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New Biotechnological Approaches to Insect Pest Management and Crop Protection; Gene Editing Approach (CRISPR-Cas system).

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Title: New Biotechnological Approaches to Insect Pest Management and Crop Protection; Gene Editing Approach (CRISPR-Cas system).

MS Project

Abstract

Since its introduction into the Integrated Pest Management (IPM) program over 20 years ago, the biotechnological approach for insect pest management, starting with transgenic *Bacillus thuringiensis* (*Bt*), appeared to have revolutionized the concept of genetic manipulation approach in insect pest management and crop protection. Transgenic (*Bt*) crops have offered great benefits in insect management for crop protection but soon after its implementation, the technology started facing the major issue of insect resistance, leading researchers to start pursuing advancements in newer biotechnological approaches to insect pest management, such as Gene Editing (the RNA interference (RNAi); Gene Drives and most recently CRISPR-Cas9 system). By 2012, scientists found a way to use CRISPR-Cas as a genome editing tool and in 2013 the CRISPR-Cas application in plants was successfully achieved, in the lab; leading to new opportunities for researchers to continue studying the CRISPR-Cas system for its potential applications and the opportunity to gain a better understanding of RNA mediated cell functions (given that RNA holds a central role in almost everything a cell does). This review will discuss the potential uses of CRISPR-Cas9 systems for agricultural and medical insect pest management as well its applications for plant diseases/crop protection. The review will also discuss the limitations, potential issues and prospects of the CRISPR-Cas9 technology as it pertains to insect pest management.

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I. Introduction

Insect pest management has been an ongoing battle for Entomologists, Farmers and Agricultural industries, with control strategies continuously evolving and becoming perhaps more effective. Control approaches have evolved throughout the years, starting with humans hand-picking and killing the insects to cultural and biological control approaches and to the discovery and wide spread use of chemical insecticides by mid -1940s, which revolutionized the Agricultural industries in the 1950s and early 1960s. Chemical control demonstrated the kind of effectiveness that had not been seen before and continued to be widely used. The public became increasingly reliant on its use as it increased our food production and removed insects that carried diseases. Such overuse had ultimately led to what was termed “Chemical crisis,” where higher and higher doses of chemicals became necessary as insects became resistant to the chemicals (“History of IPM” n.d.), until Rachel Carson brought public awareness of the environmental effects of chemical pesticides in her book, “Silent Spring,” published in 1962 (Unsworth 2010). The growing knowledge about the negative side-effects associated with excessive use of chemical pesticides had led to shifting focus to a newer, more comprehensive pest management approach, the Integrated Pest Management (IPM). The term Integrated Pest Management was first used in Agriculture beginning in the 1970s (“History of IPM” n.d.). IPM is essentially a pest management strategy that uses comprehensive approaches to reduce pest status to tolerable levels while minimizing the environmental effects (Alston 2011).

Since its development, the concept of Integrated Pest Management (IPM) has been strategically implemented, and remains arguably the “best” known form of insect pest management according to many. However, IPM has its own limitations. Most of the limitations of IPM come from

identifying the best tactics to implement in a given management strategy. These limitations have led to continuous research and the development of biotechnological approaches for insect pest management. In this review I will be exploring the new biotechnological approaches to insect pest management and crop protection, including transgenic *Bt* and gene editing approaches with more focus on the emerging CRISPR-Cas9 mediated gene editing technology. I'll explore the concept, the mechanisms and the applications of the CRISPR-Cas9 gene editing technology as well as the limitations, potential issues and future prospects of the technology.

II. Transgenic *Bacillus thuringiensis* (*Bt*) Crop Approach

Transgenic *Bt* crops are genetically engineered from spore forming bacterium (*Bacillus thuringiensis* (*Bt*)) that produces crystal-like proteins (Cry proteins). Cry proteins are toxic to many species of insects, including insects that are pests of corn, with high effectiveness in controlling the caterpillars of Lepidoptera and Coleoptera insects. The crystal toxins work by binding to specific receptors in the mid-gut of the target caterpillars, after being ingested, and create perforations in the lining of the insect's gut, leading to the insect's death. This approach is known to have no effects on humans or other vertebrates (vertebrates do not have receptors for the crystal-toxins to bind to).

Transgenic *Bt* crop technology has provided many benefits to farmers, Agricultural/seed companies and to the consumers, which has led to fewer uses of insecticides, increased yield and cheaper food prices. Corn production, for example, has greatly benefited from the use of transgenic *Bt* to control most major pests of corn including European corn borer (Lepidoptera: Crambidae), Western corn rootworm (Coleoptera: Chrysomelidae), Fall armyworm Lepidoptera: Noctuidae), and Corn Earworm (Lepidoptera: Noctuidae).

One type of transgenic *Bt*-corn expressing the Cry1Ab protein provides protection against certain Lepidopteran pests, mainly the European corn borer. For instance, prior to the use of Cry1Ab protein, European corn borer was one of the most damaging insect pests in north America, resulting in estimated damages and control costs exceeding \$1 Billion each year. The larvae feed on leaves and tunnel into the stalk, ear shanks and ear (Ostlie et al. 1997; Rice et al. 1997). Transgenic *Bt* control of the European corn borer offered dramatically superior control compared to the IPM options that were available. In earlier field tests against natural and added European corn borer infestations, the *Bt*-corn showed a more than 99% control of first generation European corn borer larvae in young (whorl-stage) corn plants compared to the chemical insecticide treatments, which typically provide 60-95% control of first generation European corn borer larvae (Ostlie et al. 1997).

The Western corn rootworm is also among the most serious pests of corn in the United States of America (Vaughn et al. 2005). Western corn rootworm larvae feed on the root tissue, weakening the root system leading to lower yield. The IPM options of chemical insecticides and crop rotations had been the only two options available to growers prior to the development of the transgenic *Bt* approach. Cry3Bb1(*Bt*) crystal toxin variant was created to target the Western corn rootworm larvae, resulting in significantly improved control of the Western corn rootworm.

Transgenic approach has also provided control of insect pests that are generally difficult to manage with synthetic insecticides. One example is the management of Fall armyworm, which is an important pest of cotton and maize. In maize, Fall armyworm can cause major defoliation damages from feeding on the early stage leaf and on the ear, causing ear injury as the corn plants reach later stages of development. Before transgenic *Bt* approach, previous methods used to

manage Fall armyworm in maize and cotton were not very efficient. The spatial distribution and concealed locations of Fall armyworm eggs and small larvae in maize and cotton can make detection and control with foliar insecticides difficult (Siebert et al. 2008). Maize hybrids and cotton varieties expressing Cry1F (*Bt* trait) have demonstrated economical levels of efficacy against Fall armyworm in field and laboratory studies, according to evaluations by Siebert et al. (2008).

