ON MOLECULAR BIOLOGY GENOME EDITING TOOL CRISPR CAS METHODOLOGY

1. ABSTRACT

"CRISPR-Cas9 genome editing tool" is highly important in molecular biology study and biotechnology due to its capability. It is a sequence that provide guidance the Cas nuclease for the identification of the targeted site. At the time when "CRISPR Cas9 Protein" is attached to the intended cell with a gRNA, this protein is added to it and then moves with the DNA strands until it identifies and attaches to a specific sequence that is similar to the segment of the gRNA sequence. Cas is a nuclease enzyme that works for the creating cleavage of the DNA attached to the desired target sequence. The CRISPR-Cas techniques involve a few steps such as CRISPR knockout and knock-in with the interference of these. The gene CRISPR knockout can be performed with high accuracy and efficiency through the algorithm-designed CRISPR used for guiding RNAs. The CRISPR knock-in into the guide RNA (gRNA) manages the function of the Cas9 nuclease enzyme to cut the genomic DNA at a target site. The multifaceted array including the genetic outcomes made it quite possible by the advancement of technologies is the result, in greater part, of their capability to effectively instigate "DNA double-strand breaks or DSBs". However, it faces some challenges and those can be mitigated with advancements in future innovations.

Keywords: gRNA sequence, genomic editing, DNA, CRISPR Cas9 Protein, CRISPR Cas9 system, knockout, knock in, molecular biology, DNA repair pathways, alteration in gene sequence

2. INTRODUCTION

Discovery of Origin

"Cluster Regularly Interspaced Short Palindromic Repeats or CRISPR" were discovered in the "DNA sequence of E. coli or Escherichia coli bacteria" by the researchers of Osaka

University, Yoshzumi Ishino and his colleagues in 1987. They generally discovered similar sequences of DNA within “the genome of E. coli” while researching genes that help in “phosphate metabolism” (Manghwar et al., 2020). “CRISPR belongs to the family of DNA sequences” which are typically found within the prokaryotic organisms' genomes (for instance archaea and bacteria). These “DNA sequences’ are mainly extracted from the “DNA fragments of bacteriophages” that had formerly affected the “prokaryote”. They are utilised to destroy and detect “DNA from bacteriophages” during the “subsequent infection”. Therefore, these sequences have a critical role to play in the “antiviral defence system of prokaryotes” (which is anti-phage) and give a specific form of acquired immunity. On the other hand, “CRISPR-associated Protein-9 or Cas9” is a specific enzyme that utilises the sequences of CRISPR as a definite guide to open up by identifying the particular DNA strands that are critically compatible with these sequences (Li et al., 2023). “Cas9 enzymes along with CRISPR sequences”, together known as the “CRISPR-Cas9” that utilized to edit genres in organisms. The “CRISPR-Cas9 genome editing technique” development was acknowledged by “the Nobel Prize in Chemistry, 2020 to Jennifer Doudna and Emmanuelle Charpentier” (Piergentili et al., 2021). Furthermore, this editing procedure has wide applications, such as in biotechnological product development, treatment of different diseases, and fundamental biological research. Therefore, it can be said that it is quite a powerful tool in terms of molecular biology that has critically revolutionised this field.

Define CRISPR and Its Components

According to Genome.gov., (2024), CRISPR is a specific technology that researchers utilise to remodify the living organisms' DNA selectively. This has been adapted for utilisation in the “laboratory” from naturally obtaining “genome editing systems” that can be “found in bacteria”. Repetitive sequences of DNA which is also known as CRISPR, were typically found in bacteria along with “spacer DNA sequences” in the middle of the repeats that are similar to the viral sequences (Arroyo-Olarte et al., 2021). It was eventually discovered that bacteria duplicate these DNA components to RNA upon significant viral infection. Later, the RNA leads to a nuclease, (it is a specific protein that splits DNA) to the “viral DNA to cut it” which provides further protection against different viruses. The nucleases are known as “Cas for CRISPR associated”. The researchers in 2012 described that “RNAs can be contrived to guide

the Cas nuclease" to any type of "DNA sequence" (Shanmugam et al., 2020). This guide-RNA can also be developed; hence it will be particular to one definite sequence which further improves the possibility that the DNA will be cleavage at that site and not any other side of the genome. Later, further research revealed that this specific system functions well in different kinds of cells, such as human cells.

"CRISPR/Cas is quite a powerful and important tool", though it has certain limitations, such as;

(i) It is not fully efficient, hence even the cells where the CRISPR/Cas is used, might not execute any activity regarding genome editing.

(ii) It is also complicated to deliver the material of CRISPR/Cas to mature cells in large quantities, which remains a serious issue for different clinical applications. In this regard, viral vectors are the critical delivery technique (Liu et al., 2022).

(iii) It is not entirely accurate and can be "off-target edits", which is quite rare, still might have some negative consequences mainly in clinical applications.

Furthermore, the CRISPR system is comprised of two chief components, which are;

Table 1: The Components of the CRISPR System

Components	Description
1. gRNA or Guide RNA	It is mainly a sequence that "guides the Cas nuclease" to the target. At the time "CRISPR Cas9 Protein" is attached to the cell with a gRNA, this protein is added with it and then moves with the DNA strands until it identifies and attaches to a specific sequence that is similar to the segment of the gRNA sequence.
2. Cas or CRISPR-associated Nuclease	It is an enzyme that attaches with and splits DNA, and the most utilised Cas protein within the research is Cas9. This specific protein can then be programmed to locate and attach

	to the “desired target sequence”, by giving a segment of RNA to lead it in its thorough search.

The “RNA-DNA heteroduplex” has been formed when gRNA attaches to the target DNA. Then the heteroduplex attaches to a specific nuclease, named Cas, which further catalyses the split of “double-stranded DNA” at a particular position near the intersection of the palindromic repeat and “target-specific sequence” in the gRNA (Zhang et al., 2020). In this particular way, the nuclease further destroys the genomes of invading pathogens. CRISPR associates with “multiple Cas Proteins” as a segment of the defence response, and hence there are individual “CRISPR-Cas defense systems”. This particular system has revolutionised the genetic engineering field by offering a more reliable and efficient way to make precise changes to the target genome.

