

Review

CRISPR/Cas genome editing: A frontier for transforming precision cassava breeding

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Improved agricultural production of essential crops through advanced breeding is important for increasing access to nutritious food for the world's rapidly growing population, which is expected to reach 9.8 billion by 2050. Recent advancements in the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein9 (CRISPR/Cas9) genome editing process, which uses single-guide RNA for genome editing, have made it easy, stable and efficient tool for targeted gene mutations, knockout and knock-in/replacement to boost crop yield. The CRISPR/Cas method is constantly being improved, and its applications have greatly expanded. It can be used to modify the genome sequence of any organism, including plants like cassava, to achieve the desired trait. As a result, CRISPR/Cas is regarded as a game-changing technology in plant biology. Here, we discuss the principles of operation, implementations and future prospects of CRISPR/Cas9 for efficient processing of individual genes in cassava cultures. Recent work on cassava crop with regards to the use of CRISPR/Cas9 for the plant improvement was also addressed.

Key words: *Manihot esculenta*, CRISPR/Cas9, genome editing, gRNA, protospacer adjacent motif (PAM).

INTRODUCTION

With a predicted global population of 9.8 billion people by 2050, the agri-food industry would need to double crop production rates (Clarke and Zhang, 2013; Ray et al., 2013). The ability to feed this rapidly growing population will soon become a critical issue that society, especially agricultural scientists, must address (Savary et al., 2019; Kumar et al., 2020). However, the five key crops that produce almost two-thirds of agricultural calories: maize, wheat, soybean, cassava and rice, currently have the production rates of 1.6, 0.9, 2.2, 1.3 and 1.0%,

respectively, as compared to the expected rate of 2.4% (Ray et al., 2013; FAO, 2020). Again, developing new varieties in any crop takes time because it is dependent on the crop's generation cycle. Factors such as diminishing cultivable land, climate change, water scarcity, and increased demand for biofuels aggravate the situation (Ricroch, 2019). Climate change has an impact on crop production by hastening ecological stresses such as soil salinity, drought, and the emergence of new pathogens and insect pests, in

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addition changing weather patterns (Godfray et al., 2010; Bhatta and Malla, 2020). Crops with higher yields, greater adaptability to changing climates, tolerance to biotic and abiotic stresses, improved nutrition, and lower resource requirements are being developed to meet this anticipated demand (Li and Xia, 2020).

Present day agriculture has evolved into an massive and complex production chain with ever increasing reliance on crop optimization via soil, water, postharvest management employing sophisticated machinery and facilities (Xingliang et al., 2018). Via self-pollination, cross-pollination, or clonal propagation, traditional breeding technologies have been used to improve crop production for important crops. Despite their use, these techniques have only been applied to a small number of crops, such as wheat and maize, which are widely grown in temperate regions around the world (Al-Khayri et al., 2015; Ansari et al., 2017), and limited diversity have further confined the amount of crop improvement that can be achieved through this process (Li et al., 2020). While conventional plant breeding is an extremely critical tool, it has its own constraints as well. To begin with, breeding can only take place between two plants that are sexually compatible. Secondly, numerous traits, including those that have negative effects on the yield potential, are transferred along with the traits of interest during crossing (ISAAA, 2020). Furthermore, in terms of edible crop crops, the incorporation of transgenes via these techniques is non-specific and is a matter of public concern. Finally, in terms of adapting elite varieties to local environmental conditions, these techniques remain time-consuming, resource-intensive, and expensive (Ghogare et al., 2020). As a result, breeding technological advancements are critical to overcoming the shortcomings of conventional breeding (Haque et al., 2018). Artificial mutagenesis, such as chemical mutagenesis, irradiation, and other modern methods, such as insertional mutagenesis by T-DNA insertion or transposon labeling, have been used to achieve these results (Xingliang et al., 2016). These modern mutagenesis have also encountered issues ranging from the random nature of induced mutations, low efficiency, time-consuming, laborious to being costly (Arora and Narula, 2017). Targeted gene technology based on homologous recombination has recently been developed, which allows for precise mutations, but they provide edits to limited number of species (Razzaq et al., 2019).

