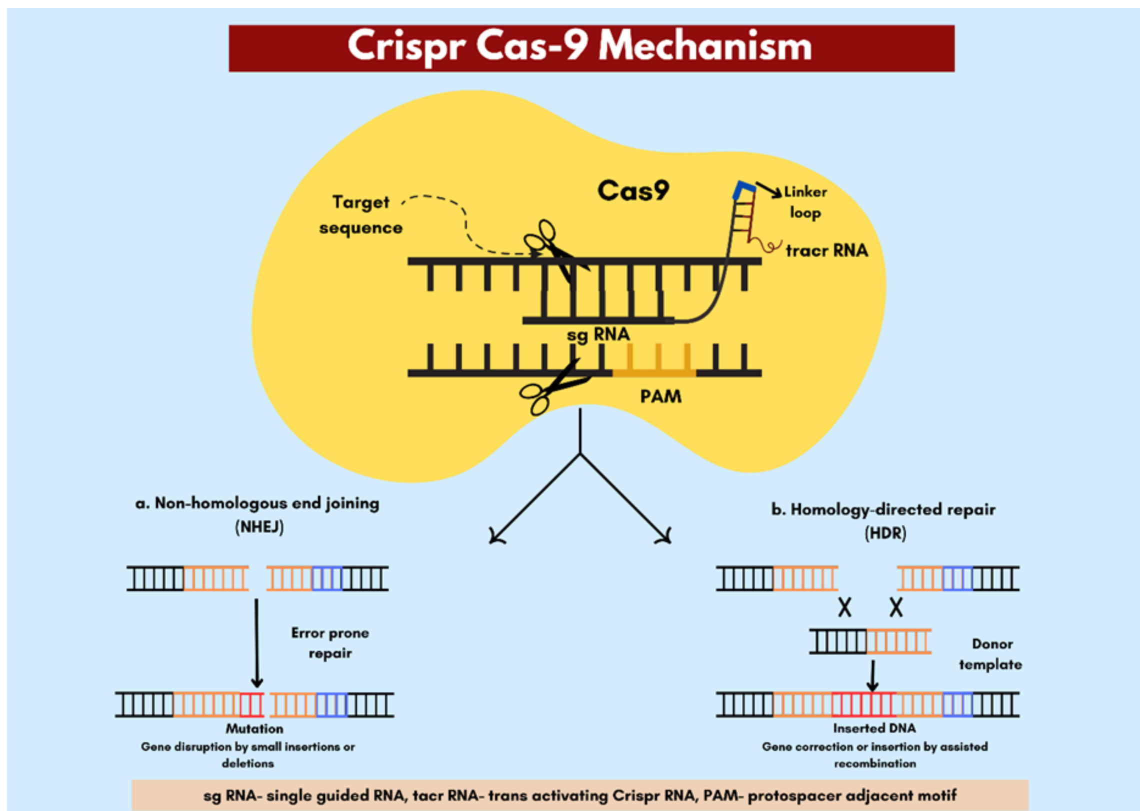

Figure 1: Showing Crispr Cas9 Mechanism(PC by Ritu Raj)