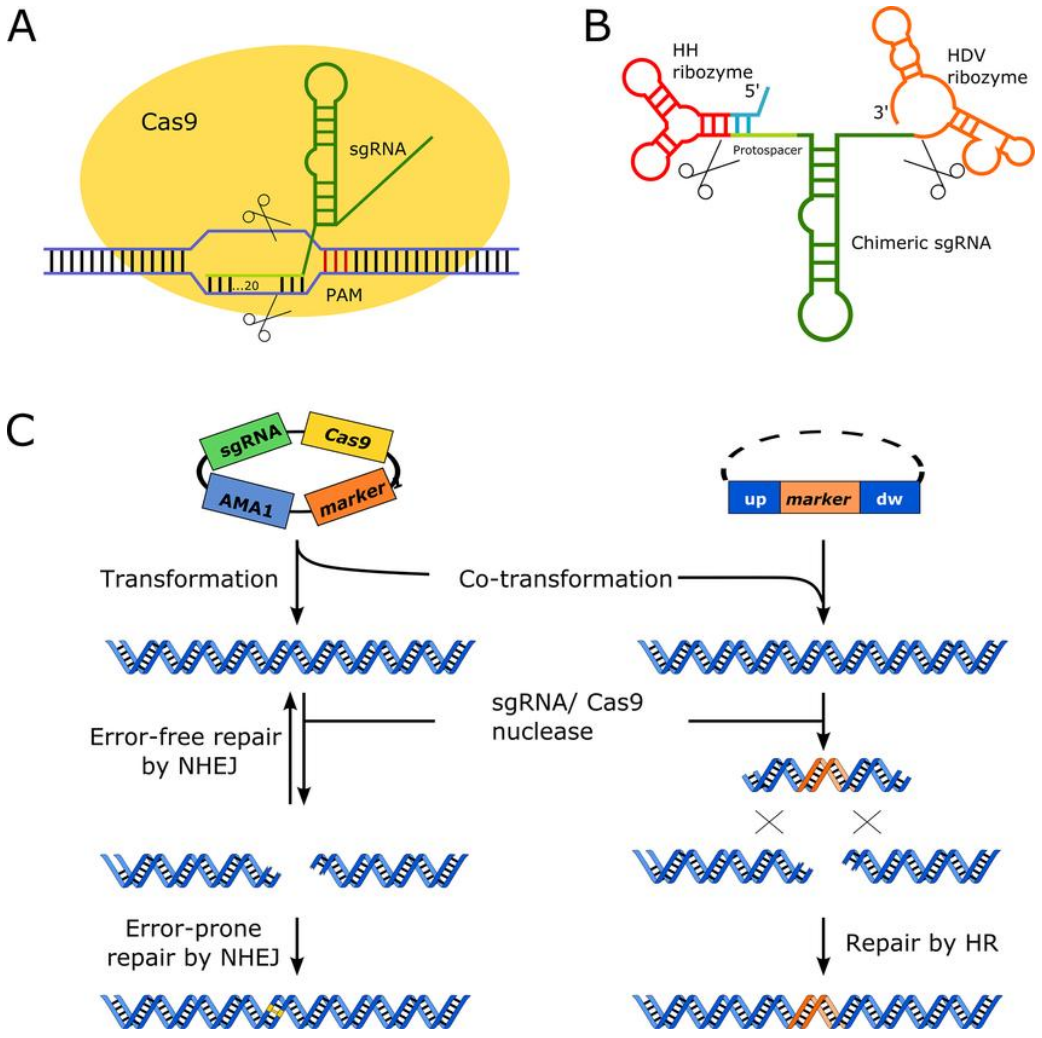

Figure 1: The CRISPR-Cas9 System Components

(Source: Khalil, 2020)

(A)The nuclease Cas9 which is guided by RNA specifically splits the “genomic target sequence”. “The scaffold and protospacer of the sgRNA” are highlighted in “green and light green, respectively”. The base pairs of the protospacer along with the associated “target sequence strand form the D-loop”. The efficient split is based on “the three bp PAM sequence presence within the target sequence”, identified straightforwardly “downstream region” which is invaded by the “specific protospacer”. “The blunt-end split” happens in the middle of the bases which is located 3 to 5 bp PAM upstream as highlighted by the scissors. (B) The sgRNA liberation, highlighted in green or light green which forms a “polymerase II transcript” through “hepatitis delta virus ribozyme and intrinsic hammerhead ribozyme” highlighted in orange and red or blue respectively. The blue segment of the “intrinsic hammerhead base pairs” along with the protospacer for effective cleavage. On the other hand, (C) The fungal AMA1-dependent vector harbouring “sgRNA and Cas9 encoding genes” are then “transformed into the fungus”. “The sgRNA or Cas9 riboprotein” induces the “site-specific DNA DNB”. Then, the DNA DNB was further repaired by NHEJ without “the homologous template for HR repair” in the left lane, also the prone or error modified by NHEJ resulted in specific mutation as highlighted by base pairs which are in yellow coloured. Specific DNA DSBs induced by Cas9 might be “repaired through HR in the presence of a circular or linear gene targeting substrate in the right lane”. This further resulted in the event of gene targeting presented as the insertion of the “orange marker gene” within this figure.

As per the opinion of Khalil, (2020), the CRISPR system worked by developing small gRNA sequences that critically correlate with the target DNA. gRNAs which are generated through CRISPR transcription, involve hairpin formations which are obtained from the palindromic repeats, that are also associated with the specific sequences of the spacer elements. Furthermore, the three major “CRISPR-Cas defense systems” include “type I, type III, and type II”. The type I system includes Cas3 protein and forms a “CRISPR-associated complex for antiviral defense or Cascade” like substance, which attaches to the gRNA and recognises the specific sequence for destruction. The type III system comprises Cas10 protein which is quite different from the other two types, regarding the targeting DNA, it critically recognises RNA

targets (Sledzinski et al., 2020). Whereas, in type III there are different proteins such as Cas9, Cas4, Cas1, and Cas2; it plays a critical role in facilitating “cellular adaptation to the pathogens”, and also takes part in the splitting of target RNA and DNA processing.

Significance of the Genome Editing Tool CRISPR CAS Methodology

The “CRISPR-Cas9 genome editing tools” are relatively crucial in biotechnology and medicine due to their capability to edit the genomes in vivo which is quite cheap, precise, and easy. According to Tyagi et al., (2020), it also can treat different genetic disorders and diseases; also, can be used to sustain the spread of viral diseases. It aids in “advancing gene therapy development” by providing a reliable as well as effective process for doing necessary alterations in the targeted genome (Taha et al., 2022). Moreover, it can be used to increase the growth as well as resilience of crops in agriculture. This can assist in “the production of crops that are quite resistant to environmental stresses as well as pressures” which further improves agricultural productivity. It is an important tool for research that permits the researchers to change gene function by modifying the DNA sequences. This can assist in acquiring a better comprehension of basic biological processes and mechanisms regarding the diseases and further helps in the development of more advanced medicines (Zafar et al., 2020). The prime technologies which are presently “used to facilitate genome editing presents in Figure 2”, which are (a) “zinc-finger nucleases or ZFNs”, (b) “clustered regularly interspaced short palindromic repeats or CRISPR”, (c) “homing endonucleases or meganucleases”, and (d) “transcription activation-like effector nucleases or TALENs”.

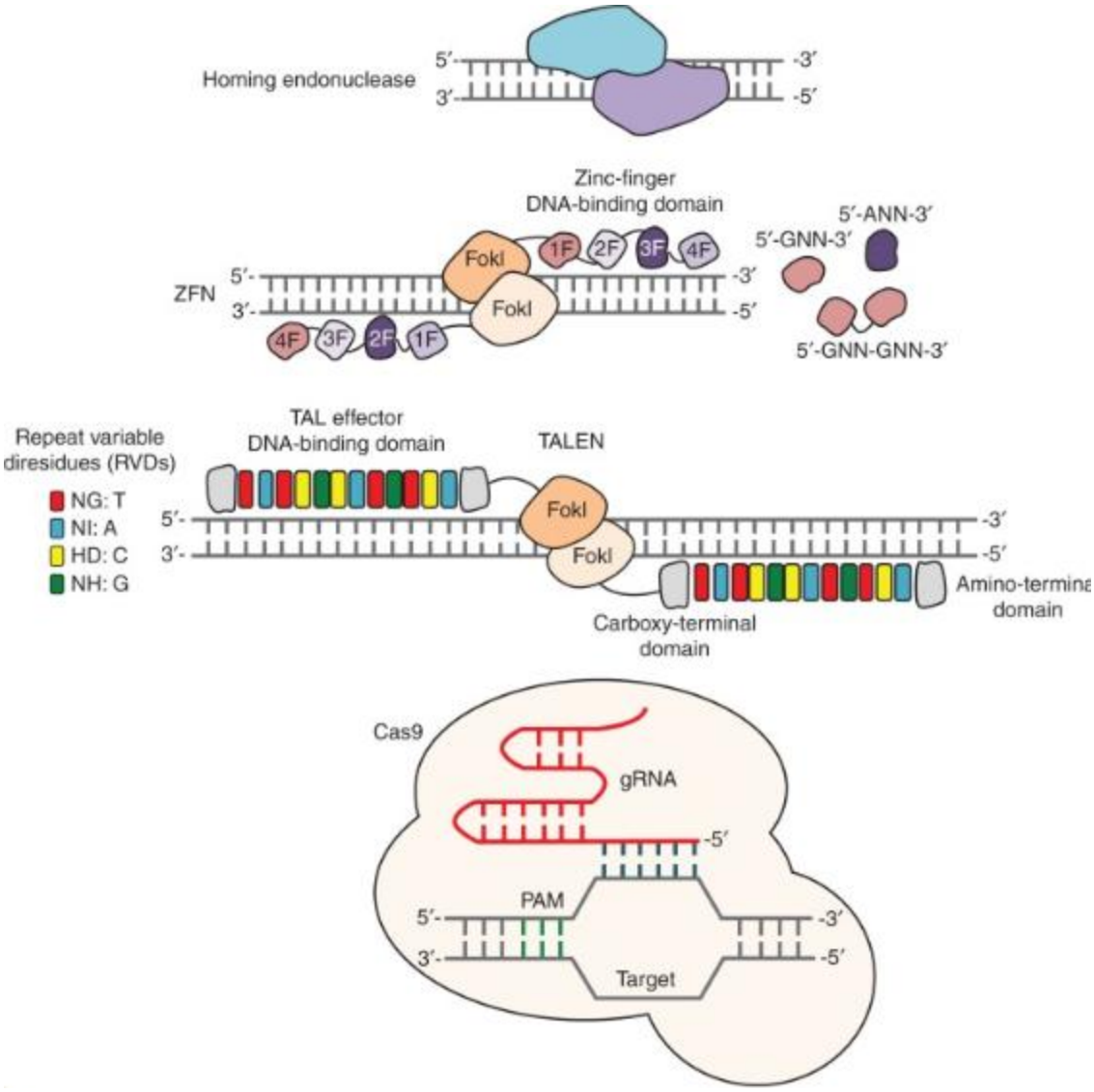

Figure 2: The Technologies of Genome-editing

(Source: Taha et al., 2022)

