Review Article

The Application of CRISPR/Cas9 Technology in the Management of Genetic and Nongenetic Plant Traits

Wilbert Mutezo [ID],[1] **Moosa M. Sedibe,**[1] **Alina Mofokeng,**[2] **Nemera Shargie,**[2] **and Tegwe Soko**[3]

[1]*Central University of Technology, Free State Private Bag X 20539, Bloemfontein 9300, South Africa*
[2]*Agricultural Research Council-Grain Crops, Private Bag X 1251, Potchefstroom 2520, South Africa*
[3]*Rattary Arnold Research Station, P.O. Box CH 142, Chisipiti, Harare, Zimbabwe*

Correspondence should be addressed to Wilbert Mutezo; wmutezo@gmail.com

Gene editing (GE) has yielded positive results in the management of genetic and nongenetic plant traits. Clustered interspaced palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) (together known as the CRISPR/Cas system) are relatively easy to use, compared to other existing genome editing technologies. The CRISPR/Cas tool produces unchanging gene mutations, with the ability to segregate from the Cas9/sgRNA construct to avoid similar modifications by CRISPR/Cas. CRISPR/Cas GE is fast and accurate; crops developed using this technique are resistant to viruses, fungi, and bacteria and resilient to abiotic and biotic stresses, while crops established using conventional methods take between 10 and 15 years to develop resistance. Therefore, CRISPR/Cas is a useful tool for sustainable agricultural production. Plant traits have been successfully manipulated using this technology. Notwithstanding the technical challenges of transferring CRISPR/Cas9 developed crops from the laboratory to the field, additional obstacles include uncertain policy systems, dispute over intellectual property ownership, and acceptability by consumers. Several Cas9-based applied techniques have gained popularity that enable researchers to enhance plants with speed and accuracy. In conclusion, the CRISPR/Cas9 system is a powerful technology for genetically modifying crops.

1. Introduction

Gene engineering enhances the heritable or nonheritable genetic material to control the genotype or phenotype of a certain cell, tissue, or organism. It is used to delete and insert a specific gene or DNA sequence to yield genetic adjustments [1]. Gene editing (GE) has benefits for the management of both genetic and nongenetic plant traits, and clustered interspaced palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) (the CRISPR/Cas system) have shown considerable advantages due to their simplicity and specificity [2–4].

CRISPR/Cas systems have been well determined and studied in animal species, but much remains to be carried out in plants [5]. The system has arisen to substitute conventional plant breeding and transgenic (GMO) strategies for plant improvement. Zinc finger nucleases (ZFNs) and TAL effector nucleases (TALENs) have traditionally been the only methods available for double-stranded DNA cleavage. However, execution of these methods is complex and hence the need for simpler and effective alternatives [6]. Recently, a less challenging method was developed, based on the bacterial type II CRISPR/Cas immune system, the CRISPR/Cas9 system. The CRISPR/Cas system allows specific DNA cleavage and DNA repair through nonhomologous end joining (NHEJ) and homologous directed repair (HDR) mechanisms [7].

2. The Evolution of CRISPR/Cas9 Technology

Considerable research has focused on CRISPR systems. The essential CRISPRs were identified decades ago by Ishino in

Escherichia coli [8]; however, the functions of the system remained unclear until the mid-2000s.

In 1993, CRISPRs were detected in *Haloferax mediterranei* [7, 9–11] and were successively recognized in a range of bacterial and archaeal genomes. In the early 2000s, the detection of sequence resemblance between the spacer regions of CRISPRs and sequences of bacteriophages, archaeal viruses, and plasmids shed light on CRISPR as an immune system [12]. This important discovery, made by Mojica and others [13], was not recognized, but was published in 2005 with the aid of three research organizations.

Proportional genomic analyses have demonstrated that CRISPR/Cas systems are bacterial cell immune response systems against viral invasion [11]. The first CRISPR was discovered in *E. coli*, using an iap gene evaluation [3]. The preserved gene sequence was repeated five times, with consistent spacing: subsequent genome analysis revealed a 14-time repeat. Jansen et al. [14] suggested the term CRISPR, and this became established as the research community worked on these sequences [7].