Speed breeding, also known as accelerated plant breeding cycle, has also risen to prominence in recent years as a modern and exciting breeding method that promises to grow new crop varieties faster, bringing hope to global food security (Ghosh et al., 2018a). This method involves growing plants in regulated growth chambers or greenhouses with optimal light intensity and quality (20 - 22 h), which speeds up various physiological processes in plants, particularly photosynthesis and flowering, and thus shortens the time of generation (Ghosh et al.,

2018b). Rapid breeding techniques can involve any combination of the following methods: plant growth environment optimization (e.g., plant density, photoperiod, and temperature), flowering pathway genetic engineering, grafting juvenile plants to mature rootstocks, use of plant growth regulators, and early seed harvest (O'Connor et al., 2013; Ceballos et al., 2017). The method has been applied to produce 4 to 6 generation per year as compared to 2 to 3 generations per year for conventional breeding technologies under standard conditions (Nocker and Gardiner, 2014). This strategy is being applied in many orphaned crops, and standardization protocols are being developed for many perennial crops including apple (Chiurugwi et al., 2019). These techniques have not been utilized to the fullest as far cassava crop production is concerned, leading to the need for a fast, precise and simple methods for cassava crop improvement to alleviate the problem of food insecurity which is be brought about by the growing world population.

Advanced genome editing technology revolutionization has shown potential improvements in crops in recent years, making it easier to produce new varieties (Abdelrahman et al., 2018). These gene-specific genome editing technologies, zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and more recently, clustered regularly interspaced short palindromic repeats (CRISPR), are important because they are fast, effective, and technically straightforward to use (Peisach et al., 2001; Christian et al., 2010; Georges and Ray, 2017). In the target DNA, the nucleases trigger double-stranded breaks. The DNA is then repaired using one of two pathways: nonhomologous end-joining repair (NHEJ) or homology guided repair (HDR); the former is the most common, resulting in insertions/deletions and substitution mutations in the target DNAs, resulting in insertions/deletions and substitution mutations in the target DNAs (Figure 1) (Savic et al., 2017). These tools also sparked the development of new transgene-free crop varieties that are difficult to differentiate from those developed using conventional breeding methods (Sharma et al., 2017). Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins genome editing technology has shown greater promise in addressing agricultural challenges than its predecessors due to its ease, versatility, and effectiveness (Gupta and Musunuru, 2014; Mohan, Shibao and Silva., 2019). This technology can be used to alter virtually any genomic sequence in species, including plants, to achieve the desired characteristics since it only requires the presence of the protospacer adjacent motif (PAM) sequence (Mekler et al., 2020).

