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THE CRISPR/CAS MEDIATED GENOME EDITING: A NOVEL INSECT PEST MANAGEMENT STRATEGY

J. SURESH[1], R. NAGANNA[2], B.S.K.NIKHIL[3], S.SRINIVASA REDDY[4], AND C.NARENDRA REDDY[5]

[1]Department of Genetics and Plant Breeding, College of Agriculture, Rajendranagar, PJTSAU, India
[2] ICAR-IIRR, Rajendranagar, Hyderabad, India
[3] Dept of Genetics and Plant Breeding, College of Horticulture, Mojerla, SKLTSHU, India
[4] Department of Entomology, College of Agriculture, Palem, PJTSAU.
[5]College of Agriculture, Rajendranagar, PJTSAU, India

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Abstract–The recent development of gene-editing technologies, such as Clustered Regularly Interspaced Short Palindromic Repeats and associated protein (CRISPR/Cas), opened new avenues for the development of novel pest control measures. The CRISPR/Cas9 system acts via a ribonucleoprotein complex, where the target recognition lobe of Cas9 directs specific binding to target DNA through interacting with homologous sgRNA and the excision lobe cuts the DNA. CRISPR-containing organisms acquire DNA fragments from invading bacteriophages and plasmids before transcribing them into CRISPR RNAs (crRNAs) to guide cleavage of invading RNA or DNA. The efficiency of target editing is dependent on intrinsic factors specific to each species, the target gene sequence, and the delivery methods of CRISPR gRNA and the Cas nuclease. The CRISPR/Cas9 cargo is delivered in different formats for genome editing in cells; Cas9 may be delivered as a DNA or mRNA molecule encoding for the cas9 gene, or it may be delivered as a functional ribonucleoprotein (RNP) into insect body for the control. In cultures, CRISPR cargo is commonly delivered by physical or non-viral and viral delivery approaches *viz.,* physical approaches; lipofection, electroporation, nucleofection, microinjection and viral vectors such as lentiviruses, adenovirus, and adeno-associated virus (AAV) are broadly used as delivery vehicles of CRISPR cargo for efficient genome editing.

INTRODUCTION

Significant challenges face agriculture in the 21st century wherein there is a need to supply quality agricultural products to support rapidly increasing global human populations without harming the environment. The damage to agricultural commodities inflicted by insect pest species causes a reduction in producer output and profitability, thereby affecting product quality, such that the development of novel and effective insect pest control tactics remains a research challenge to the farmers and scientific community. Every year, in agriculture, farmers spending billions of dollars for pest management (Krattiger, 1996). Even though, up to 40% of a crop is lost due to the insect (Oerke, 2006). Indiscriminate use of Pesticides led to resistant to insects and affect the beneficial insect populations and many harmful effects to humans and the environment (Gunning *et al*., 1991; Gatehouse *et al*., 1994; Fitt, 1994 and Haq *et al*., 2004). Nowadays, newly emerged genome editing approaches, i.e., ZFN, TALEN and especially CRISPR/Cas9, have allowed for the efficient extension of genetic modification for the management of the insect pests. CRISPR/Cas9 (clustered, regularly interspaced, short palindromic repeat/CRISPR associated) is being developed as an attractive tool in genome editing. Due to its high specificity and applicability, CRISPR/Cas9-mediated gene editing has been employed in a multitude of organisms and cells, including insects, for not only fundamental research such as gene function studies, but also applied research such as modification of

organisms of economic importance. Despite the rapid increase in the use of CRISPR in insect genome editing, results still differ from each study, principally due to existing differences in experimental parameters, such as the Cas9 and guide RNA form, the delivery method, the target gene and off-target effects (Taning *et al*., 2017). In this regard, we briefly discussed the CRISPR/Cas9 system, its basic structures, mechanism, delivery methods and applications for the management of the insect pests.