In nature, CRISPR/Cas systems function as a prokaryotic acquired immune system [13]. Insertion of a phage sequence into the spacer region of the *Staphylococcus thermophilus* CRISPR rendered this strain resistant to the equivalent phage. In-depth examination of genomic islands has revealed that Cas1-solo genes are consistently positioned within the locality of B DNA polymerases [15]; therefore, it is believed that Cas1 is the main enzyme responsible for the movement of important genetic elements, named casposons [16].

3. CRISPR/Cas9 Technology in Plant GE

The ability to develop GE crops using CRISPR/Cas is comparable to those established using conventional or mutation breeding. Depending on the situation, there may be other suitable tools; however, in general, CRISPR is recognized as a highly suitable tool.

Evolving gene modification research in crop plants involves probing gene function and rewiring regulatory signaling networks and the single-guide RNA (sgRNA) library for high-throughput loss of function screening [17–19]. The influential and innovative CRISPR/Cas system could be used to enhance plant adaptation to local environmental stresses and maximize crop yields. The Cas9 protein cleavages DNA, producing a double-strand break (DSB) and activating a mobile DNA restoration mechanism. If there is no homologous repair, the error-prone NHEJ pathway is prompted leading to random insertions/deletions or even substitutions at the DSB site, causing mutations on this region [19].

Alternatively, if a homologous template is present, the HDR pathway is instigated, resulting in gene mutations comprising gene knock-in, deletion, or mutation [20]. The use of CRISPR in plants is very topical; three reports defined the first use of Cas9/sgRNA technology in plant gene engineering [21]. These were shortly followed by a further five studies, which added gene mutations via NHEL and HDR, and generated whole plants carrying mutations at the targeted loci [9, 17, 22, 23]. Gene mutations added via NHEJ and HDR have been documented. Five of the numerous reports generated whole plants that carry mutations at the targeted loci [17].

The CRISPR/Cas tool produces unchanging gene mutations, with the ability to segregate from the Cas9/sgRNA construct to avoid similar modifications by CRISPR/Cas [4, 15, 24–26].

CRISPR/Cas technology has been applied to many plant structures to promote resistance to environmental stresses, while enhancing other agronomic characteristics. Although advances of this system have increased on-target effectiveness, most works are in its infancy and, therefore, needs improvement. Nonetheless, CRISPR/Cas9-based genome editing is a promising approach to develop "suitably edited" plants that will assist with future food security.

A different technology has been established that enables DSB and donor template-free base editing from C to T at a specific target site [27]. These base substitutions are mediated by a cytidine deaminase that converts cytidine into uridine by eliminating an amino group. Consequent DNA repair of the U-G mismatch results in a U-A base pair that is further resolved to a stable T-A base pair [27]. CRISPR/Cas-based directed evolution is a developing field, in which base editing plays a key role, and in recent years, numerous groups have established methods to improve base editing [27–29]. The viability and effectiveness of cytidine deaminase base editing (CBE) have been tested in rice at three chosen targets: one target (P2) in OsPDS, which encodes a phytoene desaturase, and two targets (S3 and S5) in OsS-BEIIb, which encode starch branching enzyme IIb. Accurate point mutations were effectively introduced into the three target sites, and the efficiencies of obtaining the desired mutations at the S5, S3, and P2 targets were 19.2%, 10.5%, and 1.0%, respectively [30].

The CRISPR/Cas base editing tool enables specific and efficient single-base conversion at targeted genomic sites and has been extensively used in several organisms, including plants [31–33]. Nonetheless, many obstacles limit the prevalent use of base editors, such as high off-target activity, limited PAM sites, and wide editing windows; various attempts have been made in recent years to reduce these limitations and improve the specificity of base editors [34, 35]. In the future, further research is required for modifying existing CBE and ABE tools, applying the RE-PAIR and RESCUE systems in plants, and developing novel base editors with the capacity of directing transversion mutations [30].