The above figure illustrates the “targeted nuclease mechanism”, here from “top to bottom” the description includes: “homing endonucleases”, ZFN, TALEN, CRISPER, and Cas9. The homing endonucleases typically split the “DNA substrates” as dimers, also do not have any specific “cleavage and binding domains”. Further, the “ZFNs” identify “target sites” that comprise “two binding sites” that “flank a 5 to 7 base pair spacer sequence” identified by the “FokI cleavage domain”. The “sites of TALE DNA binding” are identified by the TALENs that “flank 12 to 20 base pair spacer sequence” determined by the “FokI cleavage domain”. Lastly,

the “Cas9 nuclease” is further targeted to the “DNA sequence” which is interrelated with the targeting sequence in the gRNA located upper part of the “compatible protospacer adjacent motif or PAM”.

The particular “ease with which TALENs and CRISPR-Cas9” can be arranged to identify the advanced genomic sequences that have steered a revolution within “genome editing”. Furthermore, it has accelerated different scientific discoveries and breakthroughs in disciplines as various as “human gene therapy”, “synthetic biology”, neuroscience, disease modelling, the agricultural sciences, and drug discovery (Zhang et al., 2021). The multivarious array regarding the genetic outcomes made it quite possible by “the advancement of technologies” is the result, in greater part, of their capability to effectively instigate “DNA double-strand breaks or DSBs”. Furthermore, “these breaks in DNA” then facilitate the “activation of cellular DNA repair pathways” and drive the institution of the “site-specific genomic modifications” (Naeem et al., 2020). This procedure is mainly utilised to obtain gene knockout through random base deletions or insertions that can be instituted by “nonhomologous end joining or NHEJ” referred to in Figure 3.

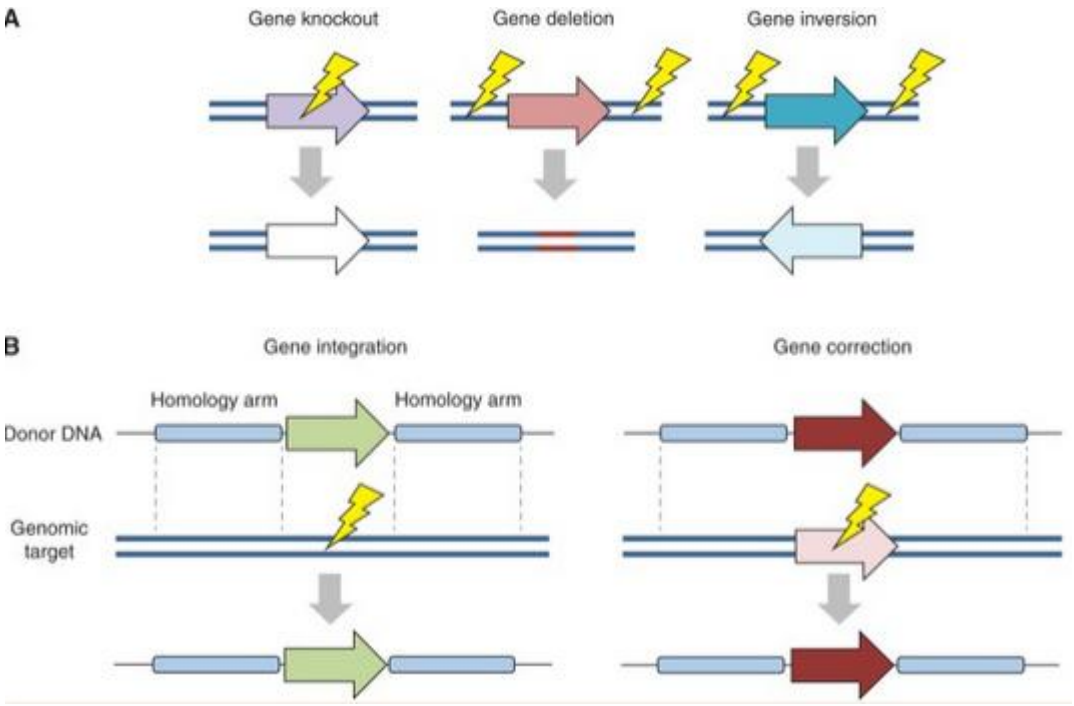

Figure 3: The Outcomes of Genome-editing

(Source: Naeem et al., 2020)

Targeted nucleases instigated DSBSs that are further repaired by "NHEJ in the presence of donor template, homology-directed repair or HD". Moreover, (A) due to the "lack of a donor template, the NHEJ institutes small base deletions or insertions" that can also result in gene disruptions. The interceding genomic sequence can be inverted or deleted when the two DSBs are added simultaneously. (B) "Recombination between the homologous DNA sequences" which are present on the chromosomal site and "donor template" site can foster targeted integration in the "presence of single-stranded or plasmid oligonucleotide".

Henceforth, it can be stated that the chief principles of genome editing, highlight several "engineering advances" that have further laid the basis for the "refinement", implementation, and creation of the present "suite of genome-modifying tools".

Recent Advancement

Genome editing is a powerful tool that includes deliberate alteration in the DNA sequences which further leads to the new age of molecular medicine against genetic disorders, cancer, and beyond. Editing methods presently available comprise TALENs, CRISPR, and ZFNs, here, CRISPR has been determined as the "antiviral immune system of bacteria and archaea", later adopted as the compatible "gene editing tool" (Wada et al., 2022). "CRISPR-Cas9" is broadly utilised other than TALENs and ZFNs as it is highly efficient and cost-effective.

Extending the Genomic Targets Range: "Genomic targeting by Cas nucleases needs a PAM sequence" within the site where the "Cas nuclease attaches DNA sequences" to "the sgRNA". "PAM for a specific Cas nuclease analysis" of whether the certain site can be edited and targeted by "CRISPR".

The specificity of DNA increasing without Impacting On-target Cleavage Effectiveness: "The activity of Off-target" is a huge issue when utilising "CRISPR tools" for "disease-related gene therapy". Therefore, constant "efforts have been made to develop high-fidelity Cas9 variants" such as HF1, evoCas9s, Sniper-Cas9, and eSpCas9. Additionally, SaCas9 which has "high fidelity" identified either by "directional screening or rational engineering"

(Asmamaw & Zawdie, 2021). The accomplishment of increased "discrimination between off-target and on-target binding" of the variants depends on the "Cas9: sgRNA: DNA" energetic destabilisation at the off-target site.

Improvements within the Precise Genome Editing: This "genome editing" is important for "gene therapy and preclinical research". Different research has been conducted to increase "the efficiency of HDR-mediated gene editing", namely the utilisation of "addition of NHEJ chemical inhibitors", and "rationally designed single-stranded oligodeoxynucleotides template" rather than double-stranded-DNA (Wang et al., 2022).

Challenges

It has been identified that "CRISPR-Cas9 is a revolutionary tool" for "genome editing" though there are different challenges; such as,

gRNA Production: The production of this RNA guides the "Cas9 protein" to the target sequence of DNA which poses critical challenges for "CRISPR-Cas9 mediated genome editing".

Off-target Mutations: One major issue regarding "the CRISPR-Cas9 mediated genome editing" is the phenomenon of "off-target mutations" (Khoshandam et al., 2024). Furthermore, these are unexpected modifications that happen in genome regions other than the actual target site which can guide adverse impacts.