Development of Genetic Manipulation in Insects

Genetic manipulation technologies in insects have been developed over the last 30 years. Combined with recent progress, these technologies can be classified into three stages of development based on their function mechanism, efficiency, and accuracy. The first stage was transposon-based transgenesis. The first successful attempt to transfer exogenous genes into insects was conducted in *Drosophila melanogaster* Meigen by P-element transposon (Rubin and Spradling, 1982). The second development stage of gene editing technologies such as ZFN (Zinc Finger Nuclease) and TALEN (Transcription Activator-Like Effector Nuclease), which are both composed of a specific DNA recognition protein (ZF or TALE) and DNA excision protein (FokI) expanded genetic modifications beyond model organisms (Bogdanove and Voytas, 2011). Compared with transposon-based technologies, ZFN and TALEN improved the efficiency and precision of targeted genetic manipulations, and have been successfully applied in Drosophila,Silkworm, and mosquitoes. The third and most promising stage of advancement has been the CRISPR/Cas9 system (Clustered Regularly Interspaced Short Palindromic Repeats). The CRISPR/Cas9 system acts via a ribonucleoprotein complex, where the target recognition lobe of Cas9 directs specific binding to target DNA through interacting with homologous sgRNA and the excision lobe cuts the DNA (Nishimasu *et al*., 2014). It is considered a revolutionary technology with high efficiency and accuracy and applicable for a wide range of species. The list of insects which genes are successfully modified by using the CRISPR/Cas9 system presented in Table 1.

The Natural Origin and Mechanisms of CRISPR Systems in Adaptive Immunity

Viruses have been a common threat to the survival of bacteria and archaea. As a result of this battle between predator and prey, an array of counter measures has been adopted by the host organism. The CRISPR system is an adaptive immune mechanism present in many bacteria and the majority of characterized Archaea genomes as a defense mechanism against invading viruses and plasmids (Barrangou and Marraffni, 2014). CRISPR-containing organisms acquire DNA fragments from invading bacteriophages and plasmids before transcribing them into CRISPR RNAs (crRNAs) to guide cleavage of invading RNA or DNA. The CRISPR/Cas technique acts in three stages: 1. Adaptation: insertion of new spacers in CRISPR locus, 2. Expression: transcription of CRISPR RNA (crRNA) and 3. Interference: Recognition and destruction of target DNA sequences (Jiang and Doudna, 2017).

CRISPR/Cas9 Structure

The CRISPR/Cas9 system is a complex composed of single guide RNA(sgRNA) and a 160kDa DNA endonuclease enzyme, Cas9, which cuts each strand of double-stranded DNA at a specific location, through its nuclease domains. The sgRNA-bound Cas9 endonuclease binds to a double-stranded DNA (dsDNA) upon site-specific recognition of a short trinucleotide Protospacer Adjacent Motif (PAM) within the DNA. Thereafter, the target DNA (tDNA) strand, which is complementary to the first 20 nucleotides (nt) of the sgRNA, forms an RNA-DNA hybrid duplex displacing the non-target DNA (ntDNA) strand from the dsDNA. The sgRNA (Figure-1C) is composed of two noncoding RNA components fused together: a CRISPR RNA (crRNA) that confers target specificity to Cas9 and a transactivating CRISPR RNA (tracrRNA) that can bind to Cas9. The crRNA contains the 20-nt long 'spacer' or 'guide' sequence at the 5'-end that forms the RNA-DNA hybrid, and a 'repeat' sequence at the 3'-end that forms a duplex with the tracrRNA. Since the crRNA cannot bind to Cas9 alone, it complexes with the tracrRNA and the resultant RNA: RNA duplex fits into Cas9. A sgRNA can be synthetically generated or obtained *in vitro* or *invivo* from a DNA template (Wang *et al*., 2016). Cas9, the RNA-guided endonuclease that cleaves target DNA in the class 2 type II CRISPR system, is the most widely used for genomic editing and regulation among the Cas proteins. The Cas9 endonuclease (Figure-1B**)** has two lobes: recognition (REC) lobe and nuclease (NUC) lobe, connected by an

Table 1. The list of insects genes are successfully modified by using the CRISPR/Cas9 system