Base editing is widely used in developing disease-resistant crops. Notwithstanding the previously described limitations above, base editing systems have the potential to simplify crop bioproduction or improvement, through high precision and efficiency [36]. Base editing could play a dynamic role in the introduction of gene mutations and directed protein evolution. Cytosine base editing lacking UGI has the capacity to make diverse mutations, other than C-to-T. This ability has already been used in CRISPR-X and targeted AID-mediated mutagenesis- (TAM-) based studies to identify known and novel mutations in mammalian cells

in cancer therapeutics targets PSMB5 and BCR-ABL, respectively [36]. Although there is an ever-increasing interest in BE and PE editing tools, there remain challenges that require further research, one major challenge being constrained target sites due to the PAM specificity of Cas proteins [31, 33].

4. Websites for Developing Constructs for CRISPR/Cas9 Genetic Engineering Procedures

For the best outcomes from CRISPR-based trials, some essential components must be considered in the experimental design. The general objective of CRISPR experiments is to acquire high rates of ideal genome mutations, low rates of off-targets or nonspecific impacts, and a suitable readout of the outcome [13]. While CRISPR has demonstrated high success rates, the fluctuations in effectiveness and particularity are not perfect, and delivery of the CRISPR framework into the natural model system of interest is challenging in certain frameworks [13]. Hence, it is important to streamline and validate exploratory designs to achieve the best outcomes.

5. Methods to Improve the Delivery of CRISPR/Cas9 in Plants

Plant transformation approaches vary significantly, and it is difficult to clearly distinguish in planta and in vitro transformation. GE provides an opportunity to create a transgene-free edited plant genome. The delivery method of the CRISPR/Cas9 segment is of critical significance for genome modification in plants [37]. Diverse delivery techniques, for example, *Agrobacterium*-mediated, bombardment or biolistic methods, floral-dip, and PEG-mediated protoplasts are often applied to crops for proficient genome editing. The efficiency of *Agrobacterium*-mediated transfer was improved by avoiding chemical selection, such as kanamycin. The absence of a chemical selection agent following *Agrobacterium* infection gives greater rates of plant regeneration, compared to using chemical selection and, therefore, likely improves mutant callus/shoot production [38].

For bombardment or biolistic methods, in planta GE has been reported, in which callus culture and regeneration are not required; shoot apical meristems (SAMs) were used that contained a subepidermal cell layer, L2, from which germ cells later develop during floral organogenesis [39]. For the biolistic delivery, gold particles coated with plasmids expressing CRISPR/Cas9 components were bombarded into SAM-exposed embryos of imbibed seeds and mutations were achieved [39]. In *Arabidopsis*, the easiest and most commonly used method of in planta transformation is the floral-dip [40]. This technique is based on the immersion of unopened flowers into an *Agrobacterium* suspension containing a strong surfactant, generally Silwet-77. Owing to the surfactant, the *Agrobacterium* cells enter flower tissues and transfer T-DNA into plant cells, predominantly into the gametes. Following fertilization, zygotes with inserted T-DNA give rise to transgenic embryos and, after germination, to transgenic plants. This method makes it very easy to generate transgenic plants, without going through in vitro mechanisms [40].

6. Mechanisms Underlying CRISPR/Cas9-Mediated Resistance

6.1. CRISPR/Cas9 and Disease Resistance. The use of resistance genes in conventional breeding has resulted in crops that use hypersensitive response mechanisms to fight disease [41]. There have been numerous advances in the use of functional mutations to create broad-spectrum disease resistance, as in the *Arabidopsis thaliana* DMR6 gene [41]. Inactivation of the AtDMR6 homologue in tomatoes confers the plant with resistance to *Pseudomonas syringae*, *Xanthomonas* spp., and *Phytophthora capsici* [41].

Wang et al. [42] developed mutagenized rice lines using CRISPR/Cas9 technology that showed significantly better blast resistance compared to wild-type rice [19]. The development of wheat varieties resistant to powdery mildew was achieved through mutation of the TaMLO-A1, TaMLO-B1, and TaMLO-D1 genes in the wheat genome using CRISPR/Cas9 [43].

