Eco. Env. & Cons. 30 (October Suppl. Issue) : 2024; pp. (S146-S162)
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ISSN 0971–765X

DOI No http://doi.org/10.53550/EEC.2024.v30i06s.023

Application of Genome Editing for Crop Improvement

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(Received 26 January, 2024; Accepted 14 May, 2024)

ABSTRACT

The population of world is increasing at an alarming rate which requires a high amount of food to feed them. Climatic conditions are also changing year by year which causes a lot of losses in case of quality with quantity of product and also resurgence of a lot of insect and pests. Traditional breeding methods were the primary approach until the 20th century. Now, plant genome editing technologies offer a significant opportunity to modify specific genes linked to desired traits. This makes it possible to develop crop varieties with higher yields, resilient to changing harsh environments, resistance to pests and have the potential to accelerate crop development significantly. This review delves deeply into the investigation of genome editing techniques, while also addressing the obstacles and prospects associated with utilising this cutting-edge technology for the specific enhancement of various crop traits, boosts yield and strengthens the crop's resistance against various pests.

Key words: *Targeted, Alteration, ZFNs, CRISPR/Cas, Harnessing.*

Introduction

Since the discovery of recombinant DNA technology in 1972 in Paul Berg's lab (Singer, 1979), genetic engineering has advanced significantly and accomplished amazing feats. As a result of extensive research into numerous molecular and genetic systems and occurrences, scientists are now able to replicate tests in vitro. Many years of research into the molecular genetics and biochemistry of bacteria and viruses have allowed scientists to develop new tools for manipulating genomic DNA through the development of various vector mechanisms and instruments for their entry into cells. All of these advancements enable the successful generation of transgenic higher creatures, such as different plant and crop species, in addition to transgenic microbes creation of practical tools. The intricacy involved with manipulating the huge genomes of higher plants is one of the problems and restrictions with conventional genetic engineering technique. An area where genetic engineering is used, has attracted substantial attention is the creation of new tools for biotechnology and plant breeding, which has speed up the development of practical instruments. The intricacy involved with manipulating the huge genomes of higher plants is one of the problems and restrictions with conventional genetic engineering technique. (Nemudryi *et al*., 2014). Currently, scientists have access to a number of technologies that aid in resolv-

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ing issues associated with accurate plant genome editing. Zinc finger nucleases (ZFNs), which cleave the DNA in vitro in precisely specified locations, were originally demonstrated to work as site-specific nucleases in 1996. They connect "zinc fingers" protein domains with *FokI* endonuclease domains (Kim *et al*., 1996). Due to the fact that each "zinc finger" domain of this chimeric protein recognizes a single triplet of nucleotides, it has a modular structure. The foundation for editing cultured cells, including model and non-model plants, was established using this technique. (Gaj *et al*., 2013 and Weeks *et al*., 2016). As a result of ongoing research and development advanced genome editing tools like TALENs (transcription activator-like effect or nucleases) and CRISPR/Cas (clustered regularly interspaced short palindromic repeats) have been created. The current availability of repeat combination modules has streamlined the design procedure for TALENs, reducing the necessity for extensive cloning, and necessitating the engineering of a new protein for each target. CRISPR, in contrast, offers simplicity and versatility, functioning effectively in crops, animal, and human cells, and has the potential to tackle various complex challenges, including the development of transgenic and mutant plants. (Noman *et al*., 2016 and Zhang *et al*., 2014). Moreover, chimeric proteins made of zinc finger domains, activation domains of other proteins, and proteins derived from the TALE DNA-binding domain were used in studies on epigenetics, gene transcription regulation, and the movement of chromosome during the cell cycle (Cong *et al*., 2013; Petolino *et al*., 2013; Wang *et al.,* 2013 and Lowder *et al*., 2016). Historically, crop variety primarily resulted from spontaneous mutations, a gradual process, until the concept of "induced mutation" surfaced, offering a faster alternative. Following this, Capecchi developed a novel idea for genome editing in the 1980s that permits the addition, deletion, or modification of genetic material at specific genomic loci (Sedeek *et al.,* 2019; Capecchi 1980 and Chen *et al.,* 2019). These steps generate dsDNA (double stranded DNA) breaks by sequence-specific endonucleases, which are expressed momentarily within the cell and either persist as non-heritable proteins or quickly degrade once their role is fulfilled (Marton *et al*., 2010; Baltes *et al*., 2014; Ali *et al*., 2015; Ilardi *et al*., 2015 and Yin *et al*., 2015). In this review, we provided a concise overview of functioning behind various genome editing tools and their application in enhancing crops. Additionally, we emphasized the numerous benefits and uses of engineered nucleases, along with addressing the biosafety and regulatory considerations concerning plants produced through engineered nuclease technologies.

Genome Editing Technologies

The utilization of sequence-specific nucleases in genome editing has gained popularity in plant research, enabling the development of high-yield crops, enhancing crop resilience to environmental challenges, and improving their nutritional content (Chen *et al*., 2019). To date, there are four genome editing techniques, namely meganucleases, ZFNs, TALENs and CRISPR-associated (Cas) protein systems.