Name of The Insect	Targeted Genes	Delivery Method	References
Ceratitis capitata	Eye Pigmentation Gene White Eye (We), Segmentation Paired Gene (Ccprd)	Microinjections: Solubilized Cas9 ribonucleoprotein complexes (RNPs) loaded with gene-specifc single guide RNAs (sgRNA) into early embryos.	(Angela *et al.*, 2017)
Anopheles stephensi	Dual Anti Plasmodium Falciparum Effector Genes	Microinjections: Embryos were injected with 100 ng/µL each of the plasmid, pAsMCRkh2, Cas9 protein, Cas9 dsRNA, and Ku70 dsRNA.	(Gantza *et al.*, 2015)
Anopheles gambiae	AGAP005958, AGAP011377 and AGAP007280	Microinjections:	(Andrew *et al.*, 2016)
Bactroceratryoni	*B. tryoni* White Eye Gene	Microinjections: The injection mixes were comprised of 0.3 µg/µl Cas9 protein, 0.2 µg/µl of each sgRNA and 1× injection buffer	(Choo *et al.*, 2018)
Plutellaxylostella	Abdominal-A homeotic gene (Pxabd-A)	Microinjections: CRISPR plasmid (300 ng/µl) for AGAP007280 (p16501) and AGAP005958 (p16505) or, for AGAP011377,	(Yuping *et al.*, 2016)
Locustamigratoria.	Odorant Receptor Co-Receptor (Orco)	Microinjection: mixture of Cas9-mRNA and Orco-Gma into the locust eggs	(Li *et al.*, 2016)
Agrotisipsilon	Yellow-Y Gene	Microinjection: Cas9 mRNA and sgRNA were mixed at nal concentrations of 300 ng/mL and 150 ng/mL, respectively.	(Chen *et al.*, 2016)
Spodopteralitura	Abdominal-A (Slabd-A) Gene	Microinjection: 5 nL of a mixture containing 300 ng/µL of Cas9 mRNA and 150 ng/µL each sgRNA	(Bi *et al.*, 2016)
Triboliumcastaneum	eGFP1	mRNA injection + DNA	(Gilles *et al.*, 2015)
Helicoverpaarmigera	HaCad	mRNA+ DNA	(Wang *et al.*, 2016)
Plutellaxylostella	Pxabd-A	mRNA+ DNA	(Huang *et al.*, 2016)

Arginine-rich linker. The REC and NUC lobes are responsible for association with the sgRNA and cleavage of the DNA, respectively. The NUC lobe consists of different domains: RuvC, HNH, and PAM-interacting (PI)(Jiang and Doudna, 2017). The RNA-DNA hybrid duplex, with a negatively charged backbone, is accommodated at the positively charged groove formed between REC and NUC lobes. The unwound ntDNA (u-ntDNA) strand is proposed to be hosted, before cleavage, at the HNH/RuvC boundary, through stabilizing electrostatic interactions between the negatively charged backbone of u-ntDNA and the positively charged amino acids of the HNH and RuvC domains. The HNH and RuvC domains perform site-specific cleavages of the tDNA and ntDNA strands, respectively, resulting in a double-strand break (DSB), thereby inducing the host DNA repair mechanisms.

Mechanism of CRISPR/Cas9 Genome Editing

Since its discovery, Cas9 has been extensively used for genome editing in multiple organisms. Cas9, like engineered ZFNs and TALENs, is a programmable, sequence-specific endonuclease. Similar to other nucleases, Cas9-mediated genome editing is achieved by a two-step process: DNA cleavage followed by DNA repair (Figure 2). The sgRNA directs Cas9 to a specific genomic locus where Cas9 creates a DSB, which triggers DNA repair through intrinsic cellular mechanisms, such as non homologous end joining (NHEJ) or homology-directed repair (HDR).NHEJ causes nearly random insertion and deletion mutations (i.e., indels) at the DSB site and, thus, may lead to gene knockout (e.g., by causing a shift in the target gene's reading frame or mutating a critical

region of the encoded protein). HDR can be exploited to generate the desired sequence replacement at the DSB site through homologous recombination guided by a donor DNA template, causing targeted gene deletion, mutagenesis, insertion, or gene correction. Thus, the CRISPR/Cas9 system provides a powerful platform for sequence-specific genome editing, including gene knockout, gene knockin, and site-specific sequence mutagenesis and corrections (Wang *et al*., 2016).