Delivery Methods: The recognition of a significant "delivery method for the CRISPR-Cas9" elements into cells is a critical challenge.

PAM Dependence: The "CRISPR-Cas9 mediated DNA cleavage specificity" needs to get sequences similar to the crRNA (CRISPR RNA) and a PAM which are placed in the "downstream of the target sequences" (Liu et al., 2020). This further sustains the sequence range that can be later targeted by the entire system.

Ethical Concerns: The characteristic of CRISPR-Cas9 to make accurate alterations to the genome further raises ethical concerns, mainly when it comes to the likelihood of "editing the human germline" (Hazafa et al., 2020).

Immune Responses: The utilisation of "CRISPR-Cas9" can further trigger the immune responses of the human body.

3. BACKGROUND

Brief History and Discovery

"CRISPR-Cas Systems", namely "CRISPR-Cas9" are typically "adaptive immune response systems" that safeguard "prokaryotes from bacteriophages". They mainly function by splitting the "nucleic acids of invading viruses", thereby safeguarding "prokaryotes" from any type of "viral infection" (Gostimskaya, 2020). The utilisation of "CRISPR-Cas9 to edit the genes" gained popularity in 2012, when Emmanuelle Charpentier, Feng Zhang, George Church, and Jennifer Dounda applied it as a specific "tool to change targeted places of genomes" (Li et al., 2023). As discussed before, the "CRISPRs" are the "repeating DNA sequences" in "the prokaryotes genome" which were identified in "E. coli in 1987 by Yoshizumi Ishino and his team". They unintentionally "cloned an unusual series of repeated sequences interspersed with spacer sequences" at the time determining a gene accountable for the modifications of alkaline phosphate. Although, without the proper information of "DNA sequence", the execution of these arrays became a mystery. "J.D. Van Embden in 1993 discovered that various *Mycobacterium tuberculosis* strains" had several spacer sequences in the middle of the DNA repeats (Khalil, 2020). They further characterised these strains depending on the spacer sequences, which is a method termed as "spacer oligonucleotide typing or spoligotyping". Moreover, "the sequences were also recognised in different other "archaeal and bacterial genomes".

"Researchers Ruud Jansen and Francisco Mojica" were the first ones to named them as "CRISPRs". They were initially thought to be the "novel DNA repair mechanism" when the CRISPR systems were discovered in thermophilic bacteria and archaea. Later Mojic and colleagues in the 2000s observed that the "spacer sequences" were relatively alike to "sequences of the plasmids, bacteriophages, and viruses" (Mushtaq et al., 2020). They further discovered that the viruses are unable to "infect bacteria with homologous spacer sequences", this means that "the sequences" have a critical role to play within "the adaptive immune system of prokaryotes". Emmanuelle Charpentier, Feng Zhang, George Church, and Jennifer Dounda in

2012 discovered that by remodifying gRNA to target certain regions within the genome, the "CRISPR-Cas9 system" can be utilised a "cut-and-paste tool" to change the structure of genomes (Zhang et al., 2021). However, "CRISPR-Cas9" has been utilised to "switch off the genes" that sustain "lipids production in microalgae", which guides high "lipid production and increased biofuel yields". CRISPR editing in 2019 was tested by treating patients who were suffering from "sickle cell disease and β-Thalassemia" (Nidhi et al., 2021). Furthermore, this success has guided many patients receiving similar treatment to get better from this condition. Although it has been identified that "CRISPR-Cas9 technology" has its advantages and disadvantages, researches continue to utilise, expand, and refine this toolkit, based on these additional tools and new applications are continuously arising.

4. CRISPR CAS SYSTEM

Basic Components of CRISPR

The chief "components of CRISPR-Cas9 systems" are "RNA-guided Cas9 endonuclease and a single-guide RNA". Furthermore, "the Cas9 protein" comprises two nucleases such as "RuvC and HNH", along with every "cleaves single strand of the target double-stranded DNA" (Asmamaw & Zawdie, 2021). The "single-stranded RNA" is a simple combination of tracrRNA and crRNA. The sgRNA and Cas9 nuclease from the Cas9 ribonucleoprotein (RNP) can be cleaved by attaching to the target DNA. Moreover, "the PAM sequence" is needed for the binding of "Cas9 protein to the target DNA".

Mechanism of Action

"The CRISPR-Cas9 system" executes in different phases, such as;

Spacer Acquisition - The CRISPR-Cas systems safeguard the organism from incoming plasmid or phage infection by integrating a small DNA stretch into the array of CRISPR through Cas2 and Cas1 protein dimers (Zheng et al., 2020). This procedure is also known as acquisition or adaptation.

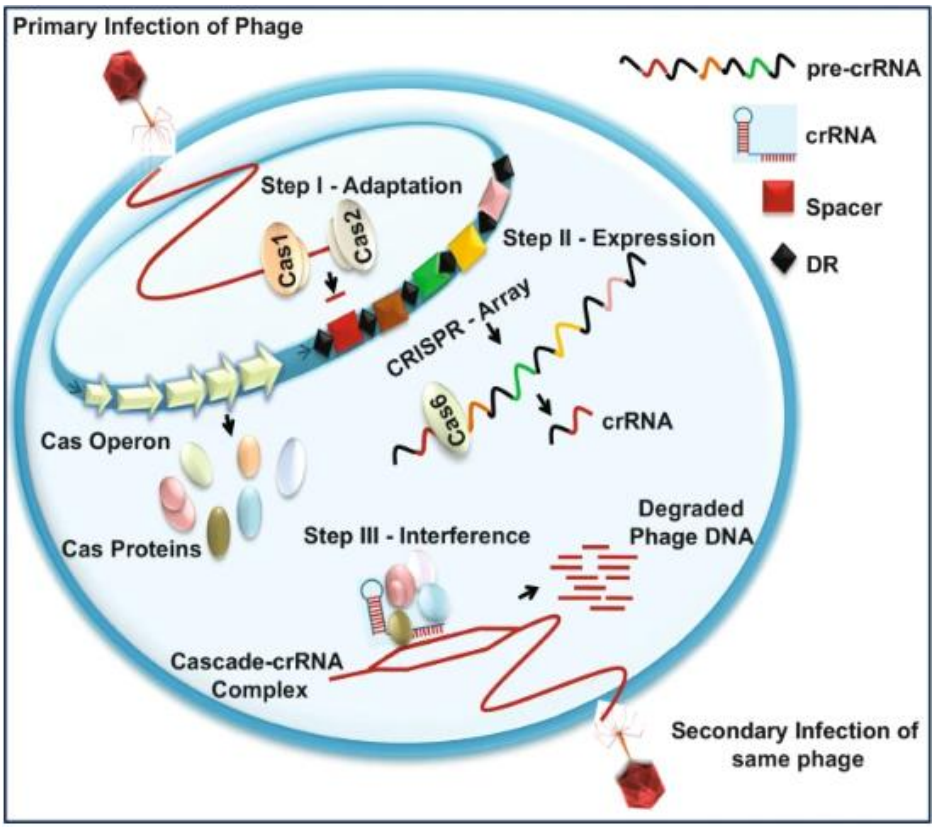

Figure 4: The Action Mechanism of CRISPR-Cas System

(Source: Devi et al., 2022)

The "mechanism of action of the CRISPR-Cas System" here illustrates the specific mechanism of "type I- E CRISPR-Cas system" action in Figure 4 (Devi et al., 2022).

The Transcription of CRISPR Array - "The CRISPR arrays" are special genes that encode the specific Cas proteins and they then transcribe into crRNA.

Trager Identification and Cleavage - "The CRISPR-Cas9 system" within its actual form is a "homing device" that generally guides "the Cas9 enzyme of molecular scissors" to the "target part of DNA" (Janik et al., 2020). Later, the "gRNA leads the Cas9 protein" to the "target DNA sequences", and together they execute to repair the gene.

This mechanism permits scientists to "manipulate the genome of any organisms" such as microbes, animals, or plants. It is an important tool for genetic engineering, though regardless of its power, the practical laboratory work is quite difficult as well as challenging.

Types of CRISPR Cas system

According to Hidalgo-Cantabrana & Barrangou, (2020), the "CRISPR Cas system" is categorised into "two distinct classes such as Class 1 and Class 2", which are as follows;

Class 1

Type I - It is recognised by the ultimate "presence of the Cas3 gene".