Meganucleases

Larger DNA sequences (>12 bp) can be cleaved sequence-specifically by specialised endonucleases called meganucleases, which are present in a variety of organisms such as bacteria, archaebacteria, fungus, yeast, algae and some plant species with a single recognition site (Chapdelaine *et al*., 2010 and Suzuku *et al*., 2020). Additionally, minor polymorphisms at the target location can be tolerated by meganucleases (Arnould *et al*., 2011). They are also referred to as homing endonucleases, and it is believed that they act as parasite elements on the host DNA. While their exact role remains uncertain, it induces dsDNA breaks at specific position within the host genome and then they spread throughout the target genome by leveraging the host's homologous repair system (Silva *et al*., 2011). It has been classified into five families on the basis of their sequence and motifs structure i.e., LAGLIDADG, PD-(D/E) XK, His- Cys box, GIY-YIG, and HNH (Silva *et al*., 2011 and Danilo *et al*., 2022). The LAGLIDADG meganuclease (LMN) family, broadly employed in genome editing, derives its name from the distinctive motif sequence found within the protein family to which this enzyme belongs. (Suzuki *et al*., 2020 and Silva *et al*., 2011). In unicellular eukaryotes, LMNs are typically expressed in the mitochondria and chloroplast, majorly as dimeric proteins, that play two distinct roles: function as specialised endonucleases to cleave exon sequences and as RNA maturases to splice their own introns. (Arnould *et al*., 2011). The NHEJ mechanism is used to fix dsDNA breaks caused by meganucleases, that results in erroneous insertions or deletions at the tar-

get site. (Arnould *et al*., 2011 and Liang *et al*., 1998). I-SceI and I-CreI are the members of this family frequently used in genome editing. I-SceI, located in *Saccharomyces cerevisiae* mitochondrial DNA's 21S rRNA gene, is hailed as the "gold standard" in genome editing for its precision and efficiency in recognizing the 18 bp sequence 52-TAGGGATAACAGGGTAAT-32. I-CreI, found within the 23S rRNA gene, was originally found in the chloroplast of the algae *Chlamydomonas reinhardtii*, having two aspartic acid residues in its catalytic region and it functions as a homodimer to recognize the 22 bp target sequence 52-CAAAACGTCGTGAGACAGTTTG-32. (Arnould *et al*., 2011 and Prieto *et al*., 2018). Meganucleases have been employed in specialized gene therapy for endonuclease engineering but face limitations in genome editing because it is puzzling to reengineer them to target another new DNA sequences (Arnould *et al*., 2011; Maeder *et al*., 2016 and Zess *et al*., 2021).

Zinc-Finger Nuclease

Zinc finger proteins (ZFNs) are made up of a DNA recognition domain and the cleavage domain of the FokI endonuclease. Each zinc finger recognises three base pairs (bp), which enables them to function by focusing on particular DNA sequences. ZFNs must be built in pairs because FokI needs dimerization to function properly, requiring the right orientation and spacing (Lee *et al*., 2018). ZFN-mediated gene editing has been successful in a various crops like maize, rice, soybeans, and wheat (Bonawitz *et al*., 2019, Shukla *et al*., 2009; Ran *et al*., 2018 and Jung *et al*., 2018). However, the expense and difficulty of creating a protein for each target site as well as the possibility of cytotoxicity from off-target cleavage limit the utilization of ZFN-mediated gene editing in crop editing (Rasheed *et al*., 2021).

The Central Repeat Domain (CRD), a DNA-binding domain made up of 33–35 amino acid repeats, is used by the pathogenic gamma proteobacterial genus Xanthomonas in nature to identify and interact with particular single-base pairs in the substrate DNA in order to deliver TAL effectors into the host plant cell (Miller *et al*., 2011). The susceptibility S gene in the promoter region, in particular, is activated by TAL effectors using the type III secretion system to enter the host plant and change gene expression in favour of the bacterium's survival strategy. This promotes pathogenesis and increases the bacterial population for the development of disease (Song *et al*., 2013).

Similar to ZFNs, TALENs consist of customized arrays of TALEs fused with the FokI cleavage domain. Except for the two hypervariable residues at 12 and 13 positions. The specific nature of TALEs is assessed by repeat variable di-residues (RVDs), with their identification in tobacco facilitated by using a â-glucuronidase (GUS) reporter (Lei *et al*., 2012). The