Delivery Methods of CRISPR/Cas9 System in Insect

Despite the great promise of the CRISPR-Cas9 technology, several challenges remain to be tackled before its successful applications. The greatest challenge is the safe and efficient delivery of the CRISPR-Cas9 genome-editing system to target cells in insect. The delivery must be specic to the desired tissues to prevent undesirable off-target gene editing events. A number of different approaches have been explored to address problem. The commonly used physical or non-viral and viral delivery approaches in eukaryote cell deliver methods are Electroporation, Microinjection, Induced transduction, Mechanical cell deformation, Hydrodynamic injection, Lipofection / Lipid Nanoparticles, Cell Penetrating Peptide (CPP), DNA nanostructure and Gold nanoparticles. These physical methods to disrupt cellular barriers, chemical modications that improve the cargo's delivery to evade the barriers and physical encapsulation of the cargo within a carrier molecule (Xu *et al*., 2019. In the laboratory environment, one of the most popular methods for achieving Cas9 expression in cell culture has been the utilization of viruses (Perera *et al*., 2018). Viruses have evolved to become extremely efcient at invading cells and inducing the expression of non-native proteins inside that host. Thevirus is able to enter a host cell and induce the expression of the transgene, but is no longer ableto replicate itself orspread to new cells.

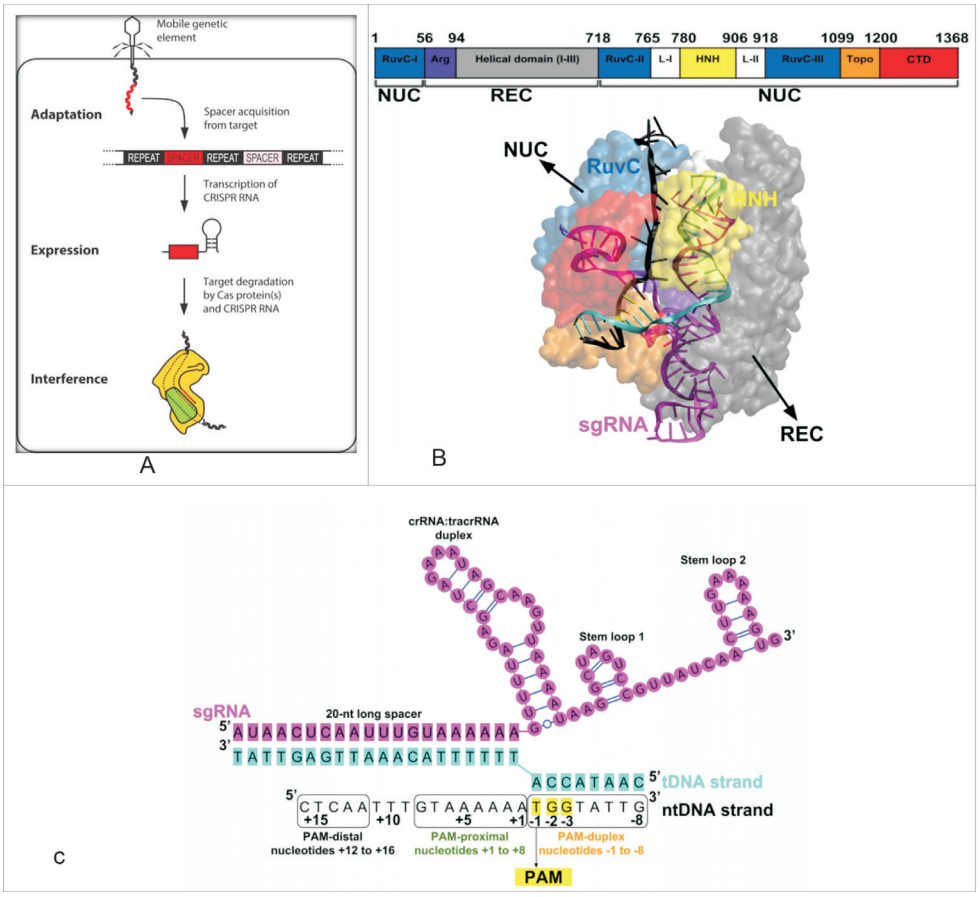

Fig. 1. A. General natural mechanisms of microbial CRISPR systems; B. The Cas9 endonuclease; C. CRISPR/Cas9 Structure. (Photos credited to authors of previously published research articles, names of these authors mentioned in this review citations and acknowledged)