Type III - It is mainly characterised by the existence of the Cas10 gene.

Type IV - Although it is part of this class, still is less distinguished when compared to the other two types such as Type III and I.

Class 2

Type II - Here, the Cas9 gene is present and is the most commonly utilised in genome editing due to its versatility and simplicity.

Type V - It uses the Cas12a protein which is formerly known as Cpf1.

Type VI - It is identified by the presence of Cas13 protein, which mainly targets RNA rather than DNA.

Each kind has individualistic uses and characteristics, and they all have a critical role in planning within "the adaptive immune system of prokaryotic organisms".

5. DESIGNING OF CRISPR CAS EXPERIMENT

This research aims to construct an ideal model for the genomic tool known as CRISPR CAS in the field of molecular biology research. The design of the experiments includes the identification of the target site first followed by the selection of Cas nuclease. The selection of these components is controlled by the goals of the research along with several gene editing technologies. The processes are described in the following sections:

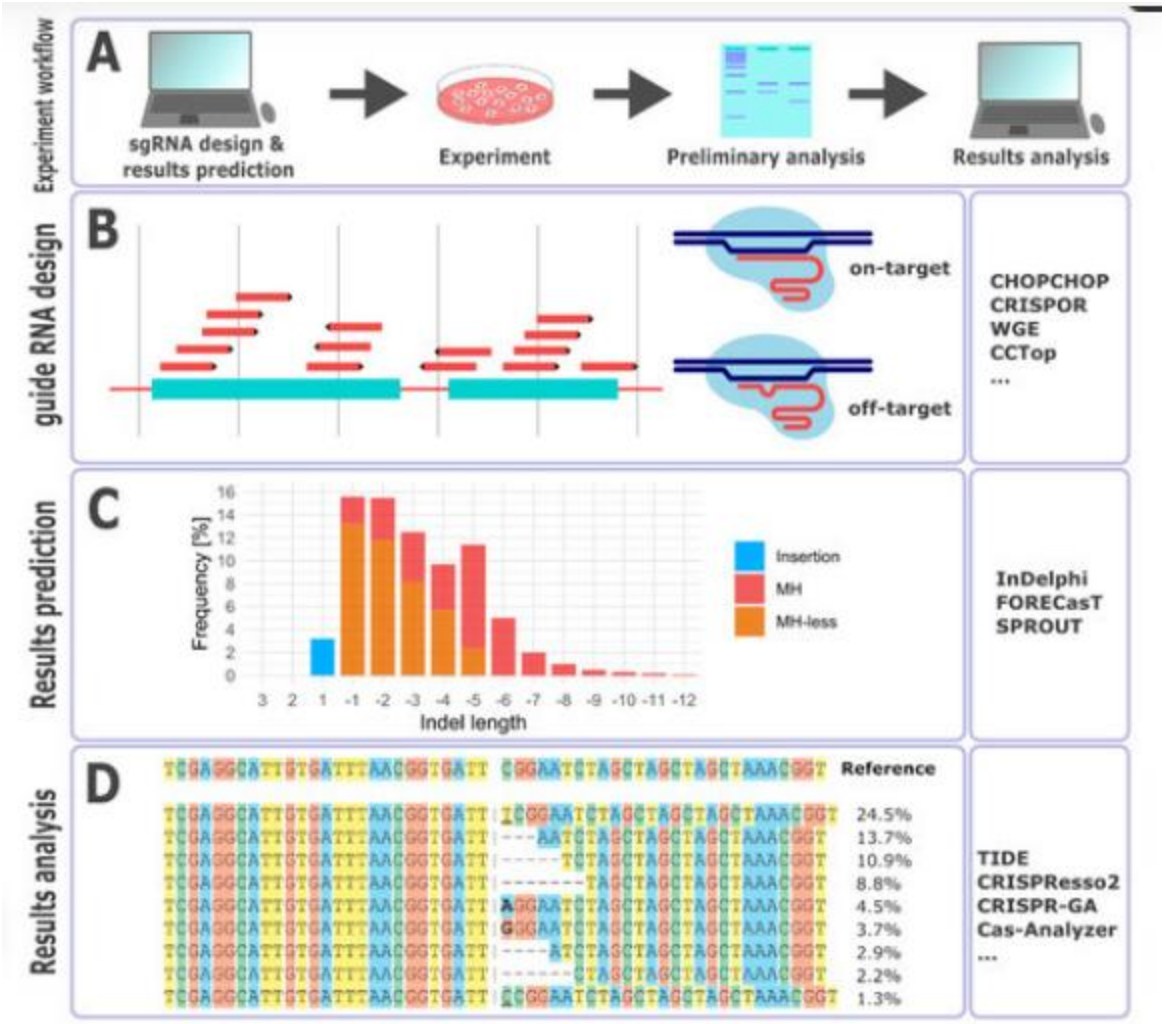

Figure 4: CRISPR/Cas9 Experiment

A) Overview of the experiment, B) Guide RNA design in both off-target and on-target, C) Outcomes of the experiment, D) Analysis of the obtained result

(Source: Sledzinski et al., 2020)

5.1 Designing

Target identification

According to the study of Bazaz & Dehghani (2022), the CRISPR techniques involve CRISPR knockout and knock-in with the interference of these. The gene "CRISPR knockout" can be performed with high accuracy and efficiency through the algorithm-designed CRISPR used for guiding RNAs. It is implemented to inactivate the function of genes by evaluating numerous phenotypes with high specificity to determine the exact target sites. Carrington et al., (2022),

suggest that the knock-in includes the introduction of correction in SNP (single nucleotide protein) mutation for adding a reporter tag to an endogenous gene. An article from 2023 by ZADabbas Shahabadi et al. says that the "CRISPR knock-in" into the guide RNA (gRNA) affects the Cas9 nuclease enzyme's ability to cut genomic DNA at a certain point. The article by Marshall et al. (2020) talks about how the goals and clinical needs of the study are used to choose which gene or genomic area to study. The "CRISPR/CAS mechanism" mostly goes after genes that show damaging mountains when it comes to some genetic diseases. This method for fixing things depends on several things, one of which is the presence of PAM sequences that help locate the CAS nuclease. What we know about the editing mechanism's genome code and how well CRISPR/CAS can get to the target spot are also important parts of the process.

Guide RNA design

The main aspect that needs concern while designing the guide RNA is to use of existing libraries of validated gRNAs (Bharathkumar et al., 2022). For instance, with the help of CRISPRInc offers valuable insights about IncRNAs for choosing effective gRNAs. Another example suggests that Addgene provides an entire list of gRNA sequences that have been validated previously (Xiang et al., 2021). Similarly, the genome-wide libraries provide information about the sgRNAs for gene knockout techniques.

5.2 Assembly

Cas9/gRNA complex formation

Currently, "SpCas9" continues to be the preferred enzyme for genetic engineers working with genomes. However, with the advancements in gene-sequencing technology, this technique now includes the use of nuclease selection based on specific criteria known as PAM (Protospacer Adjacent Motif) and cleavage pattern. S. aureus and S. thermophilus use Cas9 to pick and choose which nucleases to use for the best effects. More on this can be found in a paper from 2023 by Sarkar et al. Naeem et al. (2020) say that the Cas9 complex has an off-target effect when it cuts and joins in places it wasn't supposed to. These are places that are not the goal that make up the goal area on-site. Changes have been made to many animal and plant genes with CRISPR/Cas9 nucleases. The "CRISPR Cas9 system" links to targets more often and has bigger effects than TALENs and ZFNs, according to a number of studies (Bhardwaj & Nain, 2021). Because they are made up of two molecules each, the "ZFN" and "TALEN" groups can recognize longer target

sequences better than this one, which is made up of only one gene. If you use gRNA-specific ways, Li et al. (2022) say that the Cas9 and gRNA complex building base editing method cuts down on base editing that doesn't happen on target [1]. Things that don't go as planned come in three main types.

There are three categories of PAMs: those that have substitutions (5′-NGG-3′), those that have insertions or deletions (5′-NGG-3′), and those that cleave genomic sequences (5′-NAG-3′) (Aquino-Jarquin, 2021).