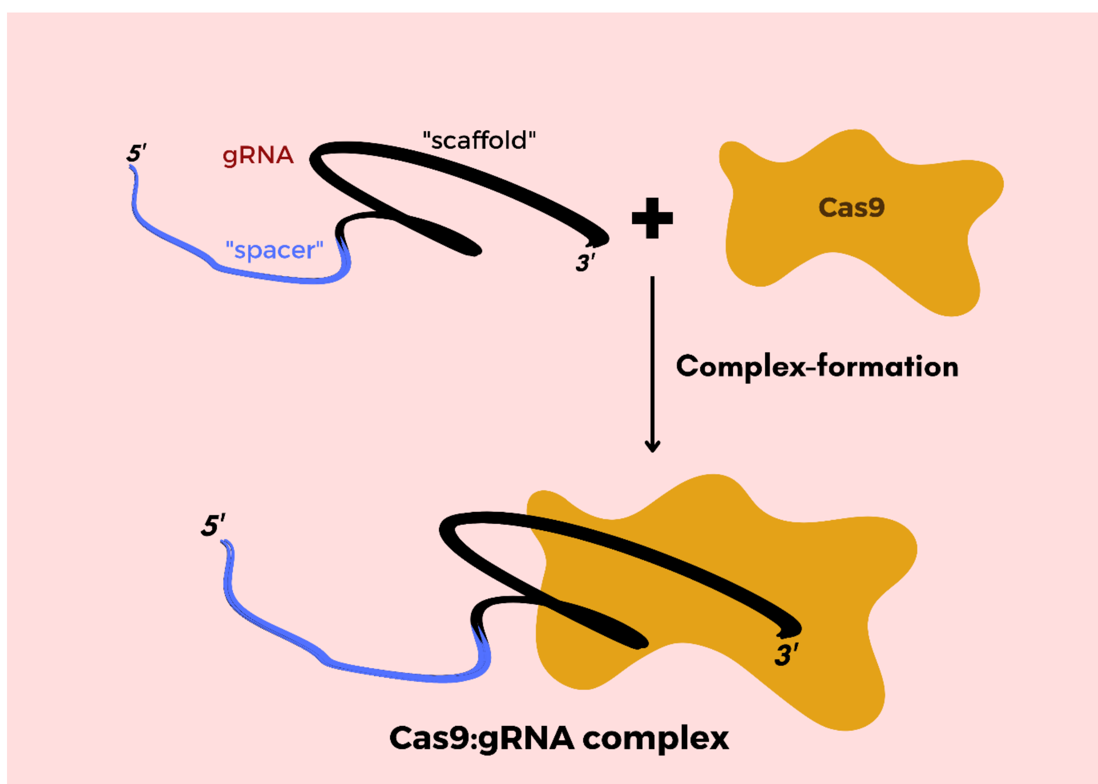

Figure2: Depicting formation of Cas9 g RNA complex formation (PC by Ritu Raj)

Applications in the field of Entomology:

Drosophilla:

In the world of genetics, the tiny fruit fly, Drosophila melanogaster, has played a crucial role in advancing insect genome editing technology (Ogaugwu et al., 2013). Thanks to the revolutionary CRISPR/Cas9 system, researchers have made significant strides in manipulating the Drosophila genome, paving the way for innovative applications and breakthroughs. In this article, we will delve into the remarkable achievements and potential of CRISPR/Cas9 in the realm of Drosophila research.

In 2013, Gratz et al. accomplished a groundbreaking feat by demonstrating how the CRISPR/Cas9 technology could delete a specific 4.6 kb section of chromosomal DNA in the Drosophila genome. This landmark study utilized a single-stranded oligonucleotide donor (ssODN) template and two target single guide RNAs (sgRNAs) to successfully carry out the deletion at the yellow locus (Gantz and Akbari 2018). The implications of this achievement were immense, as it showcased the precision and effectiveness of CRISPR/Cas9 in Drosophila genome editing.

Building upon the success of the initial breakthrough, researchers have further explored the advantages and potential applications of CRISPR/Cas9 in the creation of designer flies (Bassett and Liu 2014). In particular, a notable development was the utilization of a reintegration vector to enhance the frequency of homologous recombination (HR) (Gratz et al., 2013). This promising innovation opened doors for more efficient and targeted gene modifications in Drosophila (Gratz et al., 2013).

