Novelty Journals

ISSN 2394-966X

International Journal of Novel Research in Life Sciences
Vol. 9, Issue 2, pp: (84-103), Month: March - April 2022, Available at: www.noveltyjournals.com

APPLICATION OF CRISPR FOR PLANT GENOME EDITING: A REVIEW

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Abstract: **CRISPR-Cas9 technology is a recently created technique for genome editing that offers unparalleled variety, precision, and simplicity. CRISPRs are bacterial DNA segments with brief repeats in their nucleotide sequence.**

Genome editing usually entails identifying a section of a plant's genome that may be altered to make it more disease and herbicide resistant, improve yields, quality, or other desirable features. In vitro and in vivo model systems; CRISPR/Cas9 genome editing has been utilized to fix DNA mutations ranging from a single base pair to massive deletions. A single guide RNA (typically around 20 nucleotides) that is complementary to a target gene or locus and is anchored by a protospacer-adjacent motif achieves site-specific alteration. DNA double-strand breaks (DSBs) are induced by targetable nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) or CRISPR-associated.

The error-prone non-homologous end joining (NHEJ) or homology-directed repair mechanisms, which have evolved over time, are used to repair DSBs (HDR).CRISPR reagents are transported as DNA, RNA, or protein RNA into plant cells, where they assemble into an active site-directed nuclease (SDN) that cleaves specified DNA sequences to produce DSB. Because of its capacity to cut DNA from any genome, the CRISPR/Cas9 system has a wide range of possible applications. The CRISPR gene was discovered in Escherichia coli for the first time. The main objectives of this review shows that the application of CRISPR-Cas9 technology.

Keywords: **Gene targeting, Genome editing, Transcription activator-like effector nucleases, Zinc-finger nucleases, Homology-directed repair, Non-homologous end joining, *CRISPR/ CAS9.***

1. INTRODUCTION

CRISPR has gotten a lot of press in recent years because of its capacity to accurately edit any organism's DNA, as well as the fact that it can be utilized quickly and cheaply. In animals, microorganisms, and plants, the Cas9 protein is an RNA-guided endonuclease that creates targeted double-stranded breaks with only a short RNA sequence to impart target identification (Kumar and Jain, 2014).

Gene editing technology is a novel tool that can be used to make specific changes to the genome. Genome editing usually entails identifying a section of a plant's genome that may be altered to make it more disease resistant or resistant to specific herbicides, or to improve yields, quality, or other desirable features (Jain, 2015).

Researchers employ "molecular scissors" to cut apart and mend the genome, which is a natural process that occurs when plants are stressed and can result in new mutations that help the plant withstand further attacks. Base-pairing interactions between the sgRNA and its complementary target DNA strand help ensure Cas9–sgRNA targeting specificity. Random mutagenesis can result in a huge number of undesirable mutations and rearrangements, as well as large-scale mutant screening. It's still time-consuming and expensive (McCallum *et al.*, 2000). Because of its simplicity and ease of design, CRISPR/Cas genome editing has revolutionized genetic and molecular biology research.

Recently, three well-defined gene editing technologies have emerged: Zinc Finger Nucleases (Carroll, 2011), Transcription Activator-Like Effector Nucleases (Mahfouz *et al.*, 2011; Li, *et al.*, 2012), and Clustered Regularly Interspaced Palindromic Repeats (CRISPRs) with Cas nucleases (Cong *et al.*, 2013). To drive site-specific DNA sequence alterations, these methods create double-strand breaks (DSBs) at target loci. Sequence insertion and deletion, as well as other alterations, are carried out in the host genomes via the error-prone non-homologous end joining (NHEJ) pathway, or sequence correction or replacement is carried out by the error-free homologous recombination (HR) process (Symington and Gautier, 2011). The details of the location: the ZFN and TALEN systems use chimeric proteins to attach to DNA, whereas the CRISPR-Cas systems use an RNA molecule (Shafie and colleagues, 2014).

CRISPR/CAS9 is a ground-breaking genome editing system based on RNA-guided designed nucleases, which have already shown considerable promise in terms of simplicity, efficiency, and adaptability. The CRISPR Cas system can be used to create targeted gene mutations and gene corrections in plants, and it can also be easily designed to perform massive DNA fragment deletion and multiplex gene editing.

In the fields of biology, biotechnology, and medicine, scientists have harnessed and appropriated it for plants, animals, and human embryos since its discovery. Overall, the CRISPR/Cas9 genome editing system outperforms previous genome manipulation methods in terms of simplicity, adaptability, versatility, and efficiency. CRISPR/Cas9 is now being modified to improve its efficiency, specificity, and target range.

The CRISPR-CAS system (Haft *et al.*, 2005) is the newest gene editing technology, combining CRISPR and Cas proteins. CRISPR elements protect bacteria from a variety of viral infections. Nucleases, helicases, polymerases, and polynucleotide-binding proteins all feature functional domains that are comparable to Cas proteins (vander et al., 2009). CAS9 is an RNA-guided DNA nuclease enzyme that generates DNA double-stranded breaks (DSBs) at specified genomic loci to introduce targeted loss-of-function mutations at specific places in the genome. This technique is simple to construct, highly precise, efficient, and well-suited to high throughput and multiplexed gene editing in a number of cell types and species, including plants (Ran *et al.*, 2013).

CRISPR/Cas9 systems, which were recently found as real "do-it-yourself" genome editing tools, A designed single guide RNA (sgRNA) containing a CRISPR RNA (crRNA) and a partially complementary trans-activating RNA (tracrRNA) is required for genome editing (Mali *et al.*, 2013).

The CRISPR/Cas system produces persistent and heritable alterations that can easily separate from the Cas9/sgRNA construct, preventing subsequent CRISPR/Cas changes. In only one generation, homozygous modified transgene-free plants are developed (Brooks *et al.*, 2014; Fauser *et al.*, 2014; Feng *et al.*, 2014; Gao and Zhao, 2014; Jiang *et al.*, 2014; Schiml *et al.*, 2014; Zhang *et al.*, 2014; Zhou *et al.*, 2014).

By segregating out the transgene with self-fertilization in the T1 generation, Xu *et al.*, 2015 successfully created transgenic-free rice with the desired gene mutation. Between 2011 and 2015, CRISPR/CAS9 was used to modify the genomes of several plants, including rice (Li *et al.*, 2012), barley (Wendt *et al.*, 2013), and hexaploid wheat (Wendt *et al.*, 2015; Lor *et al.*, 2014), Sweet Orange (Wang *et al.*, 2014).

Gao *et al.* (2016) aimed to build a method for accurately identifying heritable mutations induced using CRISPR/Cas9 in the Arabidopsis T2 generation. Using simple microscopy techniques, the researchers were able to efficiently screen seeds from the altered plant that were still expressing the Cas9 protein. This will cut down on the time it takes to find plants with CRISPR/Cas9-derived mutations and lower the chance of off-target mutations elsewhere in the genome. Because of its desirable qualities such as exact specificity, multi-gene editing, minimal off-target effects, better efficiency, and simplicity, CRISPR/Cas9 is regarded as a highly promising technology for gene editing in crops (Kumar & Jain, 2014). Invading or foreign DNA fragments are acquired by this unique system in bacteria, which then uses them to detect and

digest subsequent invading DNA or RNA sequences. The primary goal of this review is to discuss the most recent advancements in CRISPR/Case9 technology and their implications for plant genome engineering.

2. LITERATURE REVIEW

2.1 CRISPRS/CAS9 FOR PLANT GENOME EDITING

2.1.1 HISTORICAL DEVELOPMENT OF CRISPRS/CAS9

Restriction endonucleases were discovered originally as a bacterial defensive mechanism (restriction-modification system) against invading phages or plasmid DNA in 1970 (Smith *et al*., 1970). CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR associated protein) was recently used to discover a similar but RNA-based adapted heritable immune system of bacteria. Sequence was first identified in Escherichia coli at the 3' end flanking region of the IAP gene coding for an amino peptidase responsible for alkaline phosphatase isozyme conversion (Ishino *et al.*, 1987).