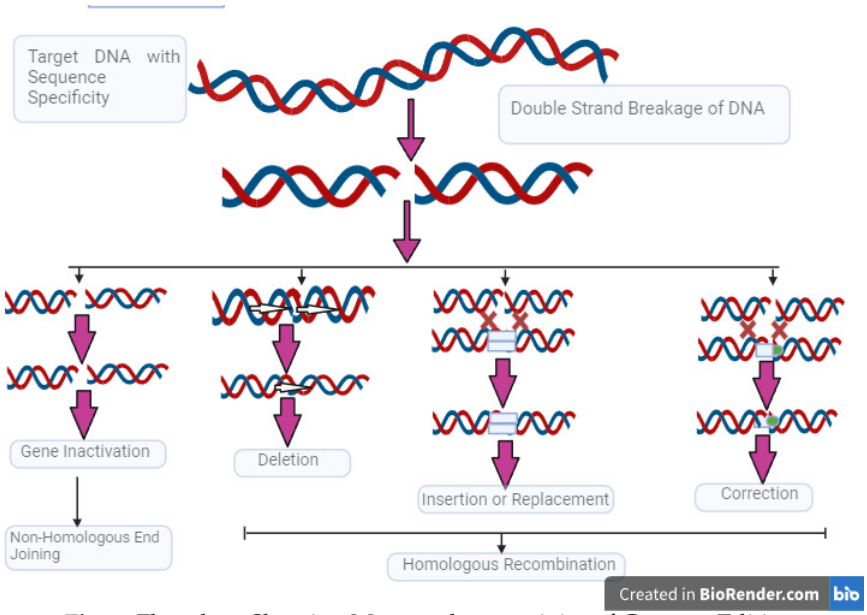

Fig. 1. Flowchart Showing Meganuclease activity of Genome Editing

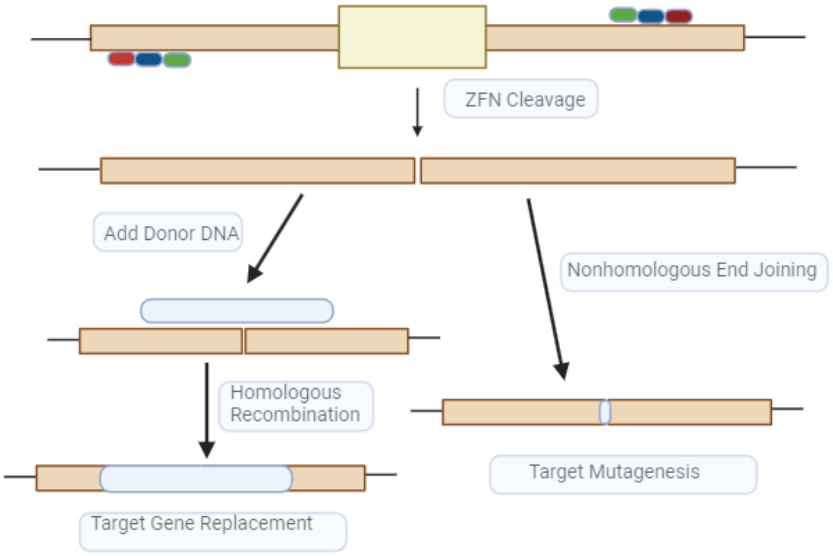

Fig. 2. Working of ZFN

twelfth residue plays a role in soothing the interaction with the nucleotide, whereas the thirteenth residue is responsible for recognition. The DNA target specificity in TALEs governed by CRD, which can differ based on the amount of repeats and the arrangement of RVDs, while the N- and C-termini of TALE repeats are flanked by secretion signals, with the C-terminus functioning as an acidic transcriptional activating domain.

The four distinct domains that are commonly found on customised TALE repeat arrays are NI (asparagine and isoleucine), NN (two asparagines), NG (asparagine and glycine) and HD (histidine and aspartic acid). These domains are each linked to the recognition of adenine guanine, thymine and cytosine, respectively. By producing recombinant arrays of RVDs that are particular to the intended genomic section, researchers have the opportunity to develop powerful genome editing tools. The bacteria *Paraburkholderia rhizoxinica* and *Ralstonia solanacearum* are useful for bioengineering because they have a better-quality of RVDs and TALE-like proteins. (Dahlem *et al*., 2012).

The introduction of TALENs, which offer recompenses over ZFNs, has propelled genome editing technology forward due to their less toxic nature and easier customization, due to single-base recognition capability of TALE DNA-binding repeats. However, because of these repetitive sequences, PCR cloning of TALE is difficult but can be solved by techniques like golden gate cloning (Song *et al*., 2013). One major drawback of TALENs is that they are larger than ZFNs, which makes it more difficult and inefficient for injecting them into the nucleus (Dunn *et al*., 2014). Genome editing using TALEN technology has been used in various animals like Xenopus (Christian *et al*., 2013), rabbit (Ansai *et al*., 2013), Zebrafish (Clasen *et al*., 2016), pig (Li *et al*., 2012), mouse (Shan *et al*., 2013) and Medaka (Char *et al*., 2015 and Li *et al*., 2016). It has also been used for targeted mutation in plants like *Arabidopsis* (Ma *et al*., 2015), rice (Shan *et al*., 2015; Haun *et al*., 2014, Li *et al*., 2016; Ma *et al*., 2015 and Haun *et al*., 2014), *Brachypodium* (Haun *et al*., 2014), potato (Clasen *et al*., 2016) and maize (Char *et al*., 2015). In soybean, researchers conducted gene targeting on the fatty acid desaturase 2 (FAD2) gene to enhance the oil quality (Haun *et al*., 2014).

CRISPR/Cas9 Genome Editing System

Several bacteria, including SaCas9 from Staphylococcus aureus, NmCas9 from Neisseria meningitides, CjCas9 from Campylobacter jejuni and StCas9 from Streptococcus thermophiles, have recently been discovered and used for plant gene editing, SpCas9 is still the method that is used the most. CRISPR is made up of conserved repeat sequences and spacers, which are distinctive variable sequences. When plasmid or phage DNA, or a spacer, get integrated into the CRISPR section of the host genome, it activates the system and grants protection against future encounters with phages or plas-