Another crucial aspect of Drosophila research involves the development of user-friendly screening platforms and comparative analysis of various gene editing techniques. In this regard, researchers have contrasted the efficacy of TALEN-based homology-directed repair (HDR) with CRISPR/Cas9-mediated HR. Furthermore, they have established three distinct HDR techniques for site-specific mutagenesis (Gratz et al., 2013). These advancements have diversified the genetic toolbox for fine-tuning the Drosophila genome and unraveling its complexities. To facilitate experimentation, two sets of parent flies were created. One group had the Cas9 gene driven by a germline-specific promoter, while the other group constitutively expressed sgRNA. Through strategic crossbreeding, these parent flies were successful in transmitting mutations to their germlines. This crossbreeding technique, depicted in Figure-3, demonstrated the inheritability of desired genetic alterations, laying the foundation for further studies.

Flies undergoing homology-directed repair (HDR) have been a subject of intense research. Scientists have adopted different methodologies to achieve this outcome (Baena-Lopaz et al., 2013; Yu et al., 2014; Kondo and Ueda 2013; Port et al., 2014; Ren et al., 2014; Sebo et al., 2014; Xue et al., 2014). For instance, transgenic Cas9 embryos were injected with a gRNA plasmid and a donor repair template (Xue et al., 2014). Likewise, donor template plasmids were injected into transgenic embryos carrying sgRNA and Cas9. Another approach involved providing non-transgenic flies with all three essential components: the donor repair template in plasmid form, sgRNA, and the Cas9 gene (Zhang et al., 2014; Gokcezade et al., 2014). These diverse strategies demonstrated the versatility and adaptability of CRISPR/Cas9 in achieving HDR in Drosophila. The CRISPR/Cas9 system's ability to induce significant gene mutations in Drosophila, affecting crucial genes such as clamp, troponin C (TpnC), Alk, Sex-lethal (Sxl), and white (w), has laid a solid groundwork for long-term pest management strategies (Knipling, 1955; Lamb et al., 2017; Urban et al., 2017; Chechenova et al., 2017; Mendoza-Garcia et al., 2017). One promising approach is the sterile insect technique (SIT), which involves releasing sterilized male insects of the target species into the wild. By combining SIT with CRISPR/Cas genome editing, optimized SIT strains can be created (Dyck et al., 2021; Kalaidzic and Schetelig 2017). This integrated approach showcases the potential of CRISPR/Cas9 in combating pest populations more effectively and sustainably.

The white gene, in particular, has proven to be instrumental in experiments that aim to modify Drosophila. Precise editing of the white gene, achieved through the direct introduction of purified Cas9 protein into *D. suzukii* embryos, streamlines the gene editing process. Recombinant Cas9 protein also holds the potential for generating heritable genetic alterations. Researchers, such as Choo *et al.,* have successfully developed genetically sexing strains of Bactrocera tryoni (Froggatt) by creating frameshift mutations using CRISPR/Cas-mediated editing. The insights gained from this research could revolutionize SIT-based pest management techniques (Choo et al., 2018). In the context of SIT, the sperm marking system serves as a valuable tool for monitoring pest populations. In 2019, researchers achieved a significant milestone by creating a sperm-marking transgenic strain in *D. suzukii*. This strain utilized endogenous promoters of *D. suzukii* to express gRNA through the U6 promoter of a small nuclear RNA gene and Cas9 through the Ds hsp70 promoter. Strikingly, the success of HRD-based gene editing was higher with co-injections of the helper plasmid compared to preformed RNPs (Ahmed et al., 2019). Notably, the modification of the white (w) gene in D. suzukii resulted in a deficiency in pigmentation within the testis sheath and disrupted copulation. These findings highlight the potential benefits of CRISPR/Cas9 in managing pest populations effectively. The utilization of CRISPR/Cas9 in Drosophila research has brought about remarkable advancements in insect genome editing technology. From precise DNA deletions to the creation of designer flies, the potential of this groundbreaking technology is vast. Moreover, the integration of CRISPR/Cas9 with pest management strategies, such as the sterile insect technique, opens up new possibilities for sustainable and effective pest control (Sim

et al., 2019; Yan et al., 2020). While there is still much to explore, the progress made thus far showcases the immense value of CRISPR/Cas9 in unraveling the mysteries of the Drosophila genome and beyond.