The CRISPR loci of phage-resistant strains were found modified at the 5'-leader end by the insertion of one to four novel spacers (protospacers) derived from phage genomic sequences. The PAM sequence is specifically recognized by Cas9 as a prerequisite for target DNA binding and possibly strand separation to allow strand invasion and R-loop formation (Jinek *et al.*, 2012).

Genome editing in vitro has been studied since the 1970's with the discovery that exogenous DNA could be taken up by yeast or bacteria and randomly integrated into the genome. Subsequently, targeted integration of DNA into the yeast (Saccharomyces cerevisiae) genome was demonstrated (Scherer *et al*., 1979). A gene of interest is targeted to modify its function, and a nuclease (an enzyme that cleaves nucleic acids) cuts that gene's DNA sequence, breaking the structure of the DNA. After cleavage, a new gene can be inserted, a change to the existing sequence can be made, or a deletion of a specific stretch of genomic DNA can be made.

Genome editing is a type of genetic engineering in which DNA is inserted, deleted, or replaced in the genome of living organisms through the use of engineered nucleases. A new wave of technology that is variously termed "gene editing," "genome editing," or "genome engineering" has emerged to address the ability to precisely and efficiently introduce a variety of genetic alterations into different cells, ranging from the knocking in of single nucleotide variants to the insertion of genes to the deletion of chromosomal regions (Rajat *et al.*, 2018). Developing a genome editing tool requires engineering endonucleases that can create highly efficient and accurate DSBs at a user-defined location in the genome and subsequently activate the cellular pathways involved in DSB repair. Processes via Homologous Recombination (HR)-mediated gene repair or Non-Homologous End Joining (NHEJ).

HR uses homologous DNA sequences as templates for precise repair. It involves strand invasion and requires a homologous DNA template to precisely edit a genomic sequence or insert exogenous DNA that results in gene knock out or gene knock in. Indel mutations are small insertions or deletions that result in small insertions or deletions (indel mutations). This process involves the re-ligation of the two broken ends at the cleavage sites and is catalyzed by DNA ligases (Basing and Alt, 2004). HDR can be utilized to insert a specific mutation with the introduction of a repair template containing the desired mutation flanked by homology arms. HDR can proceed in the same fashion as traditional homologous recombination, using an exogenous double-stranded DNA vector as a repair template (Rouet *et al.*, 1994).

2.1.2. TYPES OF NUCLEASE

The three well-defined gene editing technologies now available (CRISPRs) are Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Palindromic Repeats (CRIPRs). Each of these systems possesses an adjustable sequence-specific DNA binding domain and a nuclease domain that initiates a double-strand cleavage. In a variety of organisms, the CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR associated protein (Cas) technology is proving to be a strong genome editing tool (Gaj *et al.*, 2013).

2.1.2.1. ZINC FINGER NUCLEASES (ZFNS)

ZFNs are synthetic proteins made up of a zinc-finger (ZF) DNA-binding domain and a restriction enzyme FokI nuclease domain. The C2H2ZF domain is mostly involved in DNA binding in natural proteins. ZFNs cleave DNA into a dimer

form. Three-finger ZFNs have a target length of 18 bp, which is long enough to identify a single target site in the human genome (Wolf *et al.*, 2000). ZFNs are DNA-binding proteins that have been fused to fluorescent proteins (FPs) to allow live-cell imaging of genomic loci.

ZFNs are fusion proteins made up of a number of site-specific DNA-binding domains modified from zinc finger transcription factors and coupled to the endonuclease domain of the bacterial FokI restriction enzyme. A 3 to 4 kb DNA sequence is recognized by each zinc finger domain. ZFNs are designed as a pair to cleave a specific location in the genome by recognizing two flanking sequences, one on the forward strand and the other on the reverse strand. The pair of FokI domains dimerize and cleave the DNA at the site when the ZFNs on either side of the site bind, resulting in a double-strand break (DSB) with 5′ overhangs (Urnov *et al.*, 2010). In solution, a full-length FokI coupled to its target site can dimerize with another unattached FokI and cleave DNA (Pernstich and Halford, 2012). The nuclease domains in ZFNs, on the other hand, only efficiently dimerize and cut DNA when both ZFNs attach to their target sites separated by the spacer sequence and face one another, possibly due to their poor affinity (Smith *et al.*, 2000).

2.1.2.2. TRANSCRIPTION ACTIVATOR LIKE EFFECTORS NUCLEASES

Because of their huge size and the necessity of a pair of proteins for recognition, they are less suited than CRISPR/Cas9 (Belhaj *et al.*, 2015). TALEN is one of the genome editing tools available. TALENs appear to be more effective at genome editing and less harmful than ZFNs (Chen *et al.*, 2013). Similar to ZFNs, the specificity of TALENs was recently investigated in vitro (Guilinger *et al.*, 2014).

It's possible to make TAL effector nucleases, a new form of designed site-specific nuclease that fuses a TALE repeat domain to the foki endonuclease domain (Miller *et al.*, 2007). TALE repeats are naturally occurring tandem arrays of 10 to 30 repeats that bind and identify long DNA sequences (Bogdanove and Voytas, 2011). TALENs have been shown to be far more straightforward to construct than ZFNs. In the same manner that ZFNs can induce DSBs at a chosen target spot in the genome, TALENs can also be employed to knock out genes or introduce mutations.

TALE repeat arrays attach to desired DNA sequences with a high affinity, up to 96 percent of the time. TALENs can be created and built in as little as two days and in large quantities, as many as hundreds (Reyon *et al.*, 2011; Cermak *et al.*, 2012). A library of TALENs has been created that targets all of the genes in the genome in which DNA binding occurs. The nuclease domain of the restriction enzyme FokI, an obligatory dimer, has been combined with transcription factor domains.

2.1.2.3. CRISPRS /CAS9 SYSTEM

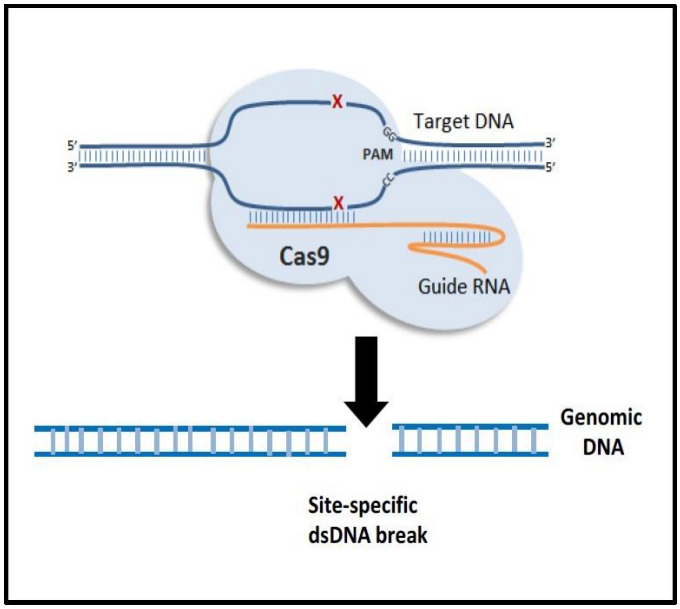

Figure. 1: CRISPR-Cas9 target recognition.

A sgRNA (which contains a CRISPR RNA (crRNA) and a partially complementary transactivating RNA (tracrRNA)) is required for RNA processing and Cas9 recognition in the CRISPR system (CRISPR-associated protein 9). Cas9 is an RNA-guided, dsDNA-binding protein that cleaves both strands of target DNA with a nuclease. With the sgRNA and the target DNA, Cas9 relies on the PAM site and base pairing (Shafie *et al.*, 2014).