Transgenic *Bt* approach has been widely used in the last 20+ years as an effective biotechnological method of insect pest management, especially in the field of Agriculture. It has provided significant environmental and economic benefits. But the sustainable use of the transgenic *Bt* technology is being threatened by the rapid evolution of insect resistance to *Bt* traits (Carrier et al. 2017).

Transgenic *Bt* Resistance

Insects evolving resistance to *Bt* crops has been one of the biggest challenges the transgenic *Bt* approach has faced. Since the first detection of field-evolved insect resistance to *Bt* crops, starting in the early 2000s, large parts of the Agricultural biotech research have been focused on resistance management with the objectives of finding ways to halt the progression of resistance or finding other approaches that can potentially replace or aid the transgenic approach.

Table 1. Seven cases of field-evolved practical resistance to single-toxin *Bt* crops (Carrière et al. 2016)

Insect	<i>Bt</i> crop	<i>Bt</i> Toxin	Country	Years of Durability	Year of initial detection
<i>Helicoverpa zea</i> (Corn Earworm)	Cotton	Cry1Ac	USA	6	2002
<i>Busseola fusca</i> (African Maize Stalkborer)	Corn	Cry1Ab	South Africa	6	2004
<i>Spodoptera frugiperda</i> (Fall Armyworm)	Corn	Cry1Fa	USA	3	2006
<i>Pectinophora gossypiella</i> (Pink bollworm)	Cotton	Cry1Ac	India	6	2008
<i>Diabrotica v. virgifera</i> (Western corn rootworm)	Corn	Cry3Bb	USA	6	2009
<i>Diabrotica v. virgifera</i> (Western corn rootworm)	Corn	mCry3A	USA	4	2011
<i>Spodoptera frugiperda</i> (Fall Armyworm)	Corn	Cry1Fa	Brazil	2	2011

Since the first detection of field-evolved insect resistance to *Bt* traits, several resistance management strategies have been identified, but the “Refuge” strategy and the theory of stacks (Pyramided) have been the two most used resistance management strategies. “Refuge” is non-*Bt* host plants or an area of the same crop that does not contain the *Bt* traits. The concept of the Refuge strategy is to reduce heritability of resistance genes by providing refuge that produces susceptible adults and promotes mating between resistant and susceptible adults to decrease the dominance of resistance (Tabashnik et al. 2004). The refuge area serves as a source of wild-type (non-mutant) insects. To implement the Refuge strategy, farmers must plant refuges of non-*Bt* host plants near the *Bt* crops, expecting that the resistant adults emerging from *Bt* crops will mate with susceptible adults emerging from the refuge plants to produce non-resistant offspring (Fig. 1.) and therefore delaying the development of resistance. This is dictated by the law of genetics (“*Bacillus thuringiensis*” n.d.).

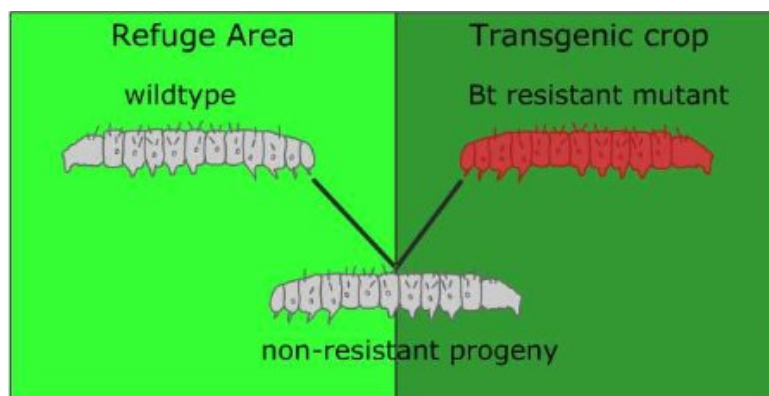


Fig 1: Concept of Refuge Strategy. **Image Source:** University of California San Diego.

The Environmental Protection Agency (EPA), in the United States, requires the farmers to plant at least 20% of refuge area within their corn acres (“*Bacillus thuringiensis*” n.d). The Refuge strategy has shown success in delaying resistance to *Bt* crops; however, this is mostly in pests

with already high susceptibility to *Bt* toxins. In order to sustain the efficacy of Refuge strategy against pests with lower susceptibility to *Bt* toxins, larger refuges are needed and *Bt* crops must be integrated with other pest management tactics (Carrière et al. 2016).

In pyramided theory, transgenic plants are engineered to carry and express two or more different types of *Bt* crystal toxins effective against the same target insect pest. This model suggested that since there are at least two modes of action, plants expressing two or more different *Bt* toxins genes have the potential to delay resistance more effectively than plants expressing a single-toxin (Zhao et al. 2003). One of the earlier case studies to test these predictions included a greenhouse study by Zhao et al. (2003) using transgenic *Bt* broccoli plants and an artificial population of Diamondback moths, *Plutella xylostella* (Lepidoptera: Plutellidae), carrying genes for resistance to the *Bt* toxins (Cry1Ac and Cry1C). After 24 generations of selection, resistance in pyramided (two-gene) plants was significantly delayed as compared with resistance to single-gene plants. Following were many other lab studies confirming delayed resistance in pyramided crops.

Though many controlled studies have proven the effectiveness of the pyramided plants and the approach has been widely used to delay evolution of pest resistance, the field performances have not consistently offered the desirable level of resistance management. Cross-resistance and antagonism between toxins used in pyramids are common (Carrière et al. 2015) and has been suggested to constitute, in part, some of the contributing factors for the limited field performances of the pyramids.

Another used resistance management strategy (often used in conjunction with the Refuge and the Pyramided strategy) is the “Seed Mixture,” also known as “Refuge in the Bag.” The “Seed Mixture” approach is when the seeds of *Bt* plants and the seeds of non-*Bt* plants are randomly

mixed in a bag of seeds and planted within a field side-by-side. One of the benefits of this approach is to help enforce farmers' compliance with the Refuge strategy.

While the Refuge and Pyramided approaches have shown some degree of success in managing evolved resistance, the insect resistance to transgenic *Bt* has not been completely resolved and has continued to spread. Consequently, newer biotechnological approaches are needed and are being pursued to potentially replace or be used in conjunction with the transgenic *Bt* approach for a more effective insect pest management, especially in the field of Agriculture – this is where the emerging new biotechnology like gene editing can play a major role.