Successful gene mutations of the phytoene desaturase gene in cassava using CRISPR/Cas9 confirmed its use as a versatile GE tool for this crop. CRISPR/Cas9-mediated modification of the cassava elF4E isoforms, nCBP-1 and nCBP-2, resulted in increased CBSD resistance [44]. CRISPR/Cas9/sgRNA technology has also been used for altering canker tolerance genes in Duncan grapefruit. This provides evidence that CRISPR/CAs technology provides a useful technique to induce resistance in plants [45].

6.2. CRISPR/Cas9 and Virus Resistance. Improved virus resistance was achieved in *Cucumis sativus* L. by disrupting the recessive eukaryotic translation initiation thing 4E (eIF4E) gene [46]. Nontransgenic heterozygous eif4e mutant plants were chosen for the production of nontransgenic homozygous T3 plants. Conversely, nonmutant and/or heterozygous plants showed susceptibility to viruses.

6.3. CRISPR/Cas9 and Parasitic Weed Resistance. Gene silencing using RNA interference (RNAi) has been successful in preventing haustorial initiation and growth in tobacco [47] and lettuce [48], respectively. In maize, RNAi did not result in *Striga* resistance [49], whereas viral-induced gene silencing in maize for *Striga hermonthica* was successful [50]. Mutant alleles at the low germination stimulant 1 (LGS1) locus significantly decrease Striga germination activity [51]. Advances in the production of reference genomes for parasitic species require greater use of GE technologies to interfere with genes responsible for parasitism [52].

7. Latest Achievements in Practical Uses of CRISPR/Cas9 Technology in Plants

The useful deletion of a candidate gene is a simple stage in plant genetics. The Cas9/gRNA technique has been used

efficiently for performing gene knockouts in plants, such as *Arabidopsis*, rice, tobacco, and sorghum [51, 53–56]. CRISPR/Cas9-mediated genome editing has been demonstrated commercially in different plants, such as maize [57–59], wheat [60–64], soybean [56], tomato [65–68], petunia [23], and sorghum [51]. Twenty-four plant species have been transformed using CRISPR/Cas technology: cereal crops (barley, maize, rice, switchgrass, and wheat), vegetables and melons (cabbage, carrot, cassava, cucumber, potato, rape, tomato, and watermelon), fruits (apple, banana, grape, grapefruit, and orange), legumes (alfalfa and soybean), technical plants (Ethiopian mustard, cotton, and flax), and coffee [18].

Using CRISPR/Cas9 technology, deletions of 200 bp have been executed with a frequency of >10% in rice protoplasts [28], and deletions of 357–761 bp have been conducted at frequencies of 4–45% in rice protoplasts and up to 6% in T0 flowers [67].

The type II CRISPR/Cas system from *Streptococcus pyogenes* and its simplified derivative, the Cas9/sgRNA system, have emerged as powerful new tools for targeted gene knockout in bacteria, yeast, fruit fly, zebrafish, and human cells. Diversification of these structures has led to expression of the Cas9/sgRNA system in two dicot plant species, *Arabidopsis* and tobacco, and two monocot crop species, rice and sorghum. *Agrobacterium tumefaciens* was used for delivery of genes encoding Cas9, sgRNA, and a nonfunctional mutant inexperienced fluorescence protein (GFP) to *Arabidopsis* and tobacco [53]. The mutant GFP gene contained target sites in its 5' coding regions that were effectively cleaved by a CAS9/sgRNA complex, along with error-prone DNA repair, resulting in functional GFP genes. DNA sequencing validated Cas9/sgRNA-mediated mutagenesis at the target site.

8. Advantages of CRISPR/Cas9 Technology in Plant Genetic Engineering

Studies of different plant systems have demonstrated the utility, versatility, and heritability of CRISPR/Cas9-mediated GE. Numerous reports have also shown that necessary crop characteristics can be altered by single-gene mutations using CRISPR/Cas9 [69]. The system is easy to develop, design, and implement, since only one nuclease is required and the gRNA is easy to assemble and transfer into cells [70]. Numerous reports have shown no off-target mutations, making CRISPR/Cas9 the tool of preference for plant genome engineering [23, 67, 71]. Compared with the time consuming and high-cost cloning procedures of ZFNs and TALENs, the CRISPR/Cas system allows for low cost and rapid adaptation to a number of sites and produces mutations of high frequencies for plant genomes [28, 37, 72, 73]. Several studies have confirmed the potential of CRISPR/Cas to generate a large range of allelic diversity at unique loci [42, 58, 73].