UNDERSTANDING CRISPR/CAS GENOME EDITING

CRISPR/Cas is a form of adaptive immunity discovered

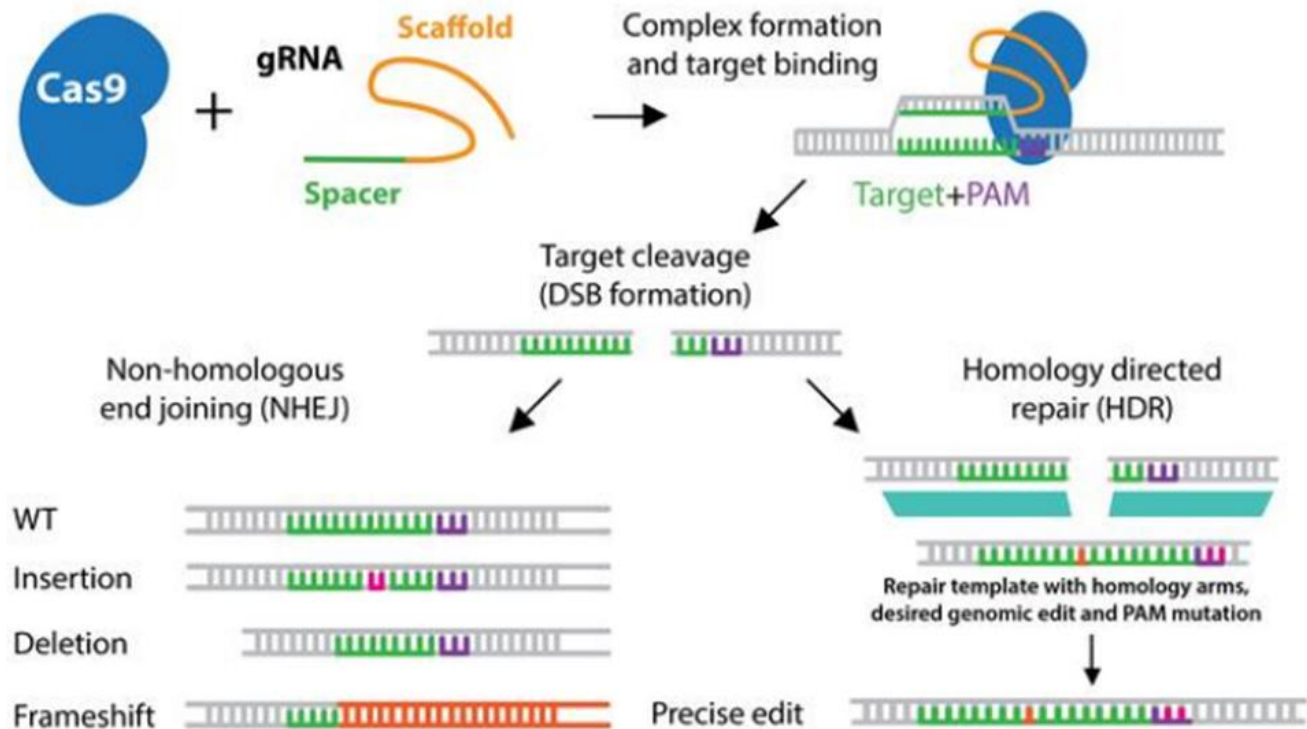


Figure 1. CRISPR/Cas9 for DSBs and NHEJ or HDR.
Source: Savic et al. (2017)

in prokaryotes in 1987 by Atsuo Nakata's group in Japan while researching the *iap* enzyme involved in isozyme conversion of alkaline phosphate in *Escherichia coli* (Ishino et al., 1987). It was not until 2005 that the CRISPR spacers were discovered to be extremely homologous with exogenous bacterial plasmids and phages enabling them to cleave foreign DNA (Watters, 2018). All at the same, the CRISPR-associated (Cas gene) genes were discovered (Jansen et al., 2002), and yields the Cas proteins (Barrangou and Horvath, 2017). CRISPR/Cas, a critical site-specific gene editing tool, was later developed in 2013, as described by two scientists named Jennifer Doudna and Emmanuelle Charpentier, who demonstrated that CRISPR can be used to alter human genes outside the body (Jinek et al., 2012; Doudna and Charpentier, 2014). To cleave complex DNA sequences, the CRISPR-Cas system employs a combination of proteins and short RNAs (Hoffmann et al., 2019). Protospacers from foreign DNA sequences are collected by the bacteria, inserted into their genome, and used to make short guide RNAs, which are then used by the CRISPR-Cas system to kill any DNA sequences that match the protospacers (Musunuru, 2017).