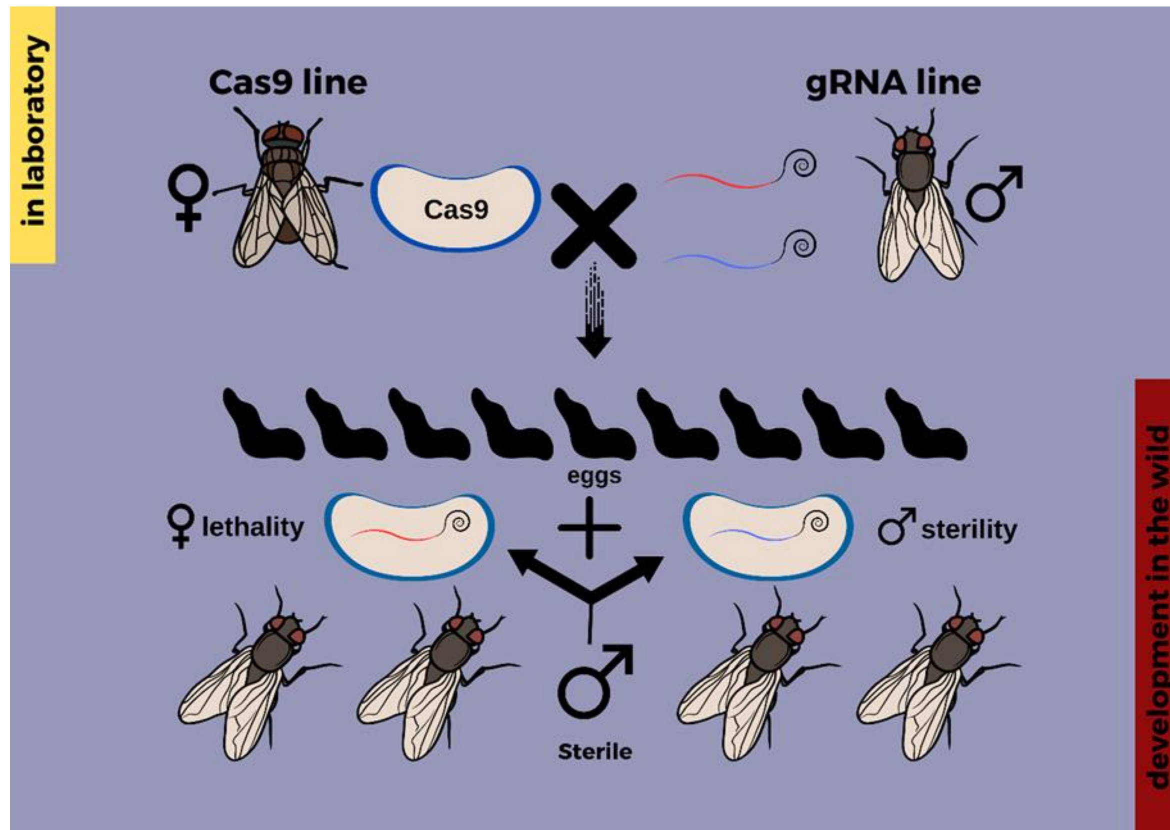

Figure 3: Depicting Sterile insect technology using Cas9 and g RNA in *Drosphilla* (PC by Ritu Raj)

Bactrocera dorsalis:

Bactrocera dorsalis, a notorious pest prevalent in various Asian regions, has recently undergone genetic modifications using the revolutionary CRISPR/Cas9 system. This groundbreaking technique targeted the white and transformer genes of the *B. dorsalis* embryos, leading to remarkable mutations. These mutations resulted in the development of abnormal internal and external reproductive organs, as well as a notable shift towards a male-biased sex ratio. The inheritance of these mutations in subsequent generations suggests that targeting the transformer gene could prove to be an effective strategy in controlling such devastating pests (Zhao et al., 2019).