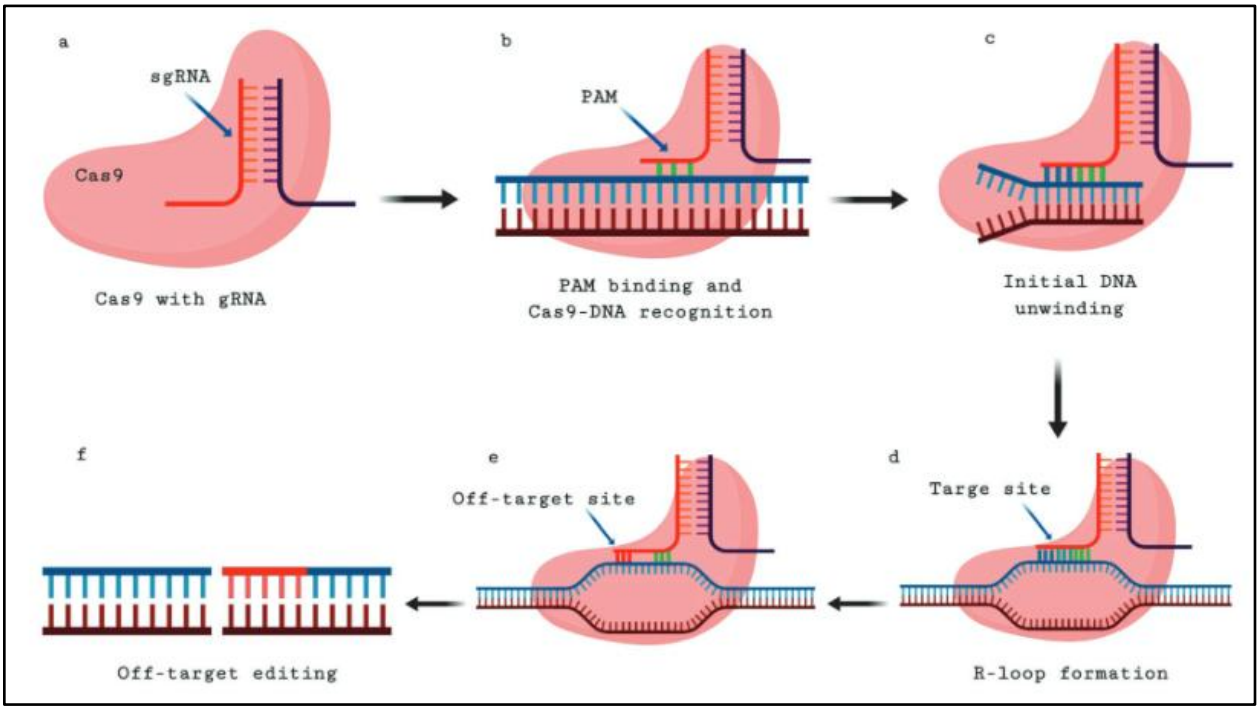

Figure 5: Process of off-target genomic editing with CRISPR/Cas9 system

(Source: Manghwar et al., 2020)

The system of “CRISPR/CAS” works with 3 or more than these mismatches in a “20-base pair (bp)” targeted “DNA sequence”. The primary association in the middle of “Cas9 and DNA” is arbitrated by identification along with the binding at the PAM site (Kang et al., 2022). This initiates the “melting of PAM proximal DNA” leading to direct probing of crRNA (CRISPR RNA) with target editing sequence resulting in the formation of a stable R loop (Kauert et al., 2023). The above figure shows the procedure of off-target editing by “the CRISPR/CAS system” recruiting the melted DNA helping bind Cas9. The “Cas9 nuclease” fells under the “class 2 type CRISPR system” in the tool used for editing of the genome. The research article of Kim et al.,

(2020) suggests that the recognition of the "PAM 5′-NGG" limits the abundance of "SpCas9" target sites in the genome of the human beings to an average of one target site for every eight base pairs. There are alterations in the PAM seen with variants of Cas9 for yielding better availability of target sites.

Name	***Protein variants***	***PAM (5' to 3')***	***Features***
'SpCas9'	***"Native Streptococcus pyogenes Cas9"***	***'NGG'***	***'Amino Acids' (1380)***
'EQR SpCas9'	***"D1135E, R1335Q and T1337R"***	***NGAG***	Alteration in variants of PAM, bacterial selection-based screen (Sattar et al., 2021)
'VRER SpCas9'	***"D1135V, G1218R, R1335E, T1337R"***	***'NGCG'***	Alteration in the variant of PAM, screening based on bacterial selection
'HiFi Cas9'	***R691A***	***'NGG'***	***"Increased specificity for ribonucleoprotein delivery" (Pedrazzoli et al., 2023)***
'CjCas9'	***"Native Campylobacter jejuni Cas9"***	***"NNNVRYM"***	***'Amino Acids' (984)***
'CasX'	***"Phyla Deltaproteobacteria and Planctomycetes"***	***"TTCN"***	***'Amino Acids' (980)***

Table: Variants of Cas9 within specific target site and altered PAM

(Source: Self-developed)

5.3 Delivery

By viral vectors /plasmid /RNP complex/nanoparticles/mutation assays

"CRISPR CAS system" is an instrument that scientists made to move things around. It lets them change the genes of target cells and help with treatments. This is what the method is all about. Putting Cas nuclease and guide RNAs (gRNAs) into certain places can change the sequence of genes (Pan et al., 202). Right now, many important things need to be done. Nanoparticles, ribonucleoproteins, mutation arrays, and Platid DNA are some of the many things on the list. According to Farzanehpour and friends (2023), the "CRISPR/CAS component" is often sent to certain places using "lentivirus" and "adeno-associated virus (AAV)". The genome and epigenome can be changed with these vectors. This can change how the genome works and what patterns it makes. It was written by Meng et al. in 2023 that lentiviral vector (LV) systems are the major way that CRISPR/Cas technologies add complex transgenes to cells and living things. In the lab, we test how well the CASPR and Cas nucleases cut DNA to show that the system works. There is a test called T7E1 that can be used to find indels during the repair process after PCR amplification (Park et al., 202). People use the CRISPR/CAS technology on live things to see how well it works. The right way to do in vitro proof is to see this happen. For their study in 2023, Singhal et al. used body cells from real people, as well as cells from mice and zebrafish. Wu et al. (2023) say that changing gene codes every day is easy and cheap to do by adding plasmid DNA to cells. Getting RNPs into cells has many benefits, such as stopping effects that aren't supposed to happen, turning on Cas for a short time, and quickly starting to change the genome. They can be polymers, liposomes, or metal nanoparticles. A lot of the CRISPR/CAS process moves because of these carrier nanoparticles. Dobey and his colleagues did a study in 2023 that shows nanocarriers can be useful for gene editing treatments since they don't spread viruses. There are many ways that CRISPR/Cas9 nanoparticles have been sent. LNPs, cationic liposomes, and cationic polymers are a few of the things that are used.

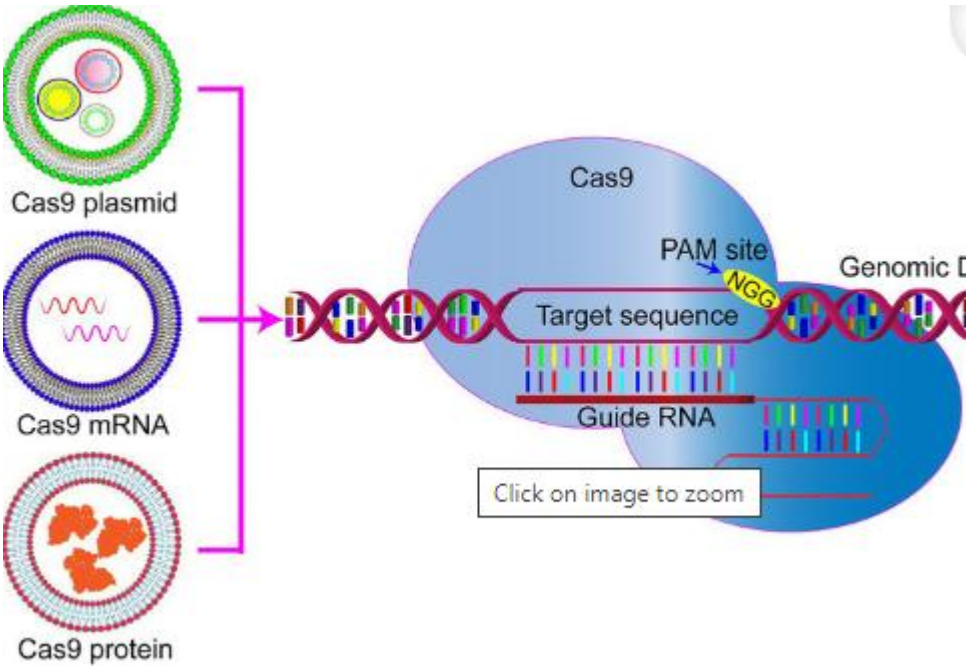

Figure 6: Delivery of the CRISPR/CAS mechanism through Nanoparticle

(Source: Duan et al., 2021)