III. Gene Editing Approach – RNAi; CRISPR- Cas9 Gene Drive system

Insects developing resistance to the *Bt* trait is an issue that has been threatening the Agricultural biotech industry, leading to biotech companies tirelessly working to identify new, cost effective and environmentally sound approaches to insect pest management, or approaches to overcome the issue of insect resistance. Gene editing has become the newer, lead technology in the biotech industries in identifying newer biotechnological approaches to insect pest management. Gene editing, also known as genome editing technology, basically takes advantage of the cell's own natural mechanisms to effectively alter the function of a gene. Genome editing is an approach in which a specific target DNA sequence of the genome is altered by adding, removing, or replacing DNA bases (Bortesi and Fischer 2014).

RNA interference (RNAi) Approach

In the past years, the RNA interference (RNAi) gene editing approach for gene silencing has been extensively researched and incorporated into the pest management and crop protection

programs. RNA interference is a gene silencing phenomenon that involves introducing a double-stranded RNA (dsRNA) into a cell to result in suppression of undesired genes and in specific cases desired, novel genes to be expressed (Kamthan et al. 2015).

The hopes for the RNAi approach were to be able to control a wider range of insects, especially the sap-sucking insects, which transgenic crops have not been able to control. Transgenic *Bt* toxins are limited to acting only in the mid-gut of susceptible target insects, mostly in Lepidoptera and Coleoptera larvae, leaving other insect orders unmanaged.

The concept of RNAi approach for insect control is for the insect to uptake the double-stranded RNA (dsRNA) into its system through feeding on plants expressing the dsRNA hairpin or through other means; with the expectation for the dsRNA to spread throughout all the cells in the body of the target insects. But achieving this effect is highly dependent on the delivery methods, as gene silencing is only limited to the cells that are affected (Katoch et al. 2013). Various delivery methods have been studied in different groups of organisms; these delivery methods include injection method, feeding, soaking, and transgenic plants expressing dsRNA (Katoch et al. 2013). The Injection method involves direct injection of dsRNA into target tissues – this method was found to be delicate and time consuming. Feeding/ingestion method involves uptake of dsRNA through insects feeding on transgenic plants expressing hairpin RNA – but the efficiency of RNAi by ingestion of dsRNA varies between different species. Also, after oral delivery, it's hard to determine the amount of dsRNA taken in by the insect, and in addition, it requires a greater amount of material for delivery (Surakasi 2011 as cited in Katoch et al. 2013; Chen et al. 2010 as cited in Katoch et al. 2013). The soaking method involves soaking the

organism (insect) in a dsRNA solution; however, the method is suitable only for specific insects and of developmental stages that readily absorb dsRNA from the solution (Katoch et al. 2013).

RNAi technology has been shown to be successful in different insect orders in knockdown of target genes, including successful effects on *Diabrotica v. virgifera*, corn rootworm larvae.

However, the technology is still being researched with current limitations, making it less sustainable as an effective approach for insect pest management.

Gene Drive Approach

Gene Drive refers to gene editing technology that enhance the probability that a specific gene will be inherited by an organism's offspring by bias inheritance (Lei et al. 2016). When an organism is engineered, the alterations made almost always harm its ability to reproduce in the wild. But if the same change is embedded within a gene drive, the inheritance advantage conferred by the drive seem to counterbalance the harmful effects (Esvelt n.d.). The idea of Gene Drives was inspired by the behavior of a class of natural genes, termed selfish genetic element, also known as "selfish genes." Selfish genes are genetic segments that can enhance and promote their own inheritability into the next generation (McFarlane et al. 2018), even if they do not confer any advantages to the organism. Gene Drive technology is essentially the use of engineered selfish genes that uses site-specific endonucleases to spread traits into populations (Hammond et al. 2016), thus Gene Drive can spread a particular gene throughout a population of the same species.

The Gene Drive approach has been identified as a potential new biotechnological strategy to insect pest management, especially for disease vectoring insects and potentially for Agricultural

insects and other invasive insect species. Gene Drives only affect sexually reproducing organisms, making it a suitable technology for managing/eliminating disease carrying/transmitting insects. The approach could also be used to potentially delete the resistance genes in insects, therefore resolving the issue of insect resistance (to the transgenic *Bt* or to insecticides).

Gene Drive technology works by tricking the rules of natural inheritance – under the law of natural inheritance (Mendelian inheritance), all sexually-reproducing organisms will inherit 50% of their genetic makeup from each of their parents. This means a particular gene only has 50% chance of being transmitted from a parent to the offspring. Gene Drive technology interferes with this process to find the wild-type version of the targeted gene and replacing it with the desired/altered gene (the Gene Drive), ensuring that more than half of the offspring will inherit the desired gene.

Gene Drive modification is achieved by attaching Gene Drives to a chosen/altered gene and delivering into an organism's genome. Thus, when an organism carrying the Gene Drive goes and mates with the wild-type organism, the wild-type version of the gene that would have been inherited from the wild-type parent will get identified and molecularly cut out (cleaved). Then the cell will repair the damage by copying the altered version of the gene (the Gene Drive) used as a template to patch up the damaged cell. The organism will now have two copies of the same gene (one in each chromosome) to pass on to the next generation. This same process will be repeated with each generation, resulting in the altered gene spreading through the population ("What's a Gene Drive?" 2015).