9. Limitations to Using CRISPR/Cas9 in Plants

Undertaking CRISPR/Cas9-mediated GE requires a number of materials upfront, for example, laboratory resources, proper identification of genomic targets, appropriate vectors that precisely and effectively cut target sites, and a robust regeneration protocol and transformation system [69]. The major setback of CRISPR/Cas9, if not properly designed and executed, is elevated off-targets [54, 74]. Some of the limitations for utilizing CRISPR in plants are as follows:

(i) Direct targeting of S genes may cause a fitness cost due to their linkage with other desirable genes, particularly genes controlling plant growth and development

(ii) "Off-target mutations" have become a mainstay in efforts to improve the CRISPR system, particularly in the production of transgene-free crops

(iii) Safety and commercialization issue are directly related to humans and other living organisms

10. Off-Target Consequences in CRISPR/Cas-Mediated GE

In terms of the frequency of off-target edits that can arise from crop genome editing, it is imperative to recognize the comparative source and scale of genome alterations that occur in plants under natural circumstances. Irrespective of the source, mutations in higher plants can result in error-prone repairs, some of which may prompt direct toxic effects to reduce protein synthesis, destroy cell membranes and photosynthetic proteins to inhibit plant growth, lead to chromosome fusions, or produce genetic changes in plant populations that may be passed on to the next generations [75, 76].

The DSBs in coding regions presented by CRISPR/Cas9, or other genome-editing reagents, are also contingent on plant endogenous repair pathways that may insert or delete fragments to prompt mutation. Consequently, genome alterations by CRISPR/Cas9, or other genome-editing reagents, are much more site-specific than related modifications by traditional plant breeding methods, and those DSBs in off-target sites are equivalent to naturally induced mutations [37, 74, 77]. In relation to process-based risks, further improvements in targeting, off-target molecular characterization, and the implementation of methodologies to avoid or characterize foreign DNA in plant germplasms in crop breeding programs will reduce the undesired outcomes of the genome-editing process. Additional characterization of downstream risks connected with a specific genome-edited crop should be conducted, as appropriate for the generated phenotype generated and its anticipated uses [78].

11. Policy and Legislation Hindering the Expansion of GE Technology

GE represents a multidimensional shift beyond "genetic modification" (GM) and is outpacing both public understanding and the capacity of regulatory organizations. Under EU law, most products produced using mutagenesis are considered GMOs by definition, but are explicitly exempted from GMO regulation. A debate exists regarding whether

mutagenesis exemption, which dates from 2001, only applies to processes available until 2001 or if the exemption also includes future processes [79]. The introduction of genome-edited products that are nearing commercialization in agriculture has underscored that the US biotechnology regulatory system has not kept pace with technological advances. Of the three agencies that regulate engineered crops and animals for agriculture, only one has indicated how it will regulate edited plants [80].

The Food and Drug Administration can regulate any plant, but has not indicated if it will single out edited plants; the US Department of Agriculture currently has no authority over edited plants when the edit is a deletion or does not contain any added DNA from a plant pest [80]; and depending on how the statutes are interpreted, the Environmental Protection Agency might be able to regulate plants edited to tolerate pests and diseases. Labeling requirements also remain undefined. Regardless, industry and some consumer groups are anxious over editing technology and may be the ultimate arbiters of whether edited products make it to market [80]. Canada's science-based regulatory system was adapted for GM crops in the early 1990s and has proven sufficiently robust in responding to new plant breeding techniques, having approved two varieties of GE canola [81].