5.4 Recognition of Binding and cutting site

The recognition of PAM and binding with it in ***"the CRISPR/CAS system"*** is a fundamental process that utilizes an interference complex known as "Cascade (CRISPR-associated complex for antiviral defense)". This is performed by the "type 1 CRISPR-CAS system" and the "type 2 CRISPR-CAS system".

5.5 Repair by NHEJ and HDR mechanism

Genomic editing nucleases effects efficiently create breaks in targeted DNA sequences to fix the damage, for instance, the efficacy of Cas9. The basic mechanism of dsbDNA (DNA double-strand breaks) includes two procedures for example NHEJ ("non-homologous end joining") and HDR ("homology-directed repair") (Shen & Li, 2022). Among these two, the NHEJ is a widely used pathway for the repairing of eukaryotic cells. The study of Bazaz & Dehghani (2022) says that the NHEJ mechanism results in deletions 9gene knock-out) or small insertions (genomic knock-in) of DNA sequences during the repair process. This process tends to result in error-prone repairing. NHEJ-mediated repair is disrupted often in coding exon regions due to frameshift mutations. This

procedure includes the indel mutation by targeting a single site in the genetic sequence and disrupting the gene expression resulting in gene knockout. The targeting of two sites on the gene sequence induces a deleterious method at the cleavage sites. This approach includes the removal of entire genomic fragments with a large number of deletions. Furthermore, NHEJ (non-homologous end joining) is a crucial cellular pathway for fixing DNA double-strand breaks (DSBs) (Stinson & Loparo, 2021). This entire process starts with the identification of DSBs and that is performed by the Ku 70 or Ku 80 heterodimer. Ku then worked as a scaffold that recruits DNA PKcs kinase and two subunits of DNA ligase (Chen et al., 2021). The broken DNA ends get ligated and repaired and before that DNA ends contain damaged nucleotides. For the preparation of the missing DNA ends NHEJ utilizes a toolbox involving a few polymerases such as Pol μ and Pol λ and nuclease such as Artemis. The CRISPR-Cas9 system employs the DSBs at specific sites on the genomic sequences (Thompson et al., 2021). NHEJ-mediated repair performs the gene knockout along with indel errors. These heterogeneous indels' s sizes range from 1 to 10 base pairs.

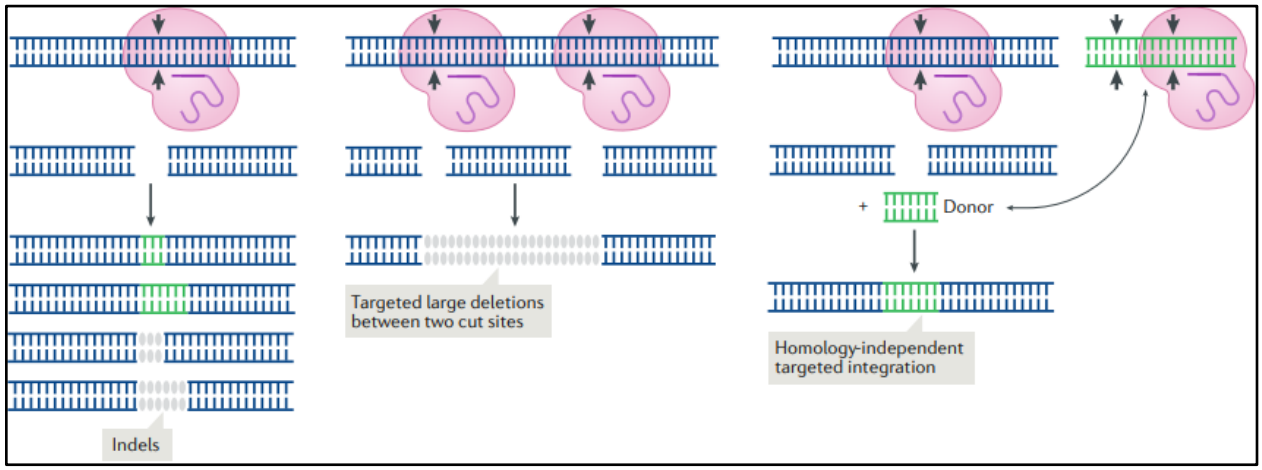

Figure 7: Mechanism of NHEJ repairing

(Source: Pickar-Oliver & Gersbach, 2021)

The HDR method works better than the NHEJ repair process for fixing the template at a homologous target site. The DNA repair process does not make any mistakes. There are special building blocks called homology-directed repair pathways (HDRs) that are used to change templates that have ssODNs or DSBs in them (Yang et al., 2020). If you change one nucleotide or add them to a longer sequence, the donor template can make it easier (Li et al., 2024; Schubert et al., 2021).

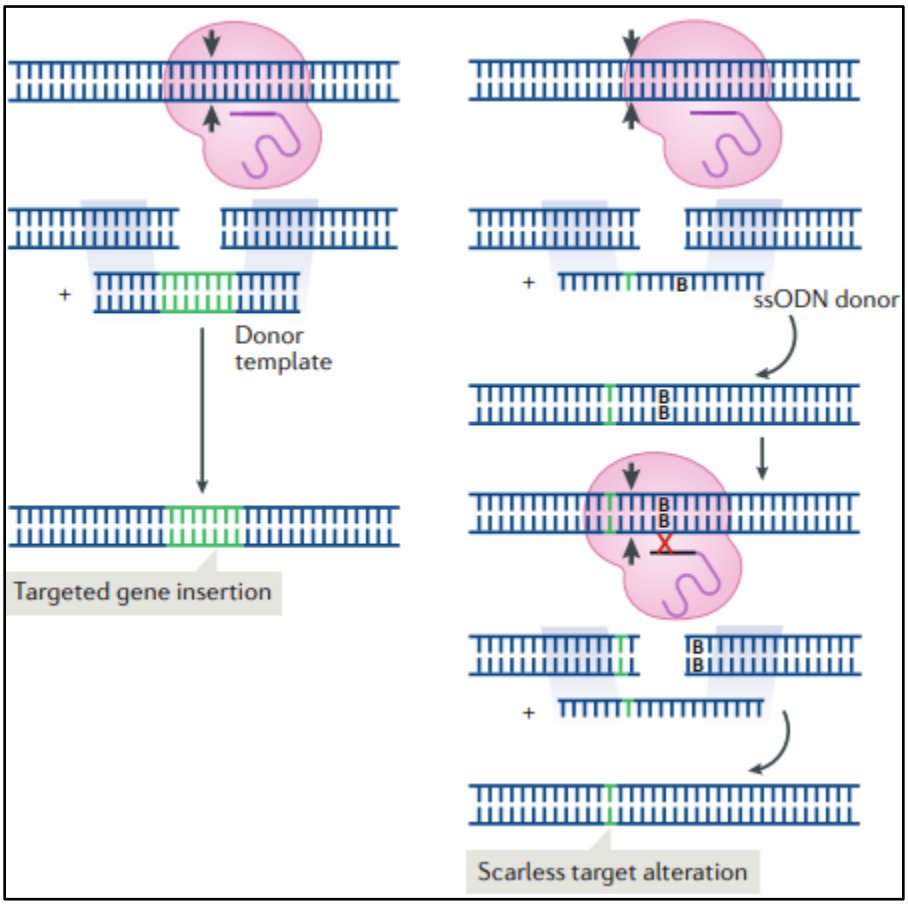

Figure 8: Mechanism of HDR repairing

(Source: Pickar-Oliver & Gersbach, 2021)

6. APPLICATIONS IN AGRICULTURE ESPECIALLY IN CROP IMPROVEMENT

This subject was investigated by Ghosh and Chatterjee in 2024. Editing tools of Genome, for instance ***"CRISPR/Cas9 technology"***, are used by scientists to find solutions. These tools are making farming more productive. A study found that genome editing (GE) can make changes that are passed down and are expected to happen in certain genomic areas. An outline of the steps needed to carry out genetic engineering (GE) is depicted in Figure 9.