Aedes aegypti, Anopheles stephensi, Anopheles gambiae

Mosquitoes are well-known carriers of deadly diseases that pose a significant threat to human health. Conditions such as filariasis, chikungunya, dengue, zika, and malaria have caused immense suffering and loss of life (Gabrieli et al., 2014; Reegan et al., 2017). Traditionally, synthetic insecticides have been employed to combat disease-carrying mosquitoes. However, the long-term use of these pesticides has led to resistance in mosquito populations and adverse environmental effects, raising concerns about their efficacy in disease control (Tikar et al., 2009; Bayen, 2012).

To effectively manage mosquito populations, it is crucial to understand the reproductive habits of female mosquitoes. These mosquitoes lay their eggs in water and require a blood meal before mating. A single female mosquito can lay eggs three to four times during her lifetime, producing an astonishing 150 to 250 offspring each time. Exploiting this knowledge can greatly assist in controlling mosquito populations and mitigating the spread of diseases (Tikar et al., 2009). Due to its role as a vector for various arboviruses, including dengue, chikungunya, and yellow fever, the *Aedes aegypti* mosquito has become a prime candidate for genetic modification (Weaver and Barrett 2004). Researchers have utilized genome editing tools, such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) (Aryan et al., 2013; Smidler et al., 2013), to manipulate the *A. aegypti* genome successfully. The CRISPR/Cas9 technology, specifically, was employed in 2015 to modify the *A. aegypti* genome, marking a significant advancement in this field (Dong et al., 2015). By precisely targeting the ECFP gene, researchers generated transgenic *A. aegypti* mosquitoes expressing the DsRed gene while suppressing the expression of the ECFP gene. Furthermore, other studies have shown that

the CRISPR/Cas9 system can induce various mutations by injecting carefully designed sgRNA-Cas9 mixtures into *A. aegypti* embryos (Kistler et al., 2015). Basu *et al.* even developed a platform to enhance gene editing efficiency through the effective screening of potential sgRNAs (Basu et al., 2015).

Bombyx mori

The lepidopteran insect *Bombyx mori* holds remarkable economic value and serves as a crucial model organism in scientific research. Researchers have long utilized genomic editing tools to understand gene functions in vivo, with B. mori being among the first insect models employed for this purpose. In 2014, Daimon et al. compared and evaluated three different genomic engineering methods: ZFNs, TALENs, and CRISPR/Cas9. Encouragingly, the outcomes demonstrated that the CRISPR/Cas9 system proved to be the most practical and effective approach (Diamon et al., 2014).

The BmBLOS2 gene served as the focus of research to evaluate the suitability of the CRISPR/Cas9 system in *B. mori*. Previous studies had shown that mutations in this gene caused the larval integument to become translucent rather than opaque, highlighting its importance (Wang et al., 2013; Liu et al., 2014). While mutation strategies typically result in small insertions or deletions (indels) in coding sequences, Liu et al. developed a technique utilizing the CRISPR system with two sgRNAs to induce variations in non-coding sequences of up to 3 kb in the *B. mori* cell line BmNs. By cleverly designing two sgRNAs separated by the 3 kb fragment, the researchers successfully induced targeted mutations in each of the designated gene loci. These findings demonstrate the immense potential of the CRISPR/Cas system in the simultaneous generation of large-fragment or multiple-gene mutations in *B. mori* (Liu et al., 2014; Ma et al., 2014). In a separate study, scientists capitalized on the distinctive mutant phenotype of the Bm-ok gene, which facilitated the straightforward identification of homozygous mutants and the determination of mutation efficiency using CRISPR/Cas9 technology (Wei et al., 2014). By adhering to ethical guidelines and leveraging the power of genetic modification techniques like CRISPR/Cas9, researchers are making staggering advancements in combatting pests and understanding complex genetic mechanisms. These innovative approaches provide hope for the development of effective strategies to control disease-carrying mosquitoes and enhance our understanding of vital organisms like *Bombyx mori*.