Many viral delivery systems have been used for delivery of CRISPR-Cas9. The commonly used viral delivery approaches are Adeno-Associated Viruses (AAV) and Lentivirus. Regardless of format; however, each of these approaches faces the same obstacle to crossing the cell membrane. Each of these delivery formats has advantages and disadvantages in overall effectiveness as well as unique delivery challenges.

Applications of CRISPR Gene-Editing in Insect Pest Management

The application of CRISPR/Cas9 in insects is still in the early stages. It has been intensely reformed for different applications in model animals, which might clarify prospective applications in insects.

Gene Drive

Gene drive refers to the increase in the frequency of particular genes by bias inheritance.Based on the CRISPR/Cas9 genome editing system, a mutagenic chain reaction (MCR) method was developed to conduct gene drive in Drosophila (Gantza *et al.*, 2015) in which MCR converted heterozygous mutations to homozygosity at the yellow locus in germlines with 96% homing efficiency by copying themselves onto the homologous chromosome through HR.

Sequence-Specic Gene Regulation

The CRISPR inference (CRISPRi) is a modified CRISPR/Cas9 system in which dCas9 paired with sgRNA can satirically hinder transcription at the

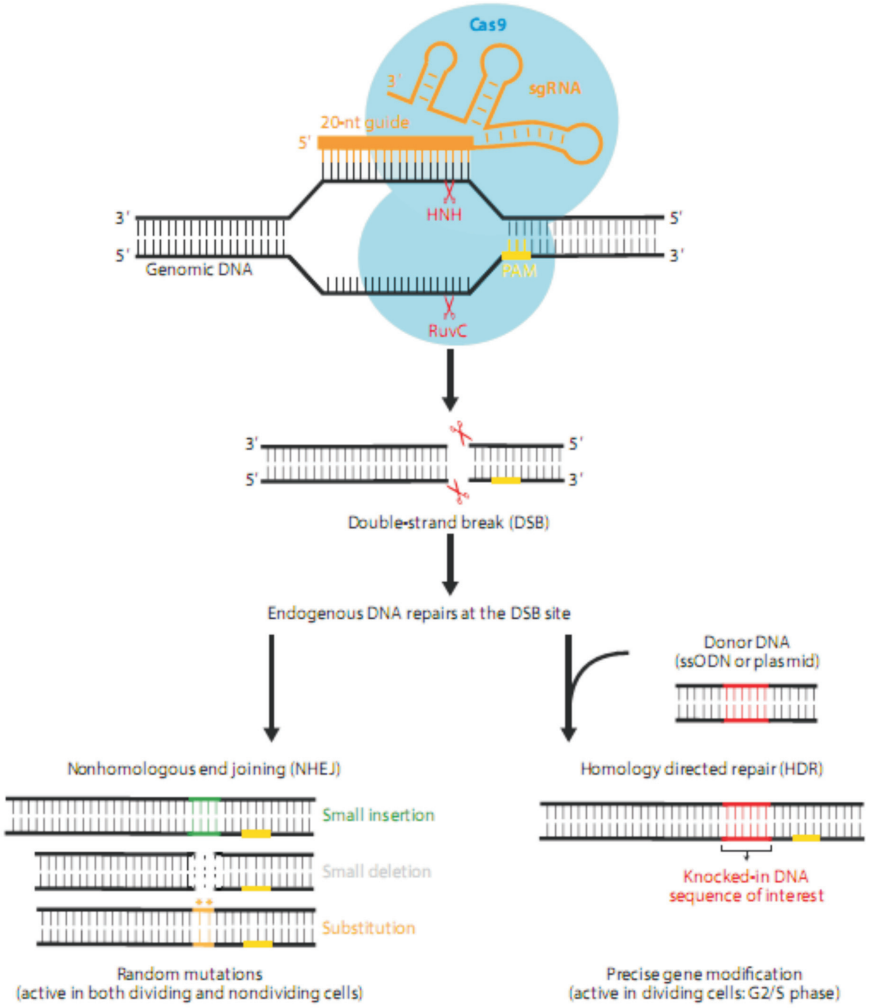

Fig. 2. Mechanism of Cas9 for genome editing: (Photo credited to authors of previously published research articles, names of these authors mentioned in this review citations and acknowledged).