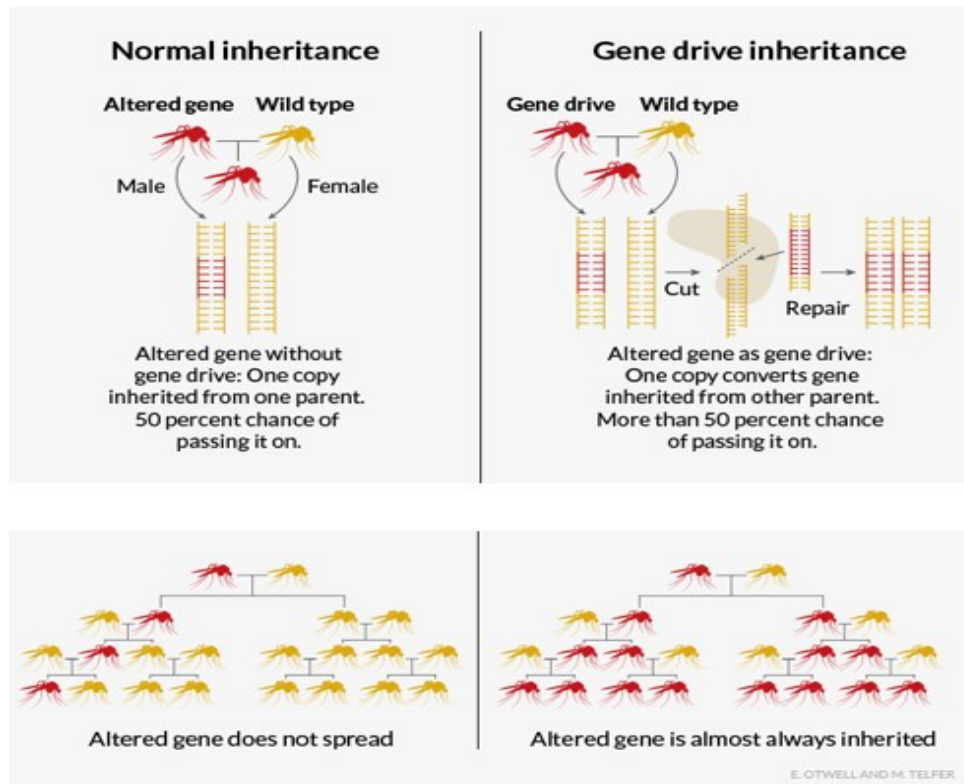


Fig. 2. Gene Drive Concept. **Image source:** E. Otwell and M. Telfer.

Unfortunately, Gene Drive technology on its own, without the CRISPR-Cas9 system, is not as effective and vice versa. Gene Drive and CRISPR-Cas9 are basically two separate technologies with the same objective, which is making gene alterations using gene editing technology to create populations of organisms that carry a desired/alterred gene. CRISPR-Cas9 technology allows scientists to make precise changes (gene alteration) to DNA, in the lab. Gene Drive technology allows scientists to push those changes through a wild-type population (“The bold plan to end malaria with a gene drive” 2018).

Prior to CRISPR-Cas9, Gene Drive technology had two major setbacks restricting its success – 1) it is hard to achieve precise, desired changes in the genome; and 2) after eventually making the desired change, the problem remains as to how to spread these changes quickly into the wild

population without having to release very high numbers of modified individuals into the wild population to have desired impact (“The bold plan to end malaria with a gene drive” 2018). Likewise, CRISPR-Cas9 technology is able to achieve precise changes in the genome and any changes made are self-propagating (able to replicate itself into every proceeding generation), but with no means to pushing these precise changes throughout the wild population without having to release very large numbers of edited individuals into the environment. However, combining the two technologies allow scientists to now have both the ability to make precise changes in the genome of almost all sexually reproducing organisms and an effective means of pushing those changes into a population. This is achieved through CRISPR-Cas9 enabled Gene Drive technology. The technology practically addressed the setbacks of both technologies, Gene Drive and CRISPR-Cas9, making it a very powerful tool in the genome editing technology.

CRISPR-Cas9 Enabled Gene Drive Approach

The CRISPR-Cas (clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas)) system is an adaptive immune system of bacteria and archaea that protects them against invading viruses (Bortesi and Fischer 2015). In the most recent years, CRISPR-Cas9 has emerged as the newest gene editing technology and as a powerful tool in the biotechnology industries, leading to numerous research work being currently conducted to explore its potential uses in insect pest management for Agricultural insects, plant protection and management of the insects that carry/transmit diseases to human. The CRISPR-Cas9 system is essentially a tool to improve the effectiveness of Gene Drives.

CRISPRs had first been described in 1987 by Japanese researchers as a series of short direct repeats interspaced with short sequences in the genome of *Escherichia coli* (*E. coli*) (Ishino et al.

1987 as cited in Doudna and Charpentier 2014). In the mid-2000s, a few laboratories began investigating CRISPRs and CRISPRs were later detected in numerous bacteria and archaea (Mojica et al. 2000 as cited in Doudna and Charpentier 2014). Before CRISPR-Cas became well known (prior to 2013), artificially engineered hybrid enzymes – zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), were the earlier tools for genome editing. Using these gene editing tools, scientists could target specific genomic sites to be altered (Bortesi and Fischer 2015, Enzmann 2018). Even though ZFN and TALEN methods enable the site-specific insertion of gene drives, they still have significant setbacks that prevent them from being widely adopted. ZFNs has limited target sites, is expensive and difficult to assemble (Lei et al. 2016). TALENs were easier than ZFNs to produce and validate according to Doudna and Charpentier (2014). However, difficulties of protein design, synthesis, and validation also remained as a barrier to its widespread adoption. CRISPR-Cas9 system has shown to be more efficient and less time-consuming compared with ZFNs or TALENs (Kumar and Jain 2014).

The CRISPR-Cas9 system presents a faster, cheaper and more efficient means for genome editing – the system allows scientists to insert, delete, replace or regulate genes in many different species, generating heritable, targeted mutations with precision that was previously not possible (“FAQ: Gene Drives” n.d; Ricroch et al. 2017). One of the main practical advantages of CRISPR-Cas technology compared to ZFNs and TALENs is the ease of multiplexing, which is the simultaneous introduction of double-stranded breaks (DSBs) at multiple sites in the genome that can be used to edit several genes at the same time (Li et al. 2013 as cited in Bortesi and Fischer 2015).

IV. CRISPR-Cas9 Mechanisms and Methods for Delivery into the organism

In bacteria, the CRISPR system is triggered after the bacterium detects the presence of virus DNA, and as part of the immune system response, guide-RNA molecules are produced to contain sequencing that matches the DNA sequence of the invading virus. The matching sequence is generated by obtaining a small piece of the invading virus's DNA to build the matching RNAs (like in the immunization process), then the guide-RNAs form a compound with the Cas9 protein (endonuclease enzyme) (Genome Editing with CRISPR-Cas9 2014). The guide-RNA and the Cas9 protein are the two main components of the CRISPR-Cas9 system – the two elements work together to form the DNA case, which can copy and paste itself into a specific position (the target site) within the genome (Courtier-Orgogozo et al. 2017). Cas9 protein is a scissor-like protein, sometimes referred to as molecular-scissors, whose job is to cut the DNA; guide-RNAs are molecules responsible for guiding the Cas9 to a specific site in the genome where the cut should be made, to cut out the old code and place in the new. CRISPR-Cas9 system is referred by some as a sophisticated biological “search/replace” system (“What's a Gene Drive?” 2015). Note that, as part of bacterial immune system, once the CRISPR cuts the matching sequence in the virus DNA, the virus will die, but in a eukaryotic cell, once a DNA is damaged, the cell repair is activated to repair the damage (Doudna 2017).