Spodoptera litura, Spodoptera frugiparda, Spodoptera exigua

The field of pest management has witnessed significant advancements with the emergence of the CRISPR/Cas9 system. This revolutionary technology enables scientists to create innovative tools by inducing specific changes in the genetic makeup of pests. One notable application of this system is the manipulation of the Slabd-A gene (Spodoptera litura abdominal-A) in lepidopteran pests. This gene plays a crucial role in the development of these insects, specifically in the segmentation of the abdomen and the identification of individual segments. By manipulating the genome of this gene, researchers were able to generate abnormal pigmentation and segmentation, providing valuable insights for effective pest management strategies (Bi et al., 2016).

Another study focused on disrupting the SlitBLOS2 gene in *S. litura*, which led to the complete eradication of yellow stripes and white spots on the larval integument. This gene knockout served as a useful marker for functional research and pest management applications (Zhu et al., 2017). Moreover, the CRISPR/Cas9 system was employed to mutate the Pheromone Binding Proteins (PBPs) gene, SlitPBP3, in *S. litura*. This allowed scientists to investigate the role of this gene in the response to sex pheromone components, revealing a decreased response in the mutant individuals compared to the wild-type (Zhu et al., 2016). Similarly, the Orco gene, responsible for olfactory reception in *Spodoptera littoralis*, was deleted using the CRISPR/Cas9 technique, resulting in insensitivity to both plant and sex odors (Koutroumpa et al., 2016).

Moving on to *S. frugiperda*, researchers examined three genes: E93, a developmental gene crucial for adult insect development, as well as two marker genes, tryptophan 2,3-dioxygenase (TO) and biogenesis of lysosome-related organelles complex 1 subunit 2 (BLOS2). By utilizing multiple sgRNA injections, this method enhanced our understanding of the complex pathways that control invasive species and pests like Fall Armyworms (FAW), opening doors for high-throughput functional genomics screening and functional gene characterization (Zuo et al., 2020). Additionally, the CRISPR/Cas9 system was employed to functionally validate the homozygous strain of S. exigua, leading to new insights into gene function within this species (Zuo et al., 2020).

Helicoverpa armigera

In an effort to combat pests, *Bacillus thuringiensis* (Bt)-based insecticides and genetically modified Bt crops have been widely implemented. However, the emergence of resistance to Bt toxins poses a

substantial hurdle to sustainable pest management (Bravo et al., 2011). Recent studies have identified the cadherin-like receptor as the binding site for the Bt Cry1A toxin in several Lepidoptera insects (Wu, 2014). This receptor plays a crucial role in the proteolytic breakdown of the Cry1Ab protoxin and the formation of a pre-pore oligomeric structure in Manduca sexta (Gomez et al., 2002). Investigations utilizing genetic linkage, cell toxicity tests, and RNA interference have shed light on the role of cadherin in Cry1Ac resistance among various Lepidopteran insects.

To further explore this phenomenon, Wang et al. (2016*)* utilized the CRISPR/Cas9 system to target the cadherin gene, initiating a targeted mutation of exon 9 in *Helicoverpa armigera.* The resulting mutants exhibited an astounding 549-fold increase in resistance to Cry1Ac, providing concrete evidence that the HaCad gene serves as a crucial Cry1Ac receptor linked to resistance. Moreover, researchers successfully mutated four pigment genes - white, brown, scarlet, and ok - in *H. armigera* using the CRISPR/Cas9 technology (Khan et al., 2017), revealing a wide range of physiological phenotypes caused by these mutations. Another innovative application of the CRISPR/Cas9 system in *H. armigera* demonstrated the maximization of fecundity through antagonist-mediated techniques, presenting a promising approach to disrupt pest mating behaviors (Chang et al., 2017).