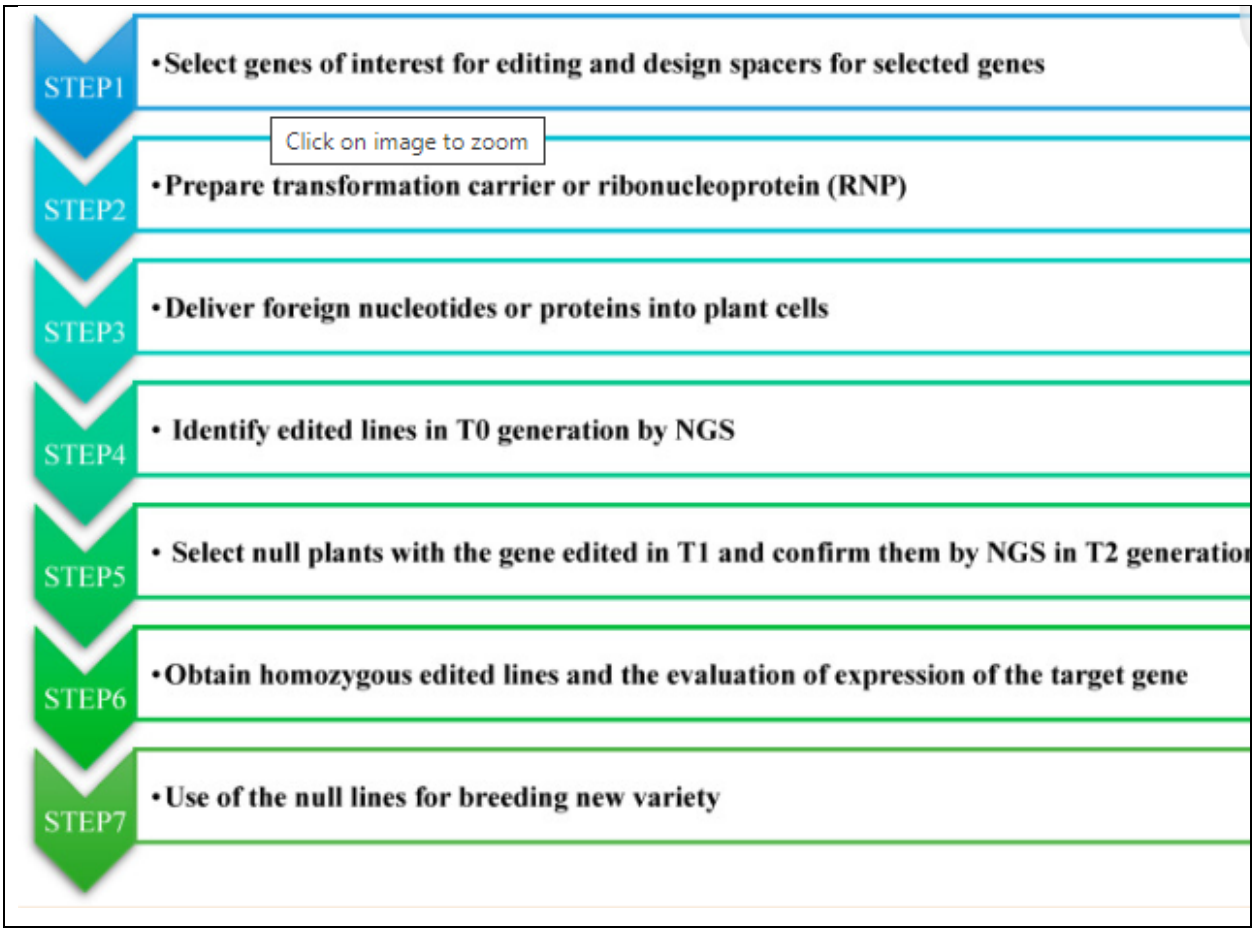

Figure 9: The flowchart of modifications in the DNA through Genomic Editing

(Source: Kang et al., 2022)

The following table will exhibit examples of plants where "the CRISPR/CAS9 mechanism" is used for the positive development of crops.

Category of crops	**Example of plants**
Fiber crops	Cotton
Feed crops	Alfalfa
Food crops	Apple, barley, carrot, chickpea, cucumber, date palm, oats, orange

Oil crops	Flax seeds, soybean, and sunflower
Crops having Ornamental uses	Lily, lotus, and rose
Crops Used in Industries	Coffee, Millet, Papaver, Parasomnia, Sorghum, Sugarcane

Table: Gene-edited crop species using CRISPR/Cas9 system

(Source: Self-made)

7. CHALLENGES AND LIMITATIONS

The research aims to recognize the overall impact and working process of ***"the mechanism of CRISPR/Cas9"*** with its limitations and provide efficient ideas for future improvement. The system faces challenges in the case of designing the effects of off-target in which Cas nucleases cleave the DNA sequence without intentions. However, the gene sequences are similar but not purely identical to the target site. These Genomic Editing systems can introduce unexpected off-target mutations. Unintended genetic changes are brought about by several effects such as detrimental repercussions including regulatory disruptions. The production of gRNA through Cas9 protein faces several challenges as well. It aids in high-quality synthesis and purification of gRNA during the binding and cleavage. The limitations of the system are dependent on the PAM that is downstream of the target sequence. The dependency on the CRISPR/Cas9 system may affect the response to the immune power in the individuals in near future and that will be challenging to mitigate. This includes both safety and efficacy of the immune system and the targeted therapy of specific diseases. There is another challenge raised within the application of the system and that involves the identification and implementation of accurate delivery methods. Hence, the research will discuss the probable solutions for mitigating these obstacles.

8. ETHICAL CONSIDERATIONS

The study of the CRISPR/Cas9 system in genomic editing (GE) involves a few aspects that must be taken under ethical considerations. These include "CRISPR/Cas9" nuclease-

mediated alteration in the sequences of gene. This leads to changes in the genetic traits that can be inherited from one generation to another. This raises a great concern and ethical dilemma in the molecular biology study field. It requires significant improvement to provide ethical frameworks with safeguard the data for preventing misuse of the information.

9. FUTURE DIRECTION AND INNOVATIONS

The upcoming research of ***"CRISPR-Cas9"*** mediated genome editing requires the lowering of the challenges and limitations. This technology holds a promising future with current advancements in molecular biology innovations in the science and medical sector. The improvement of this includes refining of gRNA design with proper algorithms. This includes the optimization of Cas9 variants with the development of novel treatment plans. The care plans integrate target-based therapy at the loci of the genome and that must be precise. The future innovations of CRISPR-Cas9 mediated genome editing system must incorporate with expansion of the CRISPR toolbox with the addition of Cas12 and Cas 13 along with Cas 9. These nucleases will work with their specific properties ranging from RNA targeting and cleavage in single-stranded DNA (ssDNA).

10. CONCLUSION

The research on the systematic approach of the CRISPR/Cas9 nuclease-mediated system summarizes that it is a groundbreaking technology that has revolutionized the genetic engineering field. It has several applications in the molecular biology research such as medicine production, biotechnology, and others. The ongoing innovations and advancements of ***"CRISPR/Cas9"*** technology have a great impact as a genomic editing tool. This works for the alliteration and manipulation of the DNA sequences and opens a new pathway for target-mediated therapies for several diseases such as cancer. The high-fidelity of Cas9 variants with NHEJ and HDR-mediated repair mechanism of the genome shows the potentiality and efficacy of the system. However, along with the advancements, there are some challenges and limitations involved. Those must be monitored through rigorous examination and removed with strategic implementation of the procedure of ***"CRISPR/Cas9"***.

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