The Cas9/sgRNA system associated with Clustered Regularly Interspaced Short Palindromic Repeats is a revolutionary targeted genome-editing approach derived from the bacterial immune system (Shen *et al.*, 2013). There are three varieties of CRISPR systems: type I, type II, and type III. Only one endonuclease, cas9, is included in Type II, making it simple and straightforward to use (Jink *et al.*, 2012). Because of this, type II is frequently utilized in genome editing experiments. In a bacterial cell, the CRISPR-cas9 system consists of crRNA (CRISPR RNA), tracrRNA (Trans CRISPR RNA), and Cas9 nuclease protein.CRISPR Cas systems often cleave DNAs that are complementary to repeat-intervening spacers derived from protospacer sequences. This spacer-guided CRISPR-Cas cleavage could cause target degradation, preventing the transmission of transmissible genomic sequences with the appropriate proto spacer.

A single guide RNA (SgRNA) can be created by fusing crRNA and transcrRNA, which enhances Cas9 DNA cleavage in vitro (Jinek et al., 2012). To combat plasmid or bacteriophage invasions, an RNA-mediated adaptive defense mechanism known as the clustered regularly interspaced short palindromic repeats (CRISPR) system exists. CRISPR/Cas9 allows bacteria to chop apart the DNA of invading bacterial viruses that would otherwise kill them. Similar to the discovery of restriction enzymes, CRISPR/Cas9 systems are new weapons in the arsenal of genome editing. The CRISPR (clustered regularly interspaced short palindromic repeats) method has recently been developed to give site-specific DNA recognition and cleavage via an RNA guide that may be customized (Cho et al., 2013).

The discovery of bacterial adaptive immune systems known as clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems has resulted in the development of the most recent set of genome-editing tools. CRISPR-Cas systems target specific DNA sequences for cleavage using a mix of proteins and short RNAs. Bacteria acquire "proto spacers" from foreign DNA sequences (such as bacteriophages), incorporate them into their genomes, and use them to express short guide RNAs, which are then employed by a CRISPR-Cas system to delete any DNA sequences that match the proto spacers. The size of the Cas9 protein is one downside of CRISPR-Cas9. The cDNA encoding S. pyogenes Cas9 is about 4.2 kb in length, slightly larger than a TALEN monomer and significantly larger than a ZF monomer (but smaller than a ZF monomer).

Dimerization is required for both TALENs and ZFNs, resulting in greater effective sizes. The complementarity between SgRNA and the target DNA determines the specificity of CRISPR Cas9.

Type II CRISPR-Cas systems offer bacteria with adaptive immunity to viruses and plasmids, and the CRISPR-Cas9 technology is derived from them. Cas9, a CRISPR linked protein, is an endonuclease that forms base pairs with DNA target sequences using a guide sequence within an RNA duplex, tracrRNA or crRNA, allowing Cas9 to cause a site-specific double-strand break in the DNA (Doudna and Charpentier, 2014).

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a genomic region that contains tandem direct repeat sequences and proto spacers, which are the gaps between repeat sequences and are both derived from invading elements (Kim and Kim, 2014). The CRISPR loci contain Cas9 genes, as well as sequences for non-coding RNA elements known as CRISPR RNA (crRNA) and short trans-encoded CRISPR RNA, also known as trans-activating crRNA (tracrRNA). When targeted by a guide RNA, the two RNA sequences crRNA and tracrRNA form a complex called guide RNA, which dictates the specificity of the cleavage of the target sequence in the nucleic acid coupled with the Proto spacer adjacent motif sequence (a 5"-NGG (Barrangou, 2013; Jinek et al., 2013). (Cong *et al.*, 2013; Mali *et al.*, 2013). At the 50-nucleotide end of the sgRNA, 20 nucleotides direct Cas9 to the corresponding target site. For innovative genome editing applications, the first 20 nucleotides of the gRNA can be changed to target any DNA sequence of the form N20-NGG (Sander and Joung, 2014).

The introduction of nuclease-driven DNA-breaks utilizing tailored engineered nucleases is used to generate desired endogenous alterations such as gene disruption, addition, or correction at one or more particular genomic sites (Kim and Kim, 2014). In a wide range of animals and cell types, the cellular recombination repair mechanism can perform the necessary alteration after a double strand break. In contrast, the newly established CRISPR–Cas system directs the Cas9 endonuclease to complementary target DNA using a short single guide RNA (sgRNA) (Gaj *et al.*, 2013).

International Journal of Novel Research in Life Sciences
Vol. 9, Issue 2, pp: (84-103), Month: March - April 2022, Available at: www.noveltyjournals.com

Table 1: A general Comparison among the sequence specific genomeediting tools

Particulars	ZFN	TALEN	CRISPR
Target specificity determineby	Zink fingerprotein	Transcription active like effectors protein	CrRNA(SgRNA)
Type of interaction	Protein DNA	Protein DNA	RNA-DNA
Type of interaction nuclease uses	FOKI	FOKI	CASE9
Specific determine length of target site	18-36 bp	30-40 bp	20 bp
Target site	Rich in G content	Start with T and end with A	End with pam sequenceslike NGG or NAG

2.2. GENE REPLACEMENT VIA HOMOLOGOUS RECOMBINATION AND NONHOMOLOGOUS END JOINING RECOMBINATION.

Genome editing is the process of creating mutations or precise alterations in a specific region of the genome using cellular DNA repair processes. Errors may arise during the repair process, resulting in a mutation at a desirable location. Exogenous DNA could be incorporated into endogenous double-strand break repair mechanisms, resulting in very particular researcher-designed modifications. To re-join severed ends, NHEJ uses DNA lipase IV. The repair may be incomplete if bases have been removed or the ends have been processed by nucleases. HDR, on the other hand, employs a template for correction, so the results are more likely to be perfect (Li *et al*., 2013).

Engineering an endonuclease that can create highly efficient and accurate DSBs at a user-defined location in the genome and then activate the cellular pathways involved in DSB repair processes via Homologous Recombination (HR)-mediated gene repair or Non-Homologous End Joining is required to develop a genome editing tool (NHEJ). For accurate repair, HR uses homologous DNA segments as templates. It includes strand invasion and necessitates the use of a homologous DNA template to accurately alter a genomic sequence or insert exogenous DNA, resulting in gene knockout or knockin. Bassing and Alt (2004) describe an error-prone ligation mechanism that results in indel mutations due to minor insertions or deletions. With the use of a repair template, HDR can be used to insert a specific mutation. The desired mutation is flanked on both sides by arms of homology. Genome cutters are made up of a unit that recognizes certain areas of DNA and an enzyme that cuts both strands of DNA in the sequence that the genetic engineer specifies. When a cell detects a double-strand DNA break, it activates the cell's machinery to repair the damage. There are two scenarios that could occur.The desired mutation is flanked on both sides by

1. Non-homologous end-joining (NHEJ), which causes a mutation at the point where the genome scissors cut the DNA, is followed by a natural repair process.

2. Homologous recombination (HR): It involves the introduction of a second DNA molecule that contains the same areas as the region in the host genome that he is attempting to modify, as well as a gene coding for the desired additional characteristic. The HDR pathway has a lower risk of error because it uses a template strand for repair, whereas the NHEJ pathway, which does not use a template strand, may result in insertion-deletions in the repaired sequence.

2.3 METHODS FOR INTRODUCING CRISPR/CAS9 PLANT CELLS.

Plasmid vectors with the Cas9 and gRNA expression cassettes make Cas9-mediated plant genome editing simple to deploy. CRISPR reagents are supplied as DNA, RNA, or protein-RNA into plant cells, where they assemble into an active site-directed nuclease (SDN) that cleaves specified DNA sequences to cause double-strand breaks (Yin *et al.*, 2017).