CRISPR-Cas9 genome editing is carried out when the Cas9 and the especially designed guide-RNA molecules complex together and run/scan along the strands of DNA, in the genome of an organism, seeking out matching sequences of genetic code using guide-RNA as a template (“What's a Gene Drive?” 2015). Once the spot where the guide-RNA match is found, the guide-RNA inserts between the two strands of the double-helix and rips them apart (creating the double-strand break), which triggers the release of the Cas9 protein to initiate the cut, precisely in that location, cutting out the double stranded helix on both sides of the DNA (“What's a Gene

Drive?" 2015). Once this happens to a cell, the cell's natural response is to initiate cell repair. The cell repair process has two repair pathways to repair a damaged DNA (non-homologous end joining (NHEJ) and the homologous recombination (HR) repair pathways). In the non-homologous end joining repair pathway, the cell essentially just grabs the two broken ends of the DNA and essentially sews them back together (Jorgensen 2016). During the cell repair process is where the CRISPR-Cas9 system is used to interfere with the process through homologous recombination (HR) repair pathway. In the homologous recombination (HR) repair pathway, after the cut is made to the matching sequence in the DNA, the cell repairs the cut by using the chromosome containing the drive cassette (guide-RNA, Cas9 enzymes and the altered gene) as a template; as a result, the drive-containing chromosome is successfully copied into the homologous wild-type chromosome (the damaged chromosome), completely replacing the wild-type DNA sequence at that position of the genome (Enzmann 2018) and consequently turning any heterozygous into homozygous.

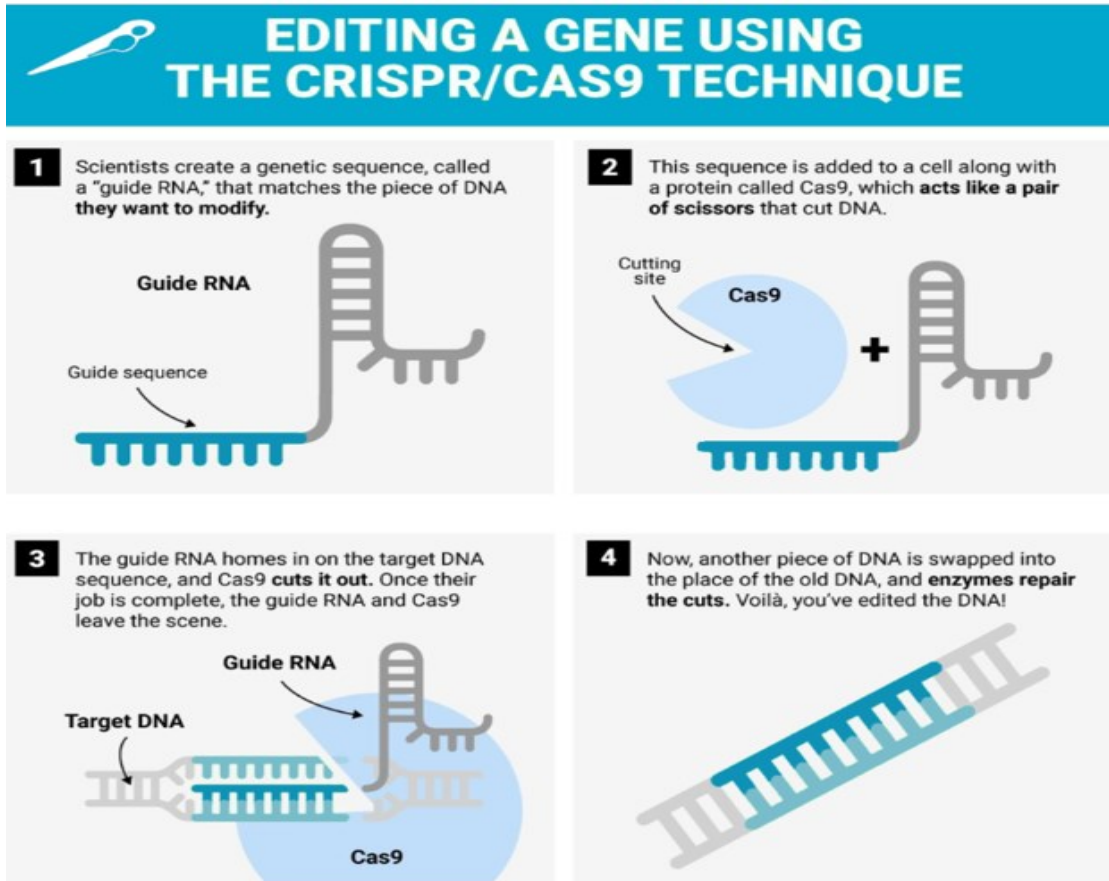


Fig. 3: CRISPR /Cas9 Gene Editing Mechanism (ZeClinics n.d.).

Image source: Nature News: Carl Zimmer.

The method relies on the fact that double-strand breaks are most frequently repaired by the homologous recombination (HR) repair pathway, (in the presence of a homologous DNA template), rather than the non-homologous end joining (NHEJ) pathway (Heyer et al. 2010), which is one of the limitations of the CRISPRs-Cas9 system that will be discussed later in this review. Also, the copying of the drive allele into wild-type chromosomes will only occur if the cell uses the homology-directed repair pathway to repair the double-strand break (Enzmann 2018). In addition, the homologous recombination (HR) repair pathway is the most suitable repair process scientists can adopt because of the homology in both ends of the broken cell and difference at the center – scientists can feed the center with the altered/desired piece of DNA

(Jorgensen 2016), creating desired mutations and other complex alterations, bypassing the mechanisms of natural evolution, and producing the desired DNA sequence that will be inherited by succeeding generations.

What makes the CRISPR-Cas9 mediated Gene Drive so efficient? In nature, one gene usually has many different versions of itself, thus when parents with different versions of a gene pass on their DNA to their offspring, each version gets inherited by only half of the offspring (Empinado 2015). In the CRISPR-Cas9 enabled Gene Drive process, the guide-RNA-Cas9 molecules allow the drive cassette to continue the “search/replace” activity within the genome until all versions of the target gene (wild-type versions of the gene) are identified and altered. This ensures that when a CRISPR-Cas9 altered organism mates with the wild-type/unaltered organism, of the same species, theoretically all its offspring will inherit the altered gene along with the CRISPR code (the guide-RNA-Cas9) that enable precise changes at the desired sites in the genome, ensuring that the desired alterations are passed on to every future generations (“What's a Gene Drive?” 2015). Researchers suggest that in theory, the release of just a few individuals within a population could lead to complete invasion of the Gene Drive cassette within 15–20 generations (Burt 2003 as cited in Courtier-Orgogozo et al. 2017).