sgRNA base-pairing genomic locus. The CRISPRi regulation can be used to achieve activation or repression by fusing dCas9 to activator or repressor modules Cas9 can serve as a unique platform to recruit protein and RNA factors to a targetedDNA site, and it has been engineered into powerful tools for sequence-specic gene regulation (Gilbert *et al*., 2013).

Genomic Imaging

Live imaging systems for visualization of genome loci are essential for studying chromatin dynamics and nuclear localization, which is important for understanding cellar processes. The CRISPR/Cas9 system can be reformed as a live imaging system by tagging dCas9 proteins with enhanced green fluorescent protein (EGFP) (Chen *et al*., 2013). An essential feature of any live imaging system is the ability to visualize more than one locus at a time. By using dCas9 from three bacterial orthologues fused with different fluorescent proteins, dCas9 and sgRNAs were found to efficiently label several target loci in live human cells (Chen *et al*., 2016).

Population Suppression

One of the simplest goals for gene-based pest control would be population suppression, i.e. decreasing the number of insects in an area, thereby reducing the damage done to crops (Esvelt *et al*., 2014). The Cas9-based gene drive could be used for population suppression is to target a gene that is essential for survival in the field but unimportant in a rearing facility (e.g. a gene essential for vision). In theory, releasing a few insects carrying a very efficient drive could be sufficient to suppress a pest population after many generations. In practice, growers would likely desire a faster response, which could be achieved by releasing more insects carrying the gene drive. For example, a ratio of 1:10, modified: wild type insects could lead to pest suppression in 10–20 generations. *Anopheles gambiae,* three female-sterility genes (AGAP005958, AGAP011377 and AGAP007280) were identified and triggered expected phenotypes with 91.4% to 99.6% efficiencies (Hammond *et al*., 2016). The reduction in female fertility has the potential to substantially reduce mosquito populations.

Insecticide Resistance Management

Another function of CRISPR/Cas9 could be to eliminate pesticide resistance. CRISPR/Cas9 can also be used to systematically knocking-down, knocking-out or over-expressing specic targeted genes which are responsible for the insectides resistance in the insect pest.CRISPR/Cas9 has recently been used to investigate the mechanism underpinning resistance to spinosad, an economically important bio-insecticide. Resistance to this insecticide has already evolved in multiple pest insects and is associated with alterations of its target, the Alpha6 subunit of the nicotinic acetylcholine receptor (nAChRs). Chemical mutagenesis experiments in D. melanogaster identied the mutation P146S in DmAlpha6 that conferred high levels of resistance to spinosad (Somers *et al*., 2015)

CONCLUSION

Crop protection against pest insects relies mostly on the widespread use of chemical pesticides that are detrimental to the environment and the development of resistance traits in insect pest populations. Additionally, IPM practices and application of biological control measures suffer from difficulties in timing of applications and high management costs. In light of these challenges, agriculture and farming have become increasingly data and technology-driven.Thus,Molecular biotechnologies allow genetic improvement, regulation, and modification across a wide range of insect pests, and have become increasingly important tools for pest management. Among the various Molecular approaches, CRISPR gene-editing has the capacity to alter the specific gene of interest. The CRISPR/Cas9 system is a revolutionary tool for both prokaryotic and eukaryotic genetics. It has been preliminarily established and developed in model and non-model insects. Highly efficient knockout and knock-in experiments have been successfully conducted in model insects such as Drosophila and silkworm, and in non-model insect. Based on CRISPR/Cas9, several well-designed systems have been developed, including gene drive and regulation and DNA/RNA tracking systems, which will have significant impact on functional studies and pest control.

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