Locust migratoria

The presence of locusts as agricultural pests poses a significant challenge that has historically been addressed through eradication measures heavily reliant on synthetic pesticides. However, these traditional methods have proven detrimental to the environment and the economy. Consequently, scientists have recognized the importance of delving into locusts at the molecular level to develop more sustainable control strategies. Research has shown that the synchronization of locust gregarious behavior, migration, and copulation is dependent on developmental synchrony. One gene, miR-276, has emerged as a crucial factor in promoting the synchronized development of locust eggs, directly impacting population density (He et al., 2016). Additionally, the sense of smell plays a vital role in insect biology, guiding behaviors such as feeding, mating, egg-laying, and foraging (Leal, 2012). In an effort to uncover functional genes associated with Locusta migratoria, researchers modified the locust genome using the CRISPR/Cas9 technology. By targeting the odorant receptor co-receptor gene (Orco), they achieved extremely high mutation rates with the injection of Cas9-mRNA and Orco-sgRNA into locust eggs, resulting in successful creation of heterozygous and homozygous Orco mutant lines. This breakthrough marked the first time CRISPR/Cas9 technology was employed to edit a locust genome, offering invaluable insights for effective locust management and serving as a blueprint for future application in related insects (Li et al., 2016).

Apis millefera

Honey bees, essential for pollination and honey production, have also become subjects of scientific research. Successful plasmid transfection procedures have been reported, one of which attempted to introduce linearized plasmids along with sperm into fertilized eggs through artificial insemination. While the transfected DNA did not integrate into the honey bee genome, it remained present for at least three generations, demonstrating the feasibility of such procedures (Robinson et al., 2000).

Insect transgenesis has utilized DNA transposons, movable DNA elements that change their location within a host genome through transposase activity. The P element, initially discovered in Drosophila, has been extensively employed in producing transgenic D. melanogaster (Hiraizumi, 1971; Spradling and Rubin 1982; Rubin and Spradling 1982). For insects beyond Drosophila, the piggyBac transposon has proven to be a valuable tool (Shinmyo et al., 2004; Handler and Harrell 1999; Sumitani et al., 2003; Toshiki et al., 2000; Eckermann et al., 2018). Researchers successfully created the first transgenic honey bee by inserting plasmids with external genes flanked by piggyBac inverted terminal repeats and piggyBac transposase mRNA into fertilized eggs (Schulte et al., 2014). This approach resulted in the integration of external plasmid sequences into the honey bee genome, effectively generating transgenic drones. While piggyBac integration should be approached with caution due to potential interference with endogenous genes, recent advancements in transposase optimization and injection techniques have increased the efficiency of genome integration, making piggyBac a useful tool for modifying honey bee genes (Figure 4).

Through these groundbreaking studies, scientists have paved the way for more effective and environmentally friendly pest management strategies. By harnessing the power of gene manipulation techniques like CRISPR/Cas9, the intricate mechanisms governing pests and invasive species can be better understood, leading to innovative approaches in mitigating their detrimental effects on agriculture.