Method of Delivery into the Organism

For a given application, scientists must choose adapted delivery methods and strategies to fulfill their objectives. For Agricultural applications, conventional *Agrobacterium*-mediated transformation using plasmid vectors containing Cas9 and guide-RNA is mainly used to deliver the CRISPR-Cas9 system into plants (Ricroch et al. 2017). The currently used delivery methods for delivery into insects include microinjections of Gene Drives into the eggs or embryos of the

target insect species. Research is still underway to identify more efficient delivery methods of the CRISPR-Cas9 system into the organisms.

V. CRISPR-Cas9 Applications in Agriculture and Medicine – for Insect Pest Management and Crop Protection

CRISPR-Cas9 can be used in numerous applications in Agriculture and the medical fields for management of insect pest and for crop protection. CRISPR-Cas9 technology can be used to build drives, for example, to block harmful pest behaviors, like swarming of *Locusta migratoria*, migratory locust (Orthoptera: Acrididae) and to sensitize pests – to make them susceptible to specific compounds, like insecticides. Drives can be built to alter or eradicate vector-borne insects, and even to eventually learn how to reprogram olfaction in insects to discourage crop consumption or to make mosquitoes dislike the way we smell (Esvelt 2015).

Agricultural Applications

One of the first reported applications of the CRISPR-Cas9 system in insects was carried out in *Drosophila*, fruit flies (Diptera: Drosophilidae), in which mutations were successfully introduced to the yellow gene (Gratz et al. 2013 as cited in Lei et al. 2016). Another successful application of the system in *Bombyx mori*, silkworm (Lepidoptera: Bombycidae), was reported, where BmBLOS2 gene was targeted (Wang et al. 2013 as cited in Lei et al. 2016), followed by other successful applications.

In a case study by Garczynski et al. (2017), CRISPR-Cas9 gene editing system was used to edit the genome of *Cydia pomonella*, Codling Moth (Lepidoptera: Tortricidae), targeting a specific gene (CpomOR1) to affect egg production and viability. Codling moth is a major, global pest of

pome fruit. The CpomOR1 gene is an odorant receptor belonging to the pheromone receptor subfamily in codling moth. Single-guide RNAs (sgRNAs), targeting nucleotides of the CpomOR1 gene, were built and injected into the early stage eggs of the codling moths. Observations showed that mutations were successfully introduced, including both deletions and insertions (Garczynski et al. 2017). The study reared emerging neonates to adulthood, and attempted to create stable populations of edited codling moths by mating males with females carrying mutations of the CpomOR1 gene. Results showed that fecundity and fertility were affected, resulting in edited females producing nonviable eggs. However, the exact role of CpomOR1 in fecundity and fertility in codling moths is currently not exactly understood.

In another case, the CRISPR-Cas9 system was used to induce a targeted heritable mutagenesis of the migratory locust. Locusts are important Agricultural pests worldwide, and their swarming behavior can cause damages to crops over wide areas at a time, often resulting in serious economic implications. In a study by Li et al. (2016), a target sequence of guide-RNA was designed to disrupt the gene encoding of the odorant receptor co-receptor (Orco). The authors then examined the roles of the odorant receptor pathway in the locust. Observations demonstrated an efficient target-gene editing, resulting in Orco gene mutants to show impaired electrophysiological responses to multiple odors, which resulted in mutant locusts to lose an attraction response to aggregation pheromones under the crowding conditions (Li et al. 2016).

For crop protection, CRISPR-Cas9 systems can be used to give crops enhanced tolerance to certain stressors (biotic and abiotic) and enhance plant quality. We know that many insects are attracted to unhealthy, diseased plants, thus maintaining good plant health is part of an Integrated Pest Management program. CRISPR-Cas9 systems can be used to edit plants such that the plant

emits or does not emit specific enzymes that can deter insect pests from contacting the plant or attracting certain insect predators to prey on the insect species that are attacking the plant.

To achieve plant viral disease resistance, for example, one of the strategies is to integrate the CRISPR-coding sequence into the host plant's genome to target and interfere with the virus genome once the plant is infected, acting as a CRISPR-like immune system in the genome of the host (Shen et al. 2018; Xu et al. 2016 as cited in Ricroch et al. 2017). According to Ji et al. (2015), a transient assay performed in *Nicotiana benthamiana* (Tobacco plant) using a CRISPR-Cas-based approach demonstrated resistance to the Gemini virus, beet severe curly top virus.

CRISPR-Cas9 systems can also be used in conjunction with transgenic technology to engineer crops that are resistant to many other plant diseases – this can be achieved through molecular breeding technology. For example, if we have a corn variety with a good yield potential (**Fig. 4. Plant B**) but this variety is also very susceptible to a plant disease, we can use CRISPR-Cas9 technology to engineer a guide-RNA with the resistance gene (from plant A) (**Fig. 4. Plant A**) and send it to find the susceptible gene in the genome of plant B, have the Cas9 cut out the susceptible gene and replace it with the resistance gene to obtain the desired corn variety (**Fig. 4. plant C**) (“CRISPR-Cas in Agriculture” 2017).

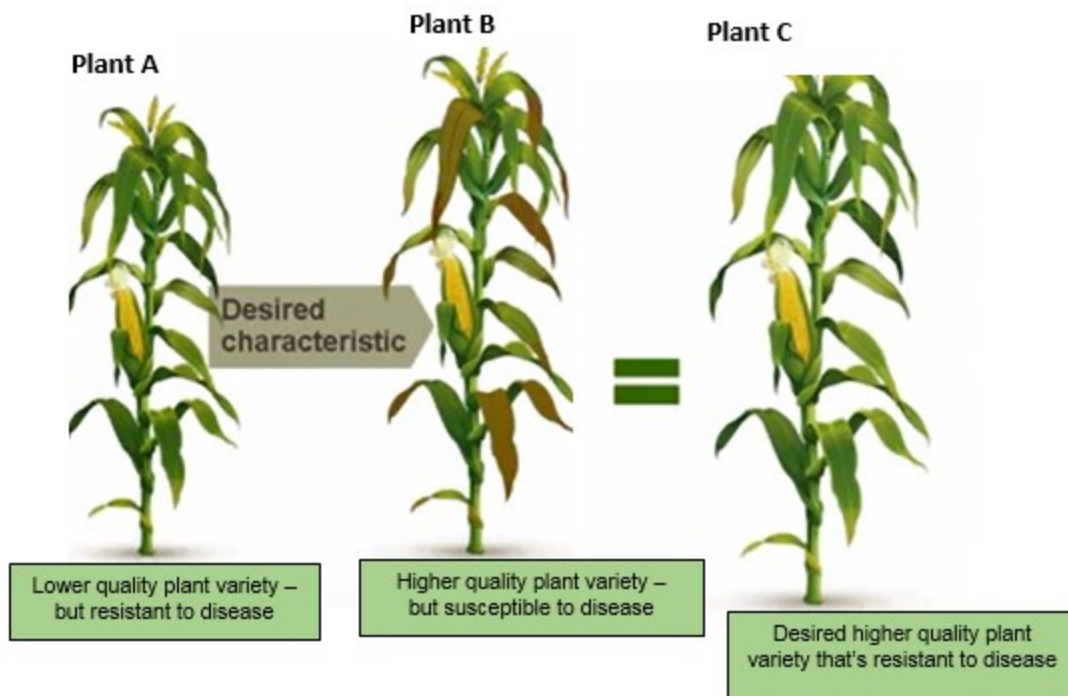


Fig. 4: CRISPR/Cas9 Mediated Transgenic Corn. **Plant Image** Source: National Press Foundation.