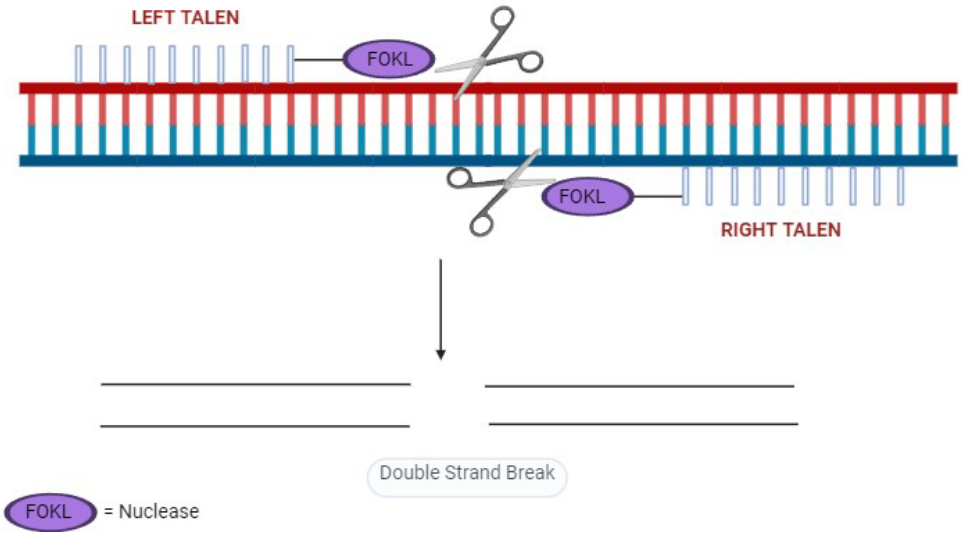

Fig. 3. Working of TALENs

mids sharing similar sequences. These elements are subsequently processed to generate precursor crRNA (pre-crRNA), a primary transcript consisting of a spacer segment flanked by repeat sequences, typically vary in length from 23 to 47 base pairs. The processing endonuclease within the CRISPR system not only cleaves the invader's targeted sequence but also identifies these distinctive CRISPR repeat sequences, which play a dual role in safeguarding against foreign DNA and contributing to crRNA maturation (Richter *et al*., 2014; Marraffini *et al*., 2010 and Stern *et al*., 2010). Following the complete integration of crRNA with Cas protein, crRNA pairs up with trans-activating crispr RNA (tracrRNA) and engages with host RNase, allowing the detection of the invading sequence, ultimately leading to its cleavage. Following the final incorporation of crRNA with Cas protein, the intrusive sequence for further cleavage is identified by hybridising crRNA with trans-activating crispr RNA (tracrRNA) to host RNase (Karyelis *et al*., 2013). The protospacer-encoded portion of crRNA instructs Cas9 to cleave the targeted complementary DNA sequence in accordance with the RNA-DNA complementary base pairing rule (Sander *et al*., 2014). The protospacer adjacent motif (PAM) sequence, typically derived from an invading virus or plasmid and required for CRISPR-Cas9 targeting, must be situated next to the target sequences (protospacers), with the PAM for SpCas9 specifically comprising the NGG sequence and allowing for any nucleotide in its composition.

A crRNA-tracrRNA duplex is artificially fused into a particular chimeric RNA known as a single-guide RNA (sgRNA or gRNA) in order to direct it to a particular target site. Cas9 undergoes conformational changes that change it from an inactive to an active DNA-binding system when it interacts with gRNA to form a nucleoprotein complex. When Cas9 cleaves the targeted DNA, which is located 3-4 nucleotides upstream of the PAM sequence, DSBs are produced. DNA repair in the cell is activated as a result of double strand break (DSB) formation, possibly through an non-homologous end joining (NHEJ) or homology directed repair (HDR) mechanism. NHEJ primarily leads to insertion or deletion mutations (INDEL), resulting in gene knockouts within coding regions, whereas genetic modification through HDR necessitates the presence of a homologous DNA template near DSB. SaCas9 from S. aureus is shorter (1053 residues) than SpCas9 from *S. pyogenes* (1368 residues), which makes it more efficient for delivery (Schaeffer *et al*., 2016). CRISPR-Cas9 was employed by Sanchez-Leon to target parts of the wheat gliadin gene family (Sanchez-Leon *et al*., 2018). This gene family encodes the major immunodominant peptide in persons with coeliac disease, caused by consuming wheat gluten proteins. They developed several sgRNAs that specifically target the DNA sequences, resulting in 21 mutant lines with a significant drop in -gliadins.

One significant achievement of this study was the screening of transgene-free lines, which resulted in the mutation of up to 35 different genes in one of the lines without any observed off-target effects. Simi-

larly, this technique was applied to rice to assess the functionality of miR396 genes, specifically targeting MIR396e and MIR396f without affecting other isoforms, resulting in enhanced panicle branching, larger grain size, increased grain yield and increased biomass even in nitrogen-deficient conditions [Zhang *et al*., 2020]. These results also suggested that rice genome editing could target miR396ef, reducing the plant's need for nitrogen fertiliser. Nishitani conducted a significant genome editing study by targeting the apple phytoene desaturase (PDS) gene, which is crucial for fruit plants in the Rosaceae family (Nishitani *et al*. 2016). Rodriguez made significant progress in the CRISPR-Cas9 system (Rodriguez *et al*., 2017), who utilized multiple sgRNAs to target different regions of the promoters of three tomato genes controlling inflorescence pattern (COMPOUND INFLORESCENCE), fruit size (CLAVATA3), and branching architecture (SELF PRUNING), resulting in the creation of new cis-regulatory alleles and variations in polygenic traits. This study proved that Cas9 can be utilised to perform complicated gene manipulation for fine-tuning quantitative features rather than just simple gene knockouts. The CRISPR tool has been used to produce numerous plants with desired gene edits, (Shi *et al*., 2017) with enhancing maize production under drought stress through the creation of ARGOS8 genome-edited variants using this technology in rice (Cas9p) (Ma *et al*., 2015), *Arabidopsis* (pcoCas9) (Li *et al.,* 2013), *Medicago truncatula*, and *Glycine max* (GmCas9) (Michno *et al*., 2015). CRISPR can address hybrid sterility and cross-incompatibility by knocking out relevant genes, potentially resolving these issues and even enabling the production of haploid plants by focusing on genes related to cell division and spindle fiber formation (Bhowmik *et al.,* 2018). Developing male sterile plants through the use of this technology is an emerging and valuable approach for gaining insights into gene functionality and regulation (Zhou *et al*., 2016). To overcome one of the primary limitations of SpCas9 system, which necessitates a strict NGG PAM sequence at the target site, researchers have developed SpCas9 variants with broader PAM specificity.

Hu employed phage-assisted continuous evolution to create xCas9, a modified SpCas9 variant capable of recognizing diverse PAM sequences such as GAA, GAT, and NG (Hu *et al*., 2018), offering enhanced PAM compatibility and reduced off-target action compared to SpCas9 (Ni *et al*., 2020). Using xCas9, Hu successfully introduced mutations in rice containing specific PAM sequences like GAT and NG (Hua *et al*., 2019), while Ge evaluated xCas9's effectiveness in Arabidopsis (Ge *et al*., 2019) for targeting the FERONIA (FER) gene with different PAMs (NGA, NGG, NGT, or NGC). Customized Cas9 nuclease domain variations, such as Nickase Cas9 (nCas9) and Nuclease catalytically dead Cas9 (dCas9), represent two examples in this context. Single-nucleotide polymorphisms (SNPs) are important for determining many agricultural traits and provide a more efficient way to protect against undesirable mutations since they can modify proteins function by introducing stop codons or substituting amino acids. The initial application of the CRISPR/dCas9 or nCas9 system in combination with cytidine deaminase to create point mutations employed by Komor (Komor *et al*., 2016). Within this system, substituting specific bases in the genome does not trigger the formation of DSBs, instead, it results in precise knockout of specific amino acids or the inactivation of a gene through the introduction of an early stop codon.