The CRISPR locus is made up of short palindromic repeats separated by "spacers," which are non-repetitive sequences. When an infection occurs, spacers are replicated and inserted into the CRISPR locus by invading sources of DNA, such as phages. A second locus contains genes that code for CRISPR-associated endonucleases (Cas), which are enzymes that cut the genome. A CRISPR RNA molecule (CRISPR RNA or crRNA) forms a complex with Cas and a trans-activating crRNA (tracrRNA) to guide the nuclease to the exogenous sequence when a repeat infection occurs from the same invading DNA. The Cas-RNA complex will recognize a complementary crRNA-targeted DNA target that is adjacent to a specific 3-nucleotide region. The invading DNA is then cut and deactivated by the complex.

CRISPR/Cas9 is an RNA-guided endonuclease (RGEN) that has revolutionized the field of genome engineering. Various approaches for delivering the two components, gRNA and Cas9, have been designed and tested in order to achieve

CRISPR/Cas9-mediated genome engineering. The two components can be injected into cultured cells and animal embryos via DNA/RNA/protein transfection or microinjection.

When numerous plasmids, plasmid and DNA fragments, single all-in-one plasmids, or RNA/protein are inserted, multiplex genome engineering can be used (Jao *et al.*, 2013; Liet *et al*., 2013b; Wang *et al.*, 2013; Guo *et al.*, 2014; Ma, *et al*., 2014; Sakuma *et al*., 2014).

In vitro, purified Cas9 protein and gRNAs can create ribonucleoproteins (RNPs), which can then be electroporated into cells (Kim *et al.*, 2014).

RNPs can be transported into cells by simply adding them to the media if a cell-penetrating peptide is added for gRNAs and coupled with Cas9 (Ramakrishna *et al.*, 2014). An artificial plasmid transformation is a viable and versatile tool for monitoring CRISPR activity against a variety of cloned targets in a variety of situations.

Forward genetics screening in cultured cells is now possible thanks to a lentiviral CRISPR/Cas9 library (Koike, et al., 2014; Shalem *et al.*, 2014; Wang *et al.*, 2014; Zhou *et al.*, 2014). Protoplast transformation or Agrobacteria infection have been used to supply for plant applications (Feng *et al.*, 2013; Li *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013).

The delivery of the CRISPR-Cas9 effector complex (DNA, RNA, or protein) and the discovery of mutations are the key differences in the techniques (PCR screen, visible markers, or obvious phenotypes). Custom guide RNA is produced in cultured cells or embryos to direct site-specific DNA cleavage by the Cas9 endonuclease in the CRISPR/Cas9 system (Cho *et al.*, 2013; Hwang *et al.*, 2013; Jiang *et al.*, 2013). Vectors are utilized to express gRNAs and Cas9, RNA injection dosages, and target site nucleotide sequences. In the presence of a short PAM-providing DNA oligonucleotide, RNA-guided Cas9 cleaves single-stranded RNA (ssRNA) targets (PAMmer). Affinity tags are not required; only a matching gRNA and corresponding PAM mer must be synthesized. sgRNAs can be given as either PCR amplicons with an expression cassette or sgRNA-expressing plasmids, depending on the application.

Transformation efficiencies are frequently calculated using input DNA samples containing a single plasmid in transformation procedures. The number of cells that have received that plasmid (transformants) per microgram of input DNA must then be normalized against equivalent data collected in an independent experiment using a reference plasmid (inferred from the number of colony forming units).

Vector constructs containing the Cas9 and sgRNA expression cassettes must be introduced into plant cells to induce genome editing in vivo. Researchers used a transient expression system to directly transport plasmids carrying the Cas9 and sgRNA expression cassettes into protoplasts via vacuum infiltration of tobacco or Arabidopsis leaves to evaluate the feasibility and efficacy of the CRISPR/Cas9 system in the early phases of testing (Jiang *et al.*, 2013).

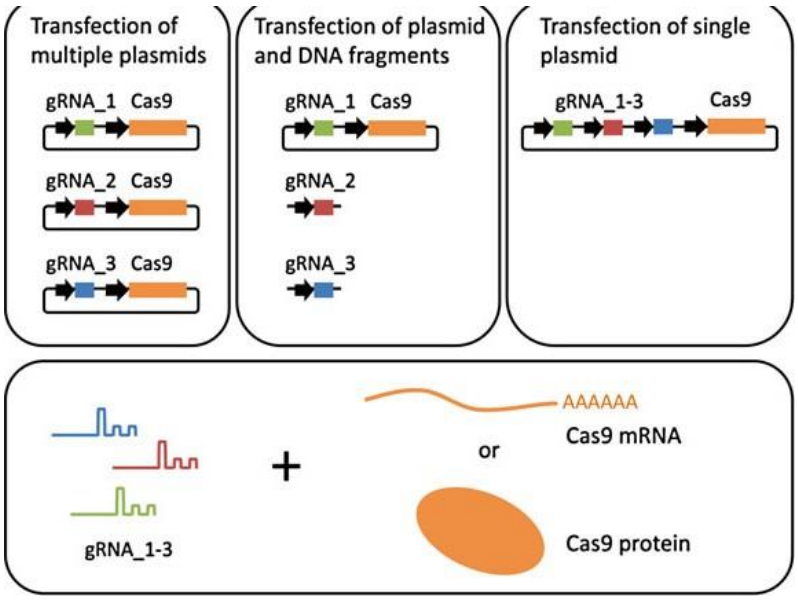

Figure. 2: Examples of transfection strategies for CRISPR/Cas9-mediated multiplex genome engineering.

For DNA transfection, several plasmids, plasmids plus DNA fragments, or single plasmids can be used (upper panels). On the other hand, Csy4-mediated cleavage of lengthy transcripts, on the other hand, can result in numerous gRNAs (Nissim et al., 2014; Tsai et al., 2014). For DNA-free transfection, many gRNAs are generated in vitro and Cas9 mRNA or protein can be employed. Immunity to genomic proto-spacers generated by CRISPR systems contained in transforming plasmids could be addressed as well.

2.4 THE CRISPR-CAS9 GENOME TARGETING MOLECULAR MECHANISM

Cas9 encourages genome editing by inducing a DSB at a specific genomic location. CRISPR-Cas is a microbial adaptive immune system that cleaves foreign genomic elements using RNA-guided nucleases. The endogenous CRISPRS/case9 system in bacteria and archaea performed adaptive antiviral immunity via three mechanisms: adaptation, crRNA synthesis, and interference (Wright et al., 2016).The acquisition of viral DNA begins with the infection of undocumented DNA. When bacteria detect bacteriophage invasion, they quickly protect themselves by inserting viral DNA, known as the protospacer, onto their chromosomes at the end of CRISPR loci (Wright *et al*., 2016). Bacteria commence the reproduction of a repeated DNA sequence—the repeat—to maintain the shape of the CRISPR array (Barrangou *et al.*, 2007).

Following that, crRNA biogenesis occurs in two steps. The CRISPR locus and the Cas gene are first translated into a single pre-crRNA and Cas protein, respectively. Different types of CRISPR systems encode different Cas proteins during this process. The type II system, in particular, is the only one known to use a single endonuclease, the Cas9 protein (Hsu *et al.*, 2014). The Cas9 protein, along with two other endonucleases, then aids in the finalization of crRNA in the type II system. A tracrRNA (trans-activating crRNA) is also encoded by a gene close to the CRISPR locus by transcription (it mates with the crRNA via the Watson-Crick base pairing rule, which is stabilized by Cas9 protein) (Wright *et al.*, 2016). Specificity in biology and applications the repeats are cleaved by an endogenous RNAase, resulting in distinct strands.

crRNAs. The repeats and parts of the spacers were then truncated by an unidentified endonuclease, signaling the end of biogenesis (Deltcheva *et al.*, 2011; Jinek *et al.*, 2012).