Applications for Managing Pests of Medical Importance

There are several opportunities for using CRISPR-Cas9 enabled gene editing techniques for effective management of insects that carry/transmit diseases to human and/or domestic animals.

The technique can be used to eradicate vector-borne diseases (malaria, dengue, Lyme disease West Nile virus, Zika Virus and other vector-borne diseases). Vectors can be altered to prevent them from carrying and transmitting diseases or directly eradicating their population.

CRISPR-Cas9 systems targeting female reproduction in the *Anopheles gambiae*, malaria mosquito vector (Diptera: Culicidae) was studied by Hammond et al. (2016). The team identified three genes (AGAP005958, AGAP011377 and AGAP007280) that are responsible for a

recessive female sterility in the *Anopheles gambiae* mosquitoes. CRISPR-Cas9 Gene Drive was designed to target and edit each of the genes. For each targeted locus, a strong Gene Drive was observed at the molecular level with transmission rates to progeny of 91.4 to 99.6% (Hammond et al. 2016). Additionally, the study showed that a CRISPR-Cas9 system targeting one of these loci, AGAP007280, meets the minimum requirement for a Gene Drive targeting female reproduction in an insect population. In another study by Gantz et al. (2015), the CRISPR-Cas9 system was developed to target *Plasmodium falciparum*, the human malaria parasite, in *Anopheles stephensi*, Asian malaria vector mosquitoes (Diptera: Culicidae), and successfully inactivating genes required for malaria parasite growth.

Other controlled studies using the CRISPR-Cas9 system to target *Aedes aegypti*, the yellow fever mosquito (Diptera: Culicidae), were completed to explore new ways to interrupt the viral disease cycles. The yellow fever mosquito is the primary vector for arboviruses that cause yellow fever, dengue, and chikungunya, which cause significant mortality and illnesses among people living in tropical regions of the world (Dong et al. 2015). A study by Kistler et al. (2015) showed that CRISPR-Cas9 can be used to engineer precise “loss-of-function” mutations, allowing for detailed genetic study of yellow fever mosquito vector. This offers scientists the knowledge of possible CRISPR-Cas9 applicability in genome modifications (Kistler et al. 2015).

Species-oriented population suppressions can be achieved using CRISPR-Cas9 systems to build and deliver deleterious Gene Drives to make genetic changes that will essentially cause the population to crash, or creating Gene Drives to bias population towards one sex (McFarlane et al. 2018). If the Gene Drive eliminates a female-specific, or male-specific genes essential for reproduction, for example, this can in theory lead to extinction of the specie (Burt 2003 as cited

in Courtier-Orgogozo et al. 2017). Other more complex alterations that can be achieved through CRISPR-Cas9 enabled Gene Drive systems include the possibility of making a population be uniquely vulnerable to otherwise harmless compounds (sensitize the population), thus allowing the development of species-specific insecticides for example. Another potential alteration includes splitting a population into reproductively incompatible groups (speciate the population) (Esvelt 2015).

Eradication of vector-borne diseases (diseases spread by insects) has been a common focus of gene drives, with special interest in developing a drive that could eradicate malaria, which is caused by *Plasmodium* parasites and transmitted by *Anopheles* mosquitoes (Enzmann 2018). Malaria kills hundreds of thousands of people each year in certain parts of the world. Researcher groups have already developed and tested CRISPR-Cas9 gene drives to spread malaria-resistance genes and drives to induce female sterility in the population of laboratory contained mosquitoes.

Reversibility – The CRISPR-Cas9 is a reversible system; this means if scientists edit a gene using the CRISPR-Cas9 technology and later don't like it, the change can be reversed by creating another CRISPR-Cas9 Gene Drive to undo the change. Esvelt (2015) indicated that such reversal has already been demonstrated in a lab experiment with yeast where the *HN3* genes was spread with Gene Drive through the lab yeast population and then a reversal Gene Drive was built that successfully undid that particular change and restored the gene that had been broken. But it's important to note that the reversal will not undo any ecological changes that resulted from the original edit (Esvelt 2015).

VI. CRISPR-Cas9 Limitations

One of the main limiting factor of the CRISPR-Cas9 technology is the fact that scientists don't yet completely understand how cells work - for instance, it's still unclear to scientists as to why some guide-RNA work well and some don't, or why some cells use one type of the repair pathway over the other (Doudna 2015). Though, we know that the CRISPR-Cas9 system relies more on the fact that cells use the homologous recombination (HR) repair pathway more frequently to repair double-strand breaks, (in the presence a homologous DNA template) rather than the non-homologous end joining (NHEJ) pathway. However, we don't know why the cell does this. Studies have shown that the frequency with which the cell chooses one repair pathway vs. the other varies between organisms, cell types and between developmental stages of the organisms (Esvelt 2015).

One of the most significant limitations of the CRISPR-Cas9 system is the appearance of certain off-target effects, pointed out by Crauciuc et al. (2017), although in a small percentage compared to previous techniques. Scientists need to understand better how to control the way DNA is repaired after it's been cut/broken and figure out how to control and limit any off-target or unintended effects (Doudna 2015). Another limitation of the CRISPR-Cas9 system is the issue of getting the system into the cell successfully (delivery method); the currently used methods are still not very effective (Gantz and Akbari 2018).

VII. CRISPR-Cas9 Potential Issues and Concerns

Currently most of the research work on CRISPR-Cas9 mediated gene editing technology are being conducted in the confinement of laboratories and have been shown to work in controlled

populations of fruit flies, mosquitoes, yeast (Noble et al. 2018) and other species. But as researchers are looking forward to applying the system into the environment, both the researchers and the public have highlighted the potential issues we could face as the result of the CRISPR-Cas9 Gene Drive system application into the environment.