The cytidine deaminase and uracil glycosylase inhibitor (UGI) fusion domains are combined in the current generation of base editors, nCas9 and dCas9, within CRISPR systems. These are produced when the enzymatic function of the Cas9 RuvC and HNH domains, or just the RuvC domain, is blocked (Wang *et al*., 2015). The complementary strand of the targeted DNA is cut by the HNH nuclease domain, whereas the non-complementary strand is cut by the RuvC domain, which results in the production of blunt ends (Belhaj *et al*., 2013). Even in single-stranded regions, nCas9 can be guided by sgRNA to target and create a nick in a single DNA strand, with cytidine deaminase subsequently changing cytosine (C) within the single-stranded DNA to uracil (U), eventually leading to its conversion into thymine (T) during the DNA repair process. Crop enhancement has been expedited by introducing gene modifications involving replacements or deletions, leading to improvements in crucial agronomic characteristics, and Cytosine-Based Editors (CBEs) and Adenine-Based Editors (ABEs), which induce C-G to T-A and A-T to G-C transitions deprived of the double-strand breaks (DSBs) or external DNA achieve this.

Recent research successfully achieved the prime genome of cereals such as rice and wheat by introducing desired gene modifications using pegRNA (prime editing-guide RNA) in conjunction with

specialised nCas9-reverse transcriptase fusion proteins (Lin *et al*., 2020). A modified nCas9 (H840A), pegRNA, and a customised M-MLV reverse transcriptase were used to create the plant prime editor (PPE) system. The maize Ubiquitin-1 (Ubi-1) promoter was used to activate optimised PPE genes for cereal crops, and the TaU3 and OsU3 (or TaU6) promoters were utilised to initiate sgRNA transcription and direct pegRNA production, respectively. They further customized pOsU3-BFP-peg01 by incorporating an RT template, enabling the conversion of ACC CAC (threonine-histidine) to ACG TAC (threonine-tyrosine) within the pegRNA-induced nick. They next created prime-edited plants with point mutations, deletions, and insertions, which can hasten crop enhancement in elite lines, by introducing PPE, pOsU3-BFP-peg01, and pUbi-BFP with sgRNA into wheat and rice protoplasts. In maize, wheat, and rice, similar research has been conducted using nCas9-PBE and dCas9-PBE to precisely exchange cytosine with thymine (Zong *et al*., 2017).

Hao developed the ABE-nCas9 tool, enabling the precise induction of A to T and G to C mutations in the rice genome without causing unintended InDels or off-target effects (Hao *et al*., 2019). Additionally, Qin *et al*. (2020) employed this system to perform accurate base editing, converting C to T and G in the cotton. They achieved this by incorporating a cytidine deaminase sequence (APOBEC) along with UGI and nCas9 into the CRISPR/Cas9 plasmid to target three specific location in the cotton genome. The integration of nCas9 (D10A), human APOBEC3A (Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3A), a cytidine deaminase-based base editor, and uracil glycosylase inhibitor (UGI) allowed for the groundbreaking application of cytidine deamination editing in potatoes in a recent study (Zong *et al*., 2018). This technique makes it possible to substitute C with T with a minimum number of undesired edits and indels. Genome editing can be employed to confer virus resistance in plants by introducing mutations which provide resistance to viruses in plants lacking natural eIF4E alleles, which play a significant role in plant viral resistance (Wang *et al*., 2012). By introducing *Pisum sativum* eIF4E virus resistance alleles into the susceptible eIF4E1 gene of Arabidopsis, researchers (Bastet *et al*., 2019) improved resistance in Arabidopsis thaliana against clover yellow vein virus. In addition, they used CRISPR-Cas9 cytidine deaminase editing to trigger the N176K substitution in Arabidopsis wild-type plants, leading to transgene-free, virus resistant seedlings. In a manner similar to this, citrus plants resistant to citrus canker can be developed using CRISPR/Cas9-mediated editing of the CsLOB1 promoter (Peng *et al*., 2017). The study demonstrated the utilization of pathogen susceptibility factors to evaluate genome editing techniques, which hold significant promise for the production of transgenes free and genetically resistant crop varieties.

Addressing weed control, scientists are exploring gene editing as a promising avenue to develop herbicide-tolerant crop varieties for sustainable agriculture. Zhang focused on the TaALS gene, responsible for encoding the essential enzyme acetolactate synthase (ALS) vital for branched chain amino acid synthesis, to develop herbicide-tolerant wheat (Zhang *et al*., 2019). Specifically, they aimed at the TaALS-P174 site, a CCC codon encoding proline found in all wheat subgenomes, by employing pnCas9-plant base editor (PBE)15 along with sgRNA TaALS-P174 constructs in the embryo of the Kenong199 wheat variety. Multiple subgenome mutations were created that gave wheat resistance to herbicides based on aryloxyphenoxy propionate, imidazolinone, and sulfonyl urea. By combining adenosine deaminase with CRISPR-nCas9, Li were able to convert A·T to G·C and develop rice plants that are resistant to herbicides (Li *et al*., 2018). Comparable studies have also been carried out in a variety of plants, including as Arabidopsis (Chen *et al*., 2017), rice (Shimatani *et al*., 2018 and Shimatani *et al*., 2017), maize (Li *et al*., 2019), rapeseed (*Veillet et al*., 2019), tomato, potato (Veillet *et al*., 2019), and watermelon (Tian *et al*., 2018), with the aim of creating mutations that confer herbicide resistance. In order to achieve gene silence, Maeder and Gilbert developed a catalytically inactive variant of Cas9 known as dead Cas9 (dCas9) (Maeder *et al*., 2013 and Gilbert *et al*., 2013). Rather than causing gene disruption, this dCas9 results in sequence-specific constitutive activation or gene silencing when combined with transcription activation or repression effectors. This strategy, called CRISPR interference (CRISPRi), minimises its effect on the transcription of non-targeted genes while permitting combinatorial control or repression of genes in a way akin to RNA interference. Moreover, Piatek effectively suppressed the transcription of protein-coding genes in *Nicotiana benthamiana* by employing the CRISPRi pathway in conjunction with dCas9 (Piatek *et al*., 2015). Several investiga-