As Farley described it (Farley et al., 2015), the GG-nucleotides bind to two amino acids in Cas9 proteins and then "pull away from their equivalent CC-nucleotides on the opposite strand of DNA," as Farley *et al.* (2015). Cas9's rock-hard double-helix structure, which is formed by double and triple hydrogen bonds between two strands, is briefly destabilized by additional contact between N-nucleotide and other amino acids.

The creation of DNA-RNA hybrid duplexes and the cutting of target DNA are required for inference, the final step in CRISPR adaptive immunity (Wright *et al.*, 2016). The protospacer adjacent motif (PAM), which is found just downstream of the CRISPR locus and has the form "Ny base Guanosine Guanosine," or simply NGG, directs crRNA recognition and is directly recognized by endonucleases, is critical in crRNA recognition.

The interaction of N-nucleotide with other amino acids in Cas9 briefly destabilizes the rock-hard double-helix structure formed by double and triple hydrogen bonds between two strands of DNA, allowing crRNA recognition if the crRNA can pair with the immediate upstream PAM. Cas9 commences its particular cleavage on target DNA following the unwinding of target DNA and the production of DNA-RNA hybrid duplexes as part of the CRISPR immunity mechanism (Bortesi and Fischer, 2015).

Bacterium can preserve a genomic record of bacteriophage infection and pass on the acquired spacers to their progenies over time, ensuring hereditary immune defense in some bacteria strains (Barrangou et al., 2007). Because of the Cas9 protein's unique structure and conformation, the CRISPR/Cas9 system provides sequence specificity. The Cas9 protein has a conserved core and a bi-lobed design with two nucleic acid binding grooves: a large recognition (REC) lobe and a small nuclease (NUC) lobe that are joined by a helix bridge. The mechanism of CRISPR-Cas9 has been further elucidated thanks to structural studies of S. pyogenes Cas9.

Cas9's molecular structure, discovered by electron microscopy and x-ray crystallography, reveals that the protein experiences a significant conformational change upon binding to the guide RNA, followed by another shift when it associates with a target double-stranded DNA (dsDNA). Cas9 nuclease's ability to bind to particular DNA locations with great fidelity makes it appealing for gene function research. To prevent DNA cleavage at the target spot, Cas9

endonuclease activity can be turned off (denoted as "dead Cas9" or "dCas9"). Cas9 is inactive in the natural world. Its REC lobe is activated when it is coupled with sgRNA. Using Watson–Crick pairing between sgRNA and targeted DNA, the Cas9-sgRNA complex scans a DNA double strand for stringent PAMs (the trinucleotide NGG). The HNH nuclease domain cleaves the RNA-DNA hybrid after anchoring at the correct PAMs, whereas RuvC cleaves the other strand to generate a double-strand break (13). In addition to mutagenesis, it plays a key role in binding the guide RNA target DNA hybrid (Jinek *et al.*, 2014; Nishimasu *et al.*, 2014).

Cas9's conformational change could be part of the mechanism of target dsDNA unwinding and guide RNA strand invasion. The PAM is required for initial DNA binding; without it, Cas9 will not identify even target sequences that are entirely complementary to the guide RNA sequence (Sternberg *et al.*, 2014). The CRISPR/Cas9 system is made up of multiple components, including target site specific CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA) or single guide RNA (sgRNA), and Cas9 endonuclease. When the Cas9 protein targets and cleaves a specific sequence within the genome, it causes a double-stranded break (1). The endogenous DNA repair process, non-homologous end joining (NHEJ), and homologous directed repair work together to mend this double-stranded break (Anders *et al.*, 2014).

Researchers used chromatin immune precipitation and high-throughput sequencing to evaluate the number and types of Cas9 binding sites on the chromosome to analyze Cas9's target-binding activity in cells. By modifying the 20-bp proto spacer of the guide RNA, CRISPR-Cas9 can be easily modified to target any genomic sequence. CRISPR has a high frequency of indel mutations that can be used to change the chromosomal target (Cradick *et al.*, 2013). CRISPR-Cas9 also allows for the simultaneous targeting of many sequences for multiplexed gene editing (Cradick *et al.*, 2013; Wang *et al.*, 2013).

The following are the general mechanisms of CRISPR/CAS9: (1) foreign DNA acquisition, (2) synthesis and maturation of CRISPR RNA (crRNA), followed by the formation of RNA Cas nuclease protein complexes, crRNA recognition, and Cas nuclease cleavage of foreign DNA

In the Type II CRISPR/Cas system (crRNAs), short pieces of foreign DNA known as "spacers" are integrated within the CRISPR genomic loci and transcribed and processed into short CRISPR RNA.These crRNAs bind to trans-activating crRNAs (tracrRNAs) and drive Cas proteins to cleave and silence pathogenic DNA based on sequence.

In prokaryotes, CRISPR/Cas9 mediated immunity is acquired. Cellular invaders, such as phage viruses, inject nucleic acid sequences into the host cell during the acquisition phase. After infection, unique DNA sequences from the cellular invaders are integrated as spacers (colored circles) bordered by repetitive sequences into the host CRIPSPR locus. As a result, when the CRISPR locus is transcribed, the newly acquired protospacer sequences are encoded by the pre-CRISPR RNAs (crRNAs). Individual crRNAs are produced once the pre-crRNA is cleaved, and these crRNAs will bind with Cas proteins. In the B.interference phase, the Cas protein uses crRNAs as guidance to silence foreign DNA that matches the crRNA sequence. As a result, the crRNA/Cas9 combination is activated the second time a bacteria encounters the same foreign DNA.

2.5 PLANT STRESS RESPONSES AND ADAPTIVE EPIGENETIC MECHANISMS

"Epigenetics is the study of heritable changes in gene expression patterns caused by changes in DNA bases, histone proteins, and/or non-coding RNA synthesis without affecting the nucleotide sequence.

Numerous studies have recently revealed new information about the epigenetic control of stress adaptation. Plants' stress-induced phenotypic responses are controlled by epigenetic factors.

Methylation Regulation is a term used to describe the process of modifying the regulation of stress-related gene expression and has been linked to epigenetic processes (Chinnusamy and Zhu, 2009). The variation in phenotype is caused by DNA in restress. Almost all genetic activities, including reproduction, are regulated by these pathways.

Transcription, replication, DNA repair, gene translocation, and cell differentiation are all processes that take place in the body. In 1994, viroid-infected tobacco plants were found to have RNA-directed DNA methylation. After autonomous viroid RNA-RNA replication, transgenic tobacco expressing the potato spindle tuber viroid showed viroid methylation. Later research revealed that heritable promoter methylation is regulated by double-stranded RNA (dsRNA), which in tobacco is cleaved into tiny 23-nt RNAs. Until the discovery of two plant-specific DNA-dependent RNA polymerases in

Arabidopsis, the transcription of highly methylated heterochromatin to yield the precursor RNA transcripts required for siRNA synthesis remained a mystery. The promoter and gene body portions of DNA are methylated, allowing the gene to exist in a suppressed state.

DNA methyltransferase and DNA demethylase enzymes regulate the overall condition of DNA methylation. Small RNAs also play a role in epigenetic control in response to abiotic and biotic stress, as well as growth and developmental signals, by suppressing transcriptional genes via RNA-directed DNA methylation (Zheng *et al.*, 2013). Several studies have been reported that support DNA methylation-mediated responses to biotic and abiotic stressors. Gene modification via the DNA methylation pathway for stress-resistant and tolerant transgenic plant development necessitates good gene selection first.