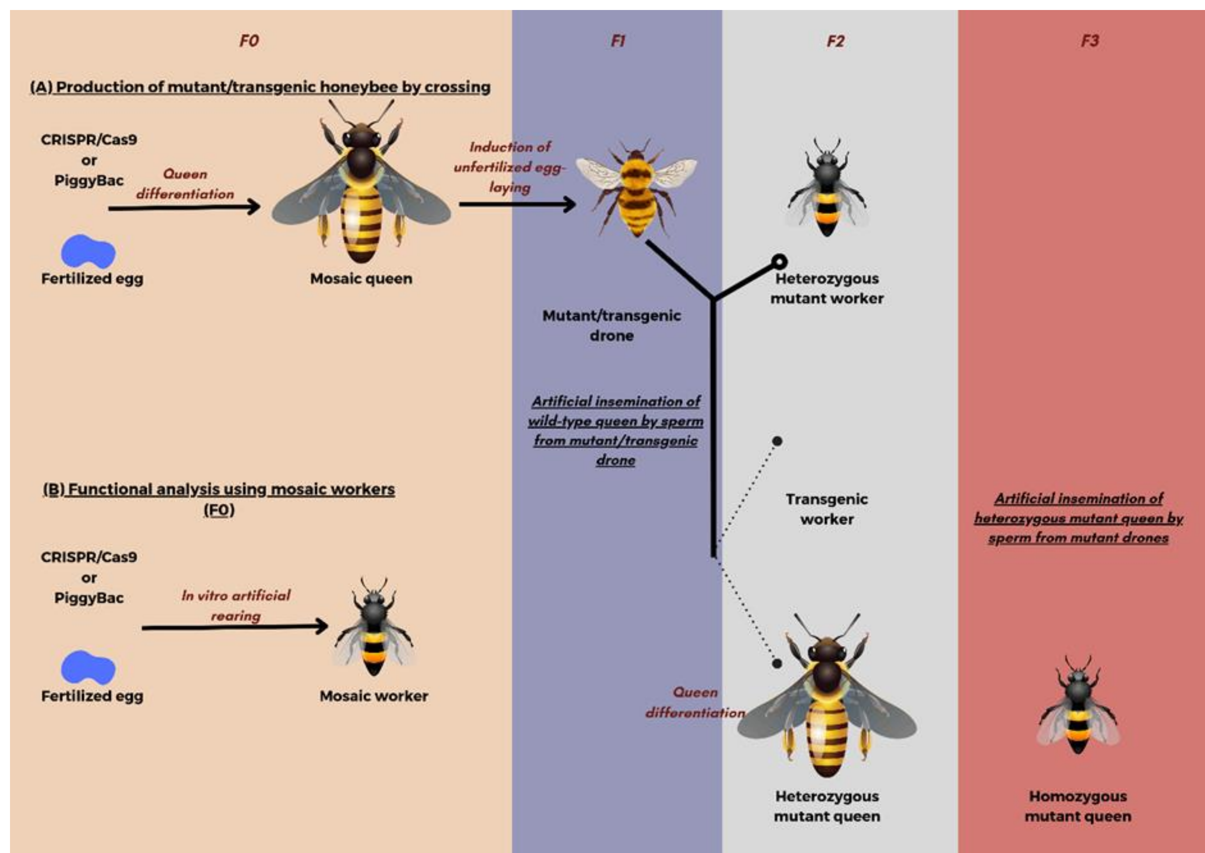

Figure 4: showing an overview of uses in honey bee in the field of Crispr Cas9. A) showing procedure to produce transgenic honey bee. B) Mosaic workers (F0) artificially reared from injected eggs by alternating methods (PC by Ritu Raj)

4. Conclusion

In conclusion, CRISPR-Cas9 technology has emerged as a groundbreaking tool in the field of entomology, offering precise and targeted genome editing capabilities. Its mechanism, inspired by the bacterial defense system, allows for the modification of specific genes in insects with remarkable accuracy. In the realm of entomology, CRISPR-Cas9 has revolutionized research and applications. Scientists can now investigate the function of crucial genes in insect physiology, behavior, and development. This knowledge contributes to a deeper understanding of insect biology and ecology. Furthermore, CRISPR-Cas9 holds immense potential for pest management and disease control. By targeting genes responsible for reproduction, insecticide resistance, or disease transmission, researchers and entomologists can develop innovative strategies to combat insect pests that threaten agriculture, public health, and ecosystems. The adaptability and versatility of CRISPR-Cas9 make it an invaluable tool in entomology, offering solutions to complex challenges in insect biology and control. As research in this field continues to advance, CRISPR-Cas9 technology will play a pivotal role in shaping the future of entomological science and its applications.

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