tions in the field of plant research have explored how different activators interact with dCas9 (Li *et al*., 2017, Lowder *et al*., 2015 and Venzquer *et al*., 2016).

The CRISPR-dCas9 system seems to be a multipurpose tool because it is also showing potential as a method for targeted epigenome editing. In addition to its primary editing function, the CRISPR-dCas9 system is being repurposed for precise genome regulation, with its utility further extended by enabling the silencing of transcription through the targeting of regulatory regions instead of the gene itself (Dominguez *et al*., 2016). The potential application of dCas9 as a tool to modify gene function by stimulating and inhibiting transcription in different organisms through epigenetic changes like histone methylation and acetylation was made possible by its ability to efficiently suppress transcription in bacteria (Qi *et al*., 2013). Thakore by integrating nuclease-deficient dCas9 with the Kruppel-associated box (KRAB) transcription repression domain—a naturally occurring element involved in assembling a complex that generates heterochromatin were able to induce gene silence by dCas9 integration with an epigenetic effector (Thakore *et al*., 2015). This resulted in the mediation of histone methylation and deacetylation, ultimately leading to the suppression of globin gene expression. In another study, Neisseria meningitidis dCas9 was combined with Lysine-Specific Histone Demethylase 1 (LSD1) to achieve gene expression repression, highlighting the system's early-stage flexibility in transcription manipulation (Kearns *et al*., 2015). Table 1 provides a brief comparison of CRISPR/Cas, TALENs, ZFNs, and meganucleases.

Application of Genome Editing in Crop Improvement

Significant real-world applications for genome editing technologies exist in the field of crops improvement, such as the development of high-yielding, high nutritious, and resistant to biotic and abiotic stresses crop varieties (Osakabe *et al*., 2016, Xiong *et al*., 2015, Kissoudis *et al*., 2014, Liu *et al*., 2014, Jain, 2015, Andolfo *et al*., 2016, Khatodia *et al*., 2016 and Nongpiur *et al*., 2016). In plant breeding, the genome editing methods has been employed for various purposes, including (1) incorporating foreign genes, (2) making small alterations to gene function, (3) introducing point mutations resembling natural SNPs, (4) controlling gene expression by either repression or activation, (5) gene knockout and gene pyramiding and (6) performing epigenetic editing. For instance, the application of ZFN technology in *Arabidopsis* (Osakabe *et al*., 2010, Zhang *et al*., 2010 and Townsend *et al*., 2009) and maize (Shukla *et al*., 2009) has resulted in the successful creation of herbicide-tolerant plant varieties by inserting herbicide resistance genes into specific region within the genome (Shukla *et al*., 2009). Additionally, the endogenous malate dehydrogenase (MDH) gene was precisely altered in plants using ZFN, leading to enhanced crop yields in the modified MDH-containing plants (Shukla *et al*., 2013).

The ODM technique has been greatly improved by the Cibus Rapid Trait Development System (RTDS) (Sauer *et al*., 2016). This technology has been successfully applied in several crops for a variety of

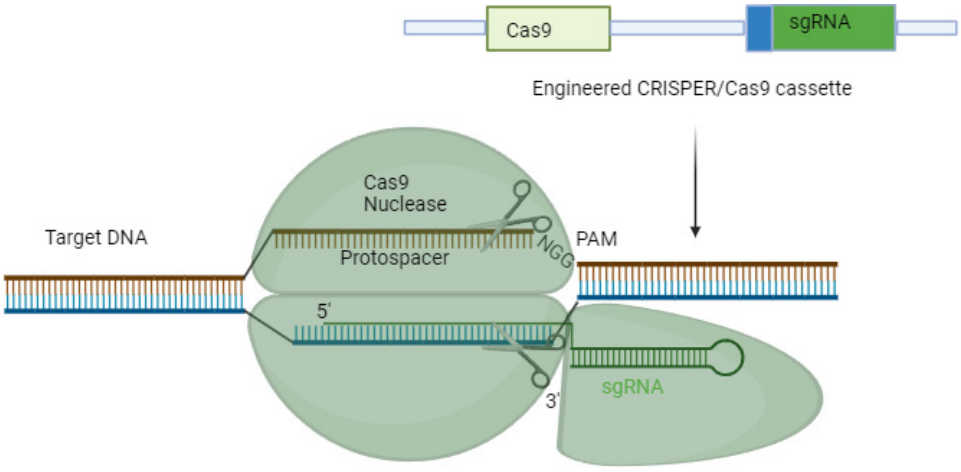

Fig. 4. Working of CRISPR/Cas9

purposes, including improved disease resistance (both bacterial and viral), insect resistance, herbicide tolerance, enhanced nutritional value, and increased yield. All of these goals have been achieved without the insertion of foreign genes, as has historically been the case in conventional genetic engineering for crop improvement (Sauer *et al*., 2016). Using ODM RTDS technology, precise editing of CAC to TAC allows for the non-transgenic conversion of the amino acid histidine (H66) in BEP to tyrosine (Y66) in GFP, a useful tool for crop breeding. (Sauer *et al*., 2016 and Abdurakhomonov, 2016).