2.6 RNA DIRECTED DNA METHYLATION AND DEMETHYLATION PLANT

RNA-directed DNA methylation (RdDM) involves the cytosine methylation of DNA sequences that are complementary to the siRNAs in RNA-directed DNA methylation (RdDM). In plants, RNA-dependent RNA polymerase 2 (RDR2) generates double-stranded RNAs (dsRNAs), which serve as precursors for the Dicer-like 3-dependent synthesis of 24-nt siRNAs. The covalent enzyme-catalyzed transfer of a methyl group from S-adenosyl methionine to the 50 position of cytosine, transforming cytosine into 5 methylcytosine, is known as cytosine methylation (5mC). Depending on the species, it can be anywhere from 6 to 25% of total cytosine (Steward et al., 2000). The chromatin structure is determined by histone variations, histone N-terminal tail modification, and DNA methylation. DNA methylation also causes a chromatin shape that is transcriptionally restrictive. Cytosine is found in plants in all sequence contexts (CpG, CpHpG, and CpHpH, where H is hydrogen).

Methylation can be done on adenine, cytosine, or thymine. In the Arabidopsis genome (Huettel et al., 2007), about 24% of CG, 6.7 percent of CHG, and 1.7 percent of CHH are methylated.

In bacteria, DNA methylation shields the host genome from restriction enzymes that cleave phage DNA, whereas in vertebrates and angiosperms, DNA methylation serves a variety of roles, including transposon silencing, transcriptional gene silencing (TGS), imprinting, and permutation (Wassenegge *et al.*, 1994). After autonomous viroid RNA-RNA replication, transgenic tobacco expressing the potato spindle tuber viroid showed viroid methylation (Huettel et al., 2007). Further research on the involvement of RdDM and the function of gene body methylation in gene regulation is required. DNA methylation is a type of epigenetic imprint that changes over time. Heterochromatic histone modifications and siRNAs cause DNA methylation at specific loci.

2.6.1. SIRNA BIOGENESIS

Heterochromatic siRNAs (hc-siRNAs), trans-acting siRNAs (tasiRNAs), and natural antisense transcript-derived siRNAs are the three types of endogenous siRNAs produced by flowering plants (nat-siRNAs). Hc-siRNAs are also known as repeat-associated siRNAs or chromatin-associated siRNAs (casiRNAs) (rasiRNAs). The presence of dsRNA precursors is required for siRNA synthesis. Sequence complementarity allows inverted repetitions, pseudo genes, and natural antisense transcripts to generate dsRNA, whereas RNA-dependent RNA polymerases convert transcripts from heterochromatic loci to dsRNA (RDRs). DsRNAs are cleaved into single-stranded 20 to 24-nt siRNAs by a Dicer-like family of ribonucleases III (DCL2, DCL3, and DCL4 in Arabidopsis). These siRNAs are loaded onto RISC (RNA-induced silencing complex) proteins that contain the argonaute (AGO) protein (Jones et al.,2006). Only NRPD1a is required for the analysis of endogenous siRNA synthesis from DNA. Pol IV can create precursor transcripts from methylation heterochromatin, transposons, and repetitive DNA, which are then converted to dsRNA.

2.7. APPLICATION OF CRISPRS/CAS9

One of the uses that have been suggested is the use of CRISPR to propagate so-called gene drives to eradicate diseases like malaria by affecting the mosquito vector's ability to transmit the disease to spread the illness or to improve crop kinds (Woo et al., 2015). CRISP could be employed in human medicine for a variety of objectives, including enhancing gene function, screening for new therapeutic targets, and direct treatments (Charpentier, 2015). CRISPR has also paved the way for the development of gene drives, which function by implanting gene-editing equipment in living things so that specific DNA gets distributed every time an organism reproduces (Gantz & Bier, 2015). Gene drives have the potential to

help control invasive species, reduce disease, and improve agriculture by addressing pesticide and herbicide resistance in insects and weeds (Esvelt *et al.*, 2014).

2.7.1. MODIFICATION OF MULTIPLE GENOMIC SITES HAS DEVELOPED A TOOLKIT FOR MULTIPLEX GENOME.

Editing in plants with a binary vector set based on CRISPR/Cas9 and a gRNA module vector set (Xing *et al.*, 2014). This makes it easier to express CRISPR/Cas9 in a variety of plant systems, and it's especially useful for high-throughput multiplex plant genome editing (Xing *et al.*, 2014). Many applications exist for simultaneous editing of many genomic loci in plants, such as investigating numerous related genes, knocking off functionally redundant genes, or genetic improvement of multiple features in crop breeding. As a result, plant genome editing requires only the transfer of two components, Cas9 and sgRNA, to the host cell via genetic transformation procedures (Baltes *et al.*, 2014).

To enable systematic reverse engineering of causative genetic variants by permitting selective manipulation of individual genetic elements, precise and efficient genome-targeting tools are required. The RNA-guided Cas9 nuclease has been used to develop a family of precision genome engineering tools (Lee et al., 2009). The adaptive immune system of type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) (Pattanayak *et al.*, 2011). The type II CRISPR locus in Streptococcus pyogenes contains four genes, including the Cas9 nuclease, and two non-coding CRISPR RNAs (crRNAs): trans-activating crRNA (tracrRNA) and a precursor crRNA (pre-crRNA) array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (Boch *et al.*, 2009).

Precision and efficient genome-targeting technologies are required to enable systematic reverse engineering of causative genetic variants by allowing selective manipulation of individual genetic elements. A suite of precision genome engineering tools has been developed using the RNA-guided Cas9 nuclease (Lee et al., 2009). CRISPR (clustered regularly interspaced short palindromic repeats) is a type II prokaryotic adaptive immune system (Pattanayak *et al.*, 2011). In Streptococcus pyogenes, the type II CRISPR locus contains four genes, including the Cas9 nuclease, as well as two non-coding CRISPR RNAs (crRNAs): a trans-activating crRNA (tracrRNA) and a precursor crRNA (pre-crRNA) array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (Boch *et al.*, 2009).

2.7.2. APPLICATION OF CRISPRS/CASE9 IN CROPS IMPROVEMENT

Modern agriculture is an excellent example of how science and technology may be combined to increase crop productivity and quality. Plant genome editing with sequence-specific nucleases has a lot of potential for crop enhancement in order to fulfill rising global food demands and provide a sustainable agricultural system (Liu et al., 2013). Traditionally, crops were enhanced using conventional and mutation plant breeding techniques, which are currently being hampered by the decline of existing genetic variation in plants, posing a threat to future food production (Chen and Gao, 2014).

Rice blast resistance was improved by creating a CRISPR/Cas9 SSN (C-ERF922) that targeted the transcription factor gene OsERF922 in rice.

There is a pressing need for effective crop enhancement strategies that use newer genome editing techniques such as the CRISPR/Cas9 system to improve existing critical functions or create new lucrative products (Zhang and Zhou, 2014). CRISPR/Cas9 and its modified forms have been intensively investigated in diverse organisms for a variety of applications, including gene mutation, gene expression repression or activation, and epigenome editing. Genome editing provides for precise and predictable changes to elite cultivars or accessions without the need for time-consuming backcrossing in traditional breeding methods. The CRISPR/Cas9 method should make pyramid breeding more efficient by allowing numerous features to be tweaked at the same time (Bortesi and Fischer, 2015).

Negative regulators of grain development and disease resistance can be tweaked to boost agricultural yield while also arming the host with pathogen resistance. Gene expression regulation and epigenetic modulation are two more ways of gene alteration that can be employed in agriculture.

A native Cas9 DSB can be repaired using either the HR or NHEJ methods (Wyman and Kanaar, 2006; Shuman and Glickman, 2007). HR-mediated repair can be used to introduce particular proteins into the body.

Point alterations such as nucleotide substitutions or by recombination of the target locus with external DNA templates to insert desired sequences (Jiang *et al.*, 2013). Instead of genome editing, the nuclease deficient catalytically inactive mutant variant of Cas9 (dCas9) has been employed for RNA-guided transcription regulation (Gilbert *et al.*, 2013; Qi *et al.*, 2013). Using dCas9 with an effector and sgRNA, this modified system has been employed for CRISPR interference (CRISPRi) and CRISPR activator (CRISPRa) for highly effective and precise gene silencing and activation, respectively. The dCas9 protein can attach to the target and integrate gRNA (*Xu et al.*, 2014).