CRISPR/Cas is faster, cheaper, and more effective at multiplexing genome editing than other previously developed genome editing tools like ZFNs and TALENs (Wang et al., 2018). CRISPR/Cas operations are rapidly

expanding as a result of these benefits (Nakayama et al., 2013). In the not-too-distant future, genome editing technologies will have a tremendous impact on agriculture, because they will allow for direct and rapid genetic modification of various crops in the field (Xuejun et al., 2017). Between 1987 and 2002, several clusters of signature CRISPR-associated (Cas) genes were discovered to be conserved and usually adjacent to repeat components, laying the groundwork for the future classification of the CRISPR/Cas system into two major classes based on effector module design principles: class I and class II, which are further divided into six categories (Makarova et al., 2011, 2015). Classifications are based on the phylogeny, sequence, locus, organizations and contents of the CRISPR array. Class 1 systems have multi-subunit effector complexes made up of several Cas proteins with uneven stoichiometry, whereas class 2 systems have a single large multi-domain protein found almost exclusively in bacteria as an effector (Yoshizumi et al., 2018).

Each of the two CRISPR-Cas classes is divided into three categories, with types I, III, and IV belonging to class 1 and types II, V, and VI in class 2. Each type is distinguished by distinct effector module architectures that include distinct signature proteins (Shmakov et al., 2015). The presence of distinct signature proteins distinguishes types I, II, and III: Cas3 for type I, Cas9 for

type II, and Cas10 for type III (Rouillon et al., 2013). The CRISPR-associated complex for antiviral defense (CASCADE) and the Csm/Cmr RAMP complexes are multimeric effector complexes of type I and type III systems, respectively, that are architecturally identical and evolutionarily related (Young et al., 2019; Molina et al., 2020). Type III systems are thought to target both DNA and RNA, while type I and II systems are thought to target only DNA (Shmakov et al., 2017a). The signature protein, Cas10, is contained in type III CRISPR-Cas systems. The functionally uncharacterized type IV systems, unlike all other developed CRISPR-Cas systems, lack the adaptation module, which consists of the nucleases Cas1 and Cas2 (Makarova et al., 2015; Pinilla-Redondo et al., 2020). Spacers produced by type I systems are known to be used in the effector modules of subtype III-B systems, demonstrating the modularity of CRISPR-Cas systems (Garrett et al., 2011). While majority of the genomes encoding type IV systems do not carry recognizable CRISPR loci, it is not excluded that crRNAs from different CRISPR arrays are used by type IV systems, similar to subtype III-B systems, once they become available (Shmakov et al., 2015). Finally, based on additional signature genes and characteristic gene arrangements, each type is subdivided into several subtypes (I-A to F and U and III-A to D in class 1; II-A to C, V-A to E and U, and VI-A to C in class 2) (Shmakov et al., 2017b).

Much of the study currently focuses on type II CRISPR/Cas9 concepts and implementations, primarily because it has a drastically reduced number of Cas proteins (Yin et al., 2015). To change genes, the CRISPR/Cas9 system employs the CRISPR-associated 9 proteins, the CRISPR RNA (crRNA) set, which encodes the guide RNA, transactivating crRNA (tracrRNA), which aids in crRNA processing, and ribonuclease III (RNase III). The use of type II CRISPR/Cas9 has resulted in significant crop improvement (Williams and Warman, 2017). The rapid production of the CRISPR/Cas9 genome editing system in plants has been addressed in many reviews (Ma et al., 2016; Liu et al., 2017; Georges and Ray, 2017; Haque et al., 2018; Manghwar et al., 2019). This paper presents a summary of recent CRISPR/Cas9 method innovations and applications in the cassava plant. Furthermore, it addresses the CRISPR/Cas method's potential growth and challenges, as well as its value for cassava crop research and enhancement.