To address such concerns, the World Health Organization (WHO) has outlined the steps that gene-drive Genetically Modified (GM) mosquitoes should go through before being deployed into the environment (“The bold plan to end malaria with a gene drive” 2018) – (Fig.5). Currently, Gene Drive research is in phase 1.

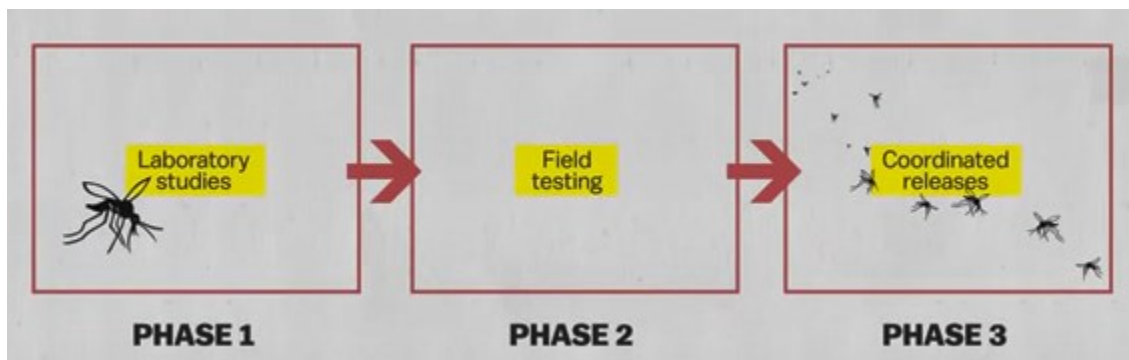


Fig. 5: WHO Phased Testing Pathway. **Image source:** Vox (2018)

Ecological impacts – These have been at the center of most discussions concerning the potential side effects of the CRISPR-Cas9 Gene Drive technology. The most discussed issue includes the potential for Gene Drives disrupting the ecosystem and thus creating unforeseen risks to other species, potentially including humans. This could happen through the mechanism of “gene-flow,” which could potentially allow drives to move beyond their target population (Esvelt 2019). Another often debated issue about the use of the CRISPR-Cas9 technology in insect management includes the concerns about potential consequences of eradicating mosquito

species, for example. Though we don't know of many real benefits mosquitoes provide to humans or to the environment, but we know that bats and some fish species use mosquitoes as a food source. Therefore, with the disappearance of mosquitoes, bats could be forced to find alternate food sources, find different niches to occupy or be forced to share niches with another species and potentially, indirectly affecting humans. Also, mosquitoes could be pollinators of some plants that we don't even know about until they disappear.

Another potential issue with the technology is the potential for the unexpected, undesired mutations that can occur – a mutation could occur in the process, which has the potential to allow in unwanted traits and therefore, potentially affecting other aspects of the ecology. Some off-target mutations can result in cell death or transformation (Zhang et al. 2014).

Ethical issues – One of the most controversial ethical issues associated with CRISPR-Cas9 technology involves its application in human and animals' reproductive cells. The United States currently does not have a ban on human reproductive cell modification (Beale 2016), nor is it regulated. However, many scientists have called for a pause on CRISPR-Cas9 work in human and animals' reproductive cells due to safety and ethical concerns (Beale 2016). In June 2016, the United States National Academies of Sciences, Engineering, and Medicine released a report on their recommendations for "Responsible Conduct" of Gene Drives ("Gene Drive Research in Non-Human Organisms: Recommendations for Responsible Conduct" 2016).

Concerns have been raised over the relationships that may exist between the use of CRISPR-Cas9 gene editing methods and Genetically Modified Organisms (GMOs). In the U.S.A., the legal status of CRISPR-Cas-induced mutations is that they are exempt from GMO laws (Ricroch et al. 2017) since the changes that are made in the gene editing process are very often ones that

could have occurred naturally without any genetic manipulations (Carroll 2017). This is unlike transgenic technology, which refers to the transfer of genetic material from one organism to another by means of genetic manipulation.

Many people fear that the current lack of regulations behind the CRISPR-Cas9 technology has presented great potential for the misuse of the technology, especially in human cells, to potentially design Gene Drives that will give genetically enhanced abilities (higher intelligence, superior muscular development, resistance to certain illnesses etc.) to the future generations of certain people. Or it could be misused to potentially create weaponized super mosquitoes (“What's a Gene Drive?” 2015) or any other dangerous animal or unethical looking creatures.

Conversely, many argue that misuse of the CRISPR-Cas9 system would not necessarily be productive, nor profitable, consequently discouraging its potential misuse. For instance, building drives to create super intelligent people – this could be counterproductive (after all, studies have shown that highly intelligent people are generally highly prone to depression).

Other ethical issues – Will it be fair for the mosquitoes, some ask. As far as we know, mosquitoes do not benefit from carrying these pathogens and therefore, altering the mosquitoes to not carry disease-causing pathogens will be fair, in the opinion of many. However, uses for the eradication of the mosquito species can be trickier to consider. Some ask if malaria is bad enough to risk it; some ask if it is ethical to not do it, given that millions of people are dying from malaria and other mosquito borne infections every year. Also, many wonder who exactly will be in charge of making such decisions – is it the scientists, the governments or the communities of those whom will be affected by the potential risks and/or benefits of the practice? (“What's a Gene Drive?” 2015).

VIII. Conclusion and Prospects

CRISPR-Cas9 enabled gene editing technology is still an emerging tool driving scientific efforts in exploring its full potential. The technology offers revolutionary efficiency in the field of genome editing. But before its full application for insect pest management or plant protection, extensive knowledge of the genome and gene functionalities of the target species is required. The application of the CRISPR-Cas9 gene editing system requires the precise definition of the target DNA sequence and the availability of good genome sequence data of the studied species to allow designs of single-guide RNAs (Ricroch et al. 2017). Understanding the homology-directed repair mechanisms that follow Cas9-mediated DNA cleavage is also needed to enhance insertion of new or corrected sequences into genomes (Doudna and Charpentier 2014).

The CRISPR-Cas9 system itself can be used as a tool to gain useful information about gene functions as mediated by RNA. Gaining these knowledges will allow scientists to apply the system with more precision, thus minimizing the off-target rate. In addition, further advances in our understanding and control of the technology will likely lead to continued optimization of the CRISPR-Cas9 technologies, allowing us to better understand and overcome potential limitations and risks (Bortesi and Fischer 2015; Gantz and Akbari 2018). With no doubt, CRISPR-Cas9 gene editing technology holds great potential for a more effective and permanent insect pest management.

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