As a result, the dCas9/sgRNA system provides a universal platform for RNA-guided DNA targeting to modulate transcription in a stable and efficient manner. There are three types of RNA-directed endonuclease applications, particularly in plants.

First, Cas9-induced DSBs were repaired using the non-homologous end joining (NHEJ) approach to generate indels, resulting in frame-shift mutations that are analogous to natural variants or those produced through physical or chemical mutagenesis as in mutation breeding (Chen and Gao, 2014; Saikaetal, 2014).

In the second category, Cas9 was utilized with a brief DNA repair template or a transgene to repair DSB using homologous recombination (HR) for point mutations, targeted transgene insertion, gene replacement, and gene tagging at predefined places. This avoids the location implications associated with genetic engineering's random insertion of genes into plant genomes. The third type employs multiplex genome editing to target numerous locations at once. Multiplex genome editing in plants can be used to examine the activities of redundant gene family members and to investigate epistatic interactions in genetic pathways (Xing *et al.*, 2014).

The CRISPR/Cas system creates heritable and persistent modifications that easily detach from the Cas9/sgRNA construct, inhibiting future CRISPR/Cas changes.

Homozygous modified transgene-free plants are created in just one generation and developed successfully (Brooks, *et al.*, 2014; Fauser *et al.*, 2014; Feng *et al.*, 2014; Gao and Zhao, 2014; Jiang *et al.*, 2014; Schiml *et al.*, 2014; Zhang *et al.*, 2014; Zhou *et al.*, 2014).

Transgenic-free rice with the desired gene mutation can be achieved by segregating out the transgene with self-fertilization in the T1 generation. Cas9 nucleases have been found to have a higher relative cleavage effectiveness against the same target sites than previously characterized TALENs and ZFNs (Gaj *et al.*, 2013; Johnson *et al.*, 2015).

As a result, plant genome editing requires only the transfer of two components, Cas9 and sgRNA, to the host cell via genetic transformation procedures (Baltes *et al.*, 2014). When the replication initiation protein gene is co-transformed with the Cas9/sgRNA construct, Gemini virus replicons (GVRs) can be employed to deliver Cas9/sgRNA to plant cells with improved mutagenesis.

In both basic and applied plant research, targeted genome alteration is critical for revealing and modifying gene functions. The efficient delivery of genome editing reagents like Cas9 nucleases, gRNAs, and homologous recombination DNA donors is critical for high-efficiency targeted genome alteration, which is difficult for most plant cells with cell walls.

One of the two main reasons for the ease with which Cas9 may be introduced into target cells is that it simply requires plasmids and the corresponding generated sgRNA. Furthermore, the CRISPR-Cas9 technology, as a tool for multiplex gene editing, not only provides a platform for scientists to analyze genomes from a broader viewpoint, but it also gives rise to new treatments for polygenic illnesses. Following the introduction of CRISPR systems, a slew of novel cellular models have emerged, demonstrating considerable benefits in epigenetic regulation and genome architecture research (Hsu *et al.*, 2014). Epigenetic alterations are crucial to biological processes because they directly regulate genomic functioning. Previous research has used zinc finger proteins and TAL effectors to target similar enzymes (Hsu *et al.*, 2014).

On the other hand, Cas9 epigenetic effectors can be used to remove or insert genetic information at specific epigenetic loci with greater flexibility. Similarly, the spatial design of many cellular constituents has a significant impact on the functional output of genomes (Hsu *et al.*, 2014). Cas9 combined with fluorescent protein allows for live-cell imaging at various loci and in multiple hues, paving the path for further research into genome architecture tools.

2.7.3. PLANT GENOME GENETIC IMPROVEMENT AND BIOFUEL DEVELOPMENT USING CRISPR/ CAS9 TECHNOLOGY

Through guide RNA (sgRNA) technology, the CRISPR/Cas9 system can be utilized to build disease resistance in plants. CrRNA and trans-encoded CRISPR RNA combine to generate SgRNA (Qi *et al*., 2013).

Plant breeding for pest resistance, disease resistance, and increased productivity aids in the production of disease-resistant crop cultivars, ensuring food security (Melchers and Stuiver, 2000). The key to long-term agriculture is increasing genetic diversity in plant crops. (Kissoudis and colleagues, 2014). Random mutagenesis approaches, like mutagenesis and radiation, are being phased out of plant biotechnology in favor of genome editing tools that allow precise manipulation of specific genomic sequences. Endogenous cell processes, primarily non-homologous end joining (NHEJ) and homology-directed repair, repair DSBs (HDR).

Plant genome editing using CRISPR/Cas9 was initially described in 2013 for monocotyledonous crop plants such as rice and wheat (Qiu et al., 2013). Streptococcus pyrones Cas9 (SpCas9) was modified to optimize the bacterial gene cordons for plant expression and was employed with the attachment of plant nuclear localization signals for dicotyledonous model plants A. thaliana and Nicotiana benthamiana (Li et al., 2013).

Another application of CRISPR/Cas9 is to integrate Cas9/sgRNA for virus resistance, which aids in the creation of virus-resistant transgenic plants.

The application of CRISPR Cas technology in biotechnology is primarily focused on the following directions, based on reverse engineering and reconstruction: improving agricultural crops, researching plant genomes, and developing a sustainable and accessible biofuel based on the discovery of new biological pathways in algae and corn (Jacob *et al.*, 2015). All investigations show that the CRISPR-Cas9 method efficiently delivers transgenes and produces numerous mutations in rice, wheat, maize, and soybeans (Li *et al.*, 2014). CRISPR/Case9 is a highly effective technique for creating "transgene-free, homozygous mutants" (Zhang *et al.*, 2016).

Base editing and plant breeding can be accomplished by creating extensive and precise point mutations in an existing plant population. Inversion is another important source of variation in plant populations and genomic sequence translocation, which can result in protein domain swaps, gene regulatory changes, and potentially new gene functions. Non-homology end joining (NHEJ) is the primary DSB repair process in many plant cell types, resulting in imprecise genome modification or fast DSB repair. Currently, HDR requires significant quantities of repair templates to be delivered. Plant species that are already well adapted to multiple settings could be domesticated with high-value features instead of expanding the environmental and disease tolerance of existing domesticated crops.

The use of CRISPR/Cas9 in plants is just getting started. In the first generation of Arabidopsis, numerous genes, including AtPDS3, AtFLS2, AtADH, AtFT, AtSPL4, and AtBRI1, are targeted with various mutational efficiencies, ranging from 1.1 percent to 84.8 percent (Schiml *et al.*, 2014). Cas9 was utilized with a brief DNA repair template or a transgene to repair DSB by homologous recombination (HR) for point mutations or targeted transgene insertion, replacement, and gene stacking at predefined places. This eliminates the location effects associated with genetic engineering's random insertion of genes into plant genomes as well as multiplex genome editing, which uses several sgRNAs and the Cas9 nuclease to target multiple distinct loci.

The targeted insertion of transgenes in the domains of metabolic engineering and molecular farming, where plants or plant cells are employed as factories for the manufacture of specific metabolites or proteins, is another application of CRISPR/Cas9 that is anticipated to increase in the future. Although the CRISPR/Cas9 system is an effective tool for genome editing, the level of off-target mutation as well as variances in cleavage efficiency among diverse but ideally matched targets need to be researched further.

2.7.4. ALLEVIATE PUBLIC CONCERNS ABOUT GM CROPS

The exciting advancement of CRISPR/Cas9 genome editing technology also presents a number of societal issues. The application of CRISPR to human germline cells poses the most pressing challenges; however, the technique has policy implications for plants and animals.CRISPR may also require a rethinking of the rules governing genetically engineered species (GMOs).