MECHANISM OF CRISPR/CAS GENOME EDITING

CRISPR/Cas genome editing technology is a third (next)-generation genome editing method that was first used in plants in 2013 and is now the most commonly used tool for gene editing. It was found to be an RNA-guided adaptive immune system for bacteria and archaea

against invasive nucleic acids (DNA or RNA) from viruses or plasmids (Carter and Wiedenheft, 2015). The CRISPR system is operated in three stages. The first being acquisition of Spacer, the second is the processing of crRNA, and the third is the degradation of interference and targets. The CRISPR/Cas device accomplishes this by identifying and killing invading foreign DNA. By inserting fragments of the invader's genetic material into their CRISPRs and spacer arrays, these systems recall the invasion history. The CRISPR/Cas system accomplishes this by identifying and destroying invading foreign DNA. These systems recall the invasion history by inserting fragments of the invader's genetic material into their CRISPRs and spacer arrays. By inserting fragments of the invader's genetic material into their CRISPRs and spacer arrays, these systems recall the invasion history (Zhang et al., 2018). It was a landmark moment in the use of CRISPR/Cas9 when Cong et al. (2013) engineered two CRISPR/Cas9 systems and demonstrated that short RNAs could direct Cas nucleases to induce precise cleavage at endogenous loci in animals.

The CRISPR/Cas9 system is based on the type II CRISPR system, which has three main components: the CRISPR-associated protein (Cas9) and two noncoding CRISPR RNAs (tracrRNA and precursor CRISPR RNA) (pre-crRNA) (Walsh and Hochedlinger, 2013). Cas9 is an endonuclease protein derived from *Streptococcus pyogenes* that has an HNH nuclease domain in the middle and a RuvC-like nuclease domain at the amino terminus. It is responsible for crRNA maturation and the introduction of targeted DSBs, which is coordinated by tracrRNA and double-stranded RNA specific RNA III (Peng et al., 2016). The HNH domain cuts the crRNA's complementary strands, while the RuvC-like domain cleaves the double-stranded DNA's opposite strand (Jinek et al., 2014; Zuo et al., 2019). In the engineered type II CRISPR/Cas9 system, two noncoding CRISPR RNAs are fused to form a synthetic dimer, single guide RNA (sgRNA). crRNA is useful for directing the nucleolytic activity of the Cas9 enzyme to degrade the target nucleic acids (Hussain et al., 2018). The molecule of sgRNA is approximately 100 nucleotides long. It has a 20-nt reference sequence at the 5' end that helps identify the target sequence, as well as the protospacer adjacent motif (PAM) sequence, which is normally the consensus NGG sequence (Tsai et al., 2015). The PAM flanking the 3'-end of the DNA target site helps Cas9 in dictating the DNA target search process and promoting self-versus non-self-discrimination because direct repeats lack PAM sites (Xiaonan et al., 2020). The loop structure at the 3' end of the sgRNA anchors the target sequence by the guide sequence and forms a complex with Cas9, which cleaves the double-stranded DNA and forms a double-stranded break (DSB) at this site (Ueta et al., 2017).

Following the formation of DSBs, the DNA repair mechanism can be activated using either error-prone

nonhomologous end-joining (NHEJ) or homology directed repair (HDR) (Figure 1) (Symington and Gautier, 2011; Stinson et al., 2020). In most cases, NHEJ is used to repair DSBs in the absence of homologous DNA, and it is a simple way to generate mismatches and gene insertions/deletions (indels), which frequently result in frameshift mutations if they occur in the coding sequence of a gene, effectively creating a gene knockout (Zaidi et al., 2018). HDR triggers unique gene replacement or foreign DNA knock-ins when an exogenous homology repair template is present (Fauser et al., 2014). These processes allow CRISPR/Cas9 to edit the genomes of a wide variety of organisms, including plants (Schenke and Cai, 2020). Numerous crops have had their genomes edited using the CRISPR/Cas9 technique, such as rice (Zhou et al., 2014; Macovei et al., 2018), tobacco (Gao et al., 2015), maize (Doll et al., 2019; Lee et al., 2019), wheat (Bhowmik et al., 2018; Okada et al., 2019), *Arabidopsis* (Zhang et al., 2018; Li et al., 2018), potato (Andersson et al., 2018), sorghum (Che et al., 2018; Char et al., 2020