12. Regulatory Issues on GM Crops and Biotechnology-Related Products in an African Context

Recognizing the important (potential) benefits of biotechnology to improving food security and rural development, governments across Africa have taken steps to establish a policy framework to support and enable the adoption of biotechnology, including GM crops and derived products. A more detailed analysis of relevant policies and regulations is presented. Examples of recent policy decisions regarding GM crops include the following: approvals for general release and commercial variety registration for insect-resistant, GM cotton hybrids in Ethiopia (2018), Kenya (2019), Malawi (2019), and Nigeria (2018). While farmers in Ethiopia started planting GM cotton on a limited scale in 2019, GM seed distribution in Kenya, Nigeria, and Malawi will start in 2020 [82, 83].

Apart from reigniting old ethical debates, GE also poses a particular challenge to legal practitioners as this new technology does not always fit into existing definitional molds, and it lacks clear legal precedent. In this article, the most salient areas of concern in the South African legal context are identified [84].

13. Why Edited Plants Should Not be Considered Transgenic

Transgene-free techniques can be achieved by CRISPR-mediated plant GE, for example, by isolating out transgenes through segregation, delivering the ribonucleoprotein complex of Cas9 and gRNA through particle bombardment or using a protoplast system, and utilizing viral vectors for editing germline cells [85]. This ensures that the end product does not carry any foreign DNA and hence cannot be regarded as transgenic. The recently acquired capacity to precisely alter plant genomes by adjusting native genes without presenting new hereditary material offers new chances to quickly exploit natural variation, create new variation, and make changes, with the objective of creating more productive and nutritious plants [86]. It has been proposed that NHEJ will be a favored methodology in crop breeding that utilizes GE, in light of the fact that the resultant plants are considered to hold no transgenes, which is one of the significant concerns of GM crops. In view of this, transgene-free plants developed through GE must not be regulated as GMO. A few specialists and interest groups have also proclaimed that such transgene-free crops should not be regulated under procedure-based GMO guidelines [87, 88].

14. CRISPR Regulatory Issues

Predictable target mutagenesis in plants, with the aid of the CRISPR/Cas9 system, opens up new avenues in plant research. Traditional arbitrary mutagenesis strategies are unable to contact every gene for inactivation due to the randomness of the gene combination.

CRISPR/Cas9 technology can assist in producing changes on unapproachable genes, transforming multiple loci, and producing huge deletions, which result in quick advances in plant breeding efforts, without introducing transgenes [11, 89–91]. While GM crops may help crop improvement, the arguments regarding the environmental and health consequences of GM plants could be prevented through GE technology (Belhaj et al., 2013; Botha & Viljoen, 2008). CRISPR-edited plants are excluded from current GMO regulations; however, this technology may present new issues, with regards to legislation and consumer acceptance of GE plants [9, 22], Shan et al., 2013.

Crops that have been engineered at the gene/genome level through the use of CRISPR/Cas9 technology are not regarded as GMO under product-based GMO regulations due to the fact that the ultimate products are not GMOs [17]. GE plants with the proper governing arrangements in place may be deemed more appropriate than plants that bring foreign DNA in their genomes. The US Department of Agriculture has specified that GE plants that do not contain foreign DNA will not be viewed as GMOs, while the European Commission is expected to soon submit their view regarding the regulatory ambiguity of genome editing (Belhaj et al., 2013). The potential for CRISPR technology to be utilized in plant research and crop breeding, and its value for global food security, is also contingent on consumer acceptance of GE crops [1, 17].

15. Conclusions and Perspectives

Recent studies have demonstrated that CRISPR/Cas9 technology is an important system for genome engineering. Several Cas9-based applied techniques are being utilized in plants, and these tools will deliver unique understanding

into plant biology and enable rapid and precise plants enhancements via breeding.

As genome-editing technology is used more extensively in plants, the safety of genome-edited plants is a point of discussion in the plant community [92]. Undoubtedly, the CRISPR/Cas9 system is one of the most powerful tools in crop genetic modification.

Abbreviations

CRISPR: Clustered random interspaced palindromic repeats
NHEJ: Nonhomologous end joining
HDR: Homologous directed repair
GE: Gene editing
GM: Genetic modification.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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