Cut and thereby eliminate the desired genetic sequence, such as a gene encoding an unfavorable characteristic linked to a disease. It is simple to use, unlike earlier gene editing technologies, in that multiple parts may be built rapidly and reliably without the need for tinkering and trial and error.

The use of the CRISPR/Cas9 technology carries the risk of producing off-target alterations, which can be harmful. These undesired sequences could be cleaved by CRISPR/Cas9, resulting in alterations that could lead to cell death or transformation.

Efficient and safe CRISPR-Cas9 delivery into difficult-to-transfect and/or infect cell types or tissuesCRISPR-Cas9 technology could be useful for creating mutants for inaccessible genes, mutating several loci, and generating huge deletions, all of which could speed up plant breeding without actually inserting a transgene. Although genetically modified crops could have been a solution for crop development, the debates over their potential environmental and health consequences could have been avoided (Hilbeck *et al.*, 2011). Using genetic engineering to manipulate the genome, a DNA construct can be directly inserted into one or more chromosomes in a random manner. The CRISPR/Cas technology makes precise changes to a plant's genome that are passed down stably, and the transgenic area can be easily deleted following target gene editing to create transgene-free plants during crop variety improvement (Mahfouz *et al.*, 2014; Gao and Zhao, 2014; Kanchiswamy *et al.*, 2015; Xu *et al.*, 2015).

2.8. THE FUTURE OF CRISPR/CAS9

Crops with improved pest resistance, increased nutritional value, and the ability to thrive in a changing climate are among the potential future crops for sustainable productive agriculture using genome editing. Climate-resilient agriculture is the future of crop improvement, using genome editing for both targeted mutagenesis-induced manipulation and the study of transcriptional control by dissecting physiological and molecular cross-talk under combined stress (Kissoudis *et al.*, 2014; Jain, 2015). Genome editing will play a critical role in the development of novel bio-energy crops that can produce a maximum yield on wastelands while also adapting to changing climates (Bosch and Hazen, 2013). In the future, inducible Cas9 systems for transcription regulation, such as split-Cas9 for chemically inducible systems and light-activated Cas9 effector (LACE), could be used to improve crops (Polstein and Gersbach, 2015; Zetsche *et al.*, 2015).

The management of stress responses at the cellular level in roots, allele replacement for QTL validation, and the epigenetic regulation of roots are all important targets for crop improvement utilizing CRISPR/Cas9 genome editing (Ahmad *et al.*, 2014). Targeted mutagenesis utilizing the CRISPR-Cas9 system has the potential to promote legume functional genomic research, particularly by creating target mutants of genes involved in roots and nodules (Sun *et al.*, 2015). To investigate whether T-DNAs confer a beneficial phenotype in naturally transgenic sweet potatoes, CRISPR/Cas9 editing technologies can be employed to delete T-DNAs for a "non-transgenic" sweet potato generation (Jones, 2015). By altering the histone proteins found in centromeric nucleosomes, this approach can also be used to produce haploid plants (Kumar and Jain, 2014).

Single sgRNA libraries could be utilized to disrupt non-coding genetic elements in the future, whereas multiplex sgRNA delivery could be used to examine the function of huge genomic regions using tiled micro deletions. Cas9 has made great progress in becoming a set of tools for cell and molecular biology research, owing to the system's simplicity, high efficiency, and adaptability. The CRISPR/Cas system is by far the most user-friendly of the designer nuclease systems now available for precision genome modification. Manipulation has also sped up research and increased researchers' ability to create genetic models. Researchers can now identify new gene functions with high sensitivity and precision thanks to advancements in the CRISPR/Cas9 toolset.

Another intriguing prospective application of the CRISPR/Cas9 technology is the direct therapy of harmful genetic illnesses through somatic cell genome editing. It may be feasible to reverse disease symptoms by correcting disease-causing mutations.

The exciting advancement of CRISPR/Cas9 technology for genome editing has also brought up certain societal issues. Some of the mechanisms underpinning it, such as CRISPR/Cas9, may turn out to be the instruments we employ in future investigations.

Novelty Journals

ISSN 2394-966X

International Journal of Novel Research in Life Sciences
Vol. 9, Issue 2, pp: (84-103), Month: March - April 2022, Available at: www.noveltyjournals.com

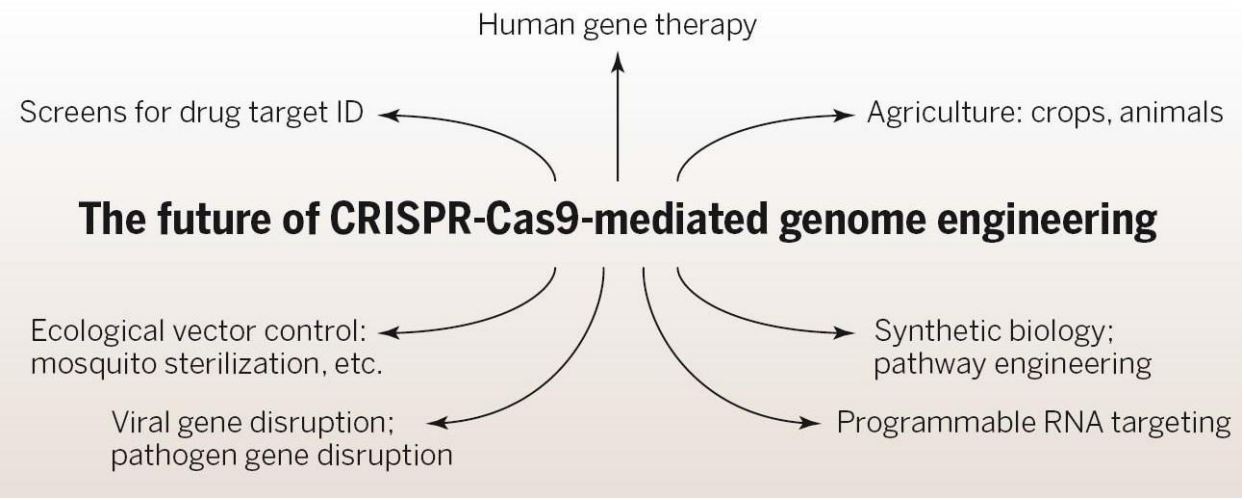

Figure. 3: The future of CRISPR- Cas9 –mediated genome engineering

Source: Jennifer A. Doudna and Emmanuelle Charpentier; The new frontier of genome engineering with CRISPR-Cas9; *Science Mag* 2014. 346**:** 6213

Off-target effects, chromatin structure, side effects on nearby genes, mechanisms underlying the different effects of different sgRNAs on mutation efficiency, and methods for efficient delivery in polyploid plants are just a few of the issues that have yet to be solved in CRISPR/Cas9-based genome editing technology. Despite these obstacles, gene editing technologies such as the CRISPR/Cas9 system will rapidly advance because of the immense excitement of the research community. In the near future, this easy, economical, and elegant genetic scalpel is projected to be widely used to improve the agricultural performance of most crops.

3. CONCLUSION

Crisper Cas9 is an efficient, highly specialized, and straightforward system. These characteristics combine to make this a promising genome editing tool for both plants and mammals. This technology has a significant influence on crop breeding. According to this study, CRISPR Cas9 will be an effective tool for practical research in plant breeding methods for crop enhancement. CRISPR is now being utilized for more than just genome editing; it's also being used to regulate gene expression and make epigenetic changes.

CRISPR–Cas could be employed for multiplex gene editing in plants, and the efficacy of CRISPR–Cas was found to vary depending on the target site. The CRISPR Cas system was effective in targeted genome engineering in monocot and dicot plants.

The CRISPR/Cas9 system and related RNA-guided endonucleases can generate targeted mutations in one or more genes as well as more complicated genomic rearrangements by introducing double-strand breaks (DSBs) at particular places in the genome. The CRISPR/Cas9 system has proven to be an effective technique in plant breeding for the production of disease-resistant cultivars.

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