Jiang engineered *Camelina sativa* seeds using CRISPR/Cas9 technology, producing a biotechnologically improved oil with an improved fatty acid composition that is more oxidation-resistant, healthier for human consumption, and more suitable for the production of particular chemicals like biofuels (Jiang *et al*., 2017).

Targeted mutation of the SP5G gene was used by Soyk to develop tomato plants with faster flowering and a denser bush structure, which eventually resulted in an earlier harvest (Soyk *et al*., 2017). By using CRISPR-induced mutation of the OST2 gene in Arabidopsis, Osakabe were able to confer salt stress resistance in plants and resulting in the generation of new alleles with this beneficial trait (Osakabe *et al*., 2016). Dwarf fruit trees with significant potential for increased productivity have been made possible by the use of genome editing techniques to modify gibberellin biosynthesis. This has allowed for denser planting and less labour, as well as a decrease in the demand for fertilisers, pesticides, and water resources (Peng *et al*., 1999). Furthermore, utilizing genome editing to suppress ethylene biosynthesis, a pivotal factor in fruit ripening or its signaling pathways, opens the door to creating novel varieties with extended shelf lives (Hollender *et al*., 2015) (Xiong *et al*., 2015). Genome editing techniques are actively used in plant breeding to develop pest and pathogen resistant plant varieties by modifying essential stages of plant immunity across a range of crops. To accomplish this objective, one can make modifications in (1) resistance genes (R-genes), (2) susceptibility genes (S-genes), (3) genes which govern how effectors and their respective targets interact and (4) the genes responsible for maintaining the balance of plant hormones (Andolfo *et al.,* 2016). For example, by focusing on the mildew resistance locus O (MLO), genome editing methods like TALEN and CRISPR/Cas9 were used to create wheat types resistant to powdery mildew disease (Wang *et al*., 2014). Similarly, by focusing on the susceptibility gene *Os11N3* (also known as *OsSWEET14*), it has been exploited to generate plants resistant to bacterial leaf blight brought on by *Xanthomonas oryzae pv. Oryzae* (Li *et al*., 2012). By employing single guide RNA/Cas9 (sgRNA/Cas9) to implement a transient transformation system and degrade or suppress the genome of the curly top virus in *N. benthamiana*, the effectiveness of the CRISPR/Cas9 system has been evaluated with respect to its capacity to interfere with geminiviruses (Ji *et al*., 2015). The CRISPR/Cas9 system was shown to produce mutations inside the targeted sequences in addition of focusing on destroying viruses in previous investigations. This happened because it interfered with the ability of viruses to replicate freely when specific sgRNAs matching sequences of the bean yellow dwarf virus (BeYDV) or tomato yellow leaf curl virus (TYLCV) were inserted into *N. benthamiana* plants that expressed the Cas9 endonuclease and then exposed to

Table 1. Comparison between different genome edition techniques (Eid *et al*., 2016, Guha *et al*., 2017, Li *et al*., 2020 and Janik *et al*., 2020).

Feature	Meganucleases	ZFNs	TALENs	CRISPR/Cas
Target site length	12-40 bp	18-36 bp	28-40 bp	20-22 bp
Recognition	Protein recognize DNA	Protein recognize DNA	Protein recognize DNA	RNA protein complex recognizes DNA
Dimerization	Not required	Required	Required	Not Required
Multiplexing	Challenging	Challenging	Challenging	Feasible
Nuclease protein	I-SceI	FokI	FokI	Cas
Repair events	HDR	NHEJ	HDR	NHEJ
Efficiency	Moderate	Low	Moderate	High
Cost	High	High	Moderate	Low
Ease of engineering	Low	Low	Moderate	High
Specificity	High	Moderate	High	Low

the corresponding viruses (Andolfo *et al*., 2016).

The inactivation of ethylene-responsive factor (ERF) is a crucial tactic used by genome editing technologies to alter metabolic pathways that control hormonal balance, thereby improving the immunomodulatory component of the plant immune system. For example, CRISPR/Cas9-mediated mutations of the rice target OsERF922 gene have effectively altered the gene, leading to heightened resistance against *Magnaporthe oryzae* (Liu *et al*., 2012 and Wang *et al*., 2016). To make *Cucumis sativus* resistant to viruses including Papaya ring spot mosaic virus-W (PRSV-W), cucumber vein yellowing virus (CVYV), and zucchini yellow mosaic virus (ZYMV), it was used to remove the eIF4E gene, which encodes the eukaryotic translation initiation factor (Fang *et al*., 2016). Furthermore, the effectiveness of CRISPR/Cas9 was illustrated as a swift and efficient genome editing system in *Phytophthora sojae*, an oomycete pathogen that affects soybeans. This was accomplished through the modification of the pathogenicity gene (*Avr4/6*), offering a promising avenue for conducting essential functional genomics research in *Phytophthora sojae*, ultimately contributing to efforts aimed at controlling this pathogen (Fang *et al*., 2016). In a similar vein, it has been used to create plants resistant to herbicides. For instance, by modifying the ALS2 gene, a crucial component of plant amino acid biosynthesis, a mutant maize plant resistant to chlorsulfuron was created (Svitashev *et al*., 2015). One exciting area of biotechnology where the CRISPR/Cas9 system has great potential is the development of plants that can produce human proteins like albumin, which is used to treat conditions like burns, hemorrhagic shock, cirrhosis and hypoproteinemia, or insulin for patients with diabetes mellitus (Hasting *et al*., 1992).

In response to the increasing global demand of

Table 2. Agronomic traits -improved by gene-editing technologies

Crop	Locus/Gene	SDN	Phenotypic Change	Remarks
Maize	MTL	TALEN	Haploid Production	Maternal haploid induction when used as pollinator
Rice	CSA	CRISPR-Cas9	Reverse photo-period sensitive genic male sterility (rPGMS)	Male fertility in short-day conditions in comparison to the long-day requirements for the initial PGMS line discovery
	Tms	CRISPR-Cas9	Temperature-sensitive genic male sterility (TGMS)	TGMS lines comparable to the parental lines in morphology, promising for hybrid seed production
Tomato	SP5G	CRISPR-Cas9	Early flowering	Located in a QT, suppresses flowering, compact and determinate in habit
	SLS	CRISPR-Cas9	Inflorescence branching	A range of different inflorescence branching patterns generated

Table 3. Some examples of gene editing for abiotic and biotic stress resistance

Crop	Gene	SDN	Phenotypic Change	Remarks
Wheat	TaMLO-A1, B1, D1	TALEN	Powdery mildew resistance	Edited plants resistant to powdery mildew, resistance broad-based
Maize	ARGOS8	CRISPR-Cas9	Drought resistance	Increased yields under drought stress at flowering time
	NLB18	CRISPR-Cas9	Resistance to northern leaf blight	Sequence replacement by HDR
Rice	OsERF922	CRISPR-Cas9	Resistance to rice blast	Decreased lesion size
	EPSPS	CRISPR-Cas9	Resistance to herbicide Glyphosate	Two mutations introduced in the second exon of the gene by intron targeting
Tomato	SLMLo1	CRISPR-Cas9	Powdery Mildew Resistance	
Cucumber	eIF4E	CRISPR-Cas9	Virus resistance	

500 tons per year for albumin, despite the limited supply of human plasma used for its production, genomic engineering techniques have been utilized to insert the human albumin gene into the rice genome to address the growing requirements (He *et al*., 2011). When these proteins are produced and refined, they can be extracted from the tissues of plants and animals and used in medicine. As such, these novel genome editing techniques are widely used for agricultural improvement, including the development of new bioenergy crops (Bosch *et al*., 2013). Table 2 describing agronomic traits -improved by gene-editing technologies. Table 3 describing some examples of gene editing for abiotic and biotic stress resistance. Table 4 representing list of institutes in India working on genome editing.

Challenges for Genome Editing

Whole plantlets are regenerated from the transformed cells after the SDNs are introduced into plant cells or protoplasts. But in many crops species, dependable protocols for plantlet regeneration are not available.

SDNs do induce off target effects albeit at low or even negligible frequencies. Whenever such effects are produced, additional time, effort and cost will be imposed by the breeding schemes used for eliminating these effects form the mutant lines.

Conclusion

Genome editing techniques have emerged as a prominent choice within the realm of molecular biology, offering valuable tools for both understanding plant biology at a functional level and elevating crop improvement efforts. This trend is underscored by numerous ongoing endeavors where gene editing systems are employed to facilitate comprehensive investigations into diverse aspects of plant biology. Additionally, they play a pivotal role in en-

Table 4. List of institutes in India working on genome editing

Institutions	Specific areas of interest
National Institute of Plant Genome Research (NIPGR)	CRISPR-Cas9-mediated genome editing for nutritional enhancement of Indian mustard. RICE/Maize-CRISPR/Cas9/Cpf1 based genome editing technology to enhance and modify root architecture and the response of the plant to abiotic stress and nutrients. Biofortification of banana for a-carotene, b-carotene and lutein. (Collaboration with NABI, Mohali)
Bose Institute, Kolkata	Developing an enhanced toolkit for inducible genome editing and regulating gene expression in tomato: consequences for modifying complex traits through synthetic biology methods
Junagadh Agricultural University	Genome editing in groundnut for high oleic acid and low linoleic acid. Gene editing in Saurashtra's main field crops: a breeding programme utilising CRISPR-Cas9 technology
ICAR-National Institute for Plant Biotechnology	Genome editing of potatoes to produce true potato seeds (TPS) specific to a variety Genome editing using CRISPR-Cas9 of several negative regulators for rice blast resistance
IARI—New Delhi (cooperating centres ICGEB, New Delhi, NRRI, Cuttack	Genetic improvement of rice through RNA guided genome editing (CRISPR/Cpf1) for yield, WUE,NUE, biotic and abiotic stress resistant. Incorporation of known/novel alleles of TB1, CKX2, DEP1, SPL14, DST, PP2Cs, Os8N3, miR169a and eF4g genes through genome editing in elite mega rice varieties which will be beneficial for donors in breeding programmes or directly released for commercial cultivation.
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)	Utilising a two-pronged strategy to develop double herbicide-tolerant Arhar (*Cajanuscanjan*) for better weed management: haplotype mining in native germplasm and genome editing by CRISPR/Cas9.
Tamil Nadu Agricultural University (TNAU)	Using genome editing to improve rice's nutritional value and resistant to disease Coordination with ICAR-NASF to develop "Thermo-sensitive genic male sterile lines (TGMS)" in rice by CRISPR-mediated genome engineering: locus of TMS5
National Agri-Food Biotechnology Institute (NABI)	LCY- bananas are rich in beta-carotene, the team is concentrating on metabolic engineering of banana and wheat to improve their nutritional value. Pro-vitamin A (beta-carotene) biofortification of bananas

hancing crop yields through the rapid and precise introduction of mutations and the subsequent utilization of associated breeding methods. The remarkable attributes of genome editing, including its simplicity, efficiency, high accuracy, and versatility in multiplexing applications, are fundamentally reshaping the landscape of crop breeding. These techniques are not just a leap forward but a quantum leap in the realm of crop improvement, setting the stage for the next generation of breeding innovations.

Conflict of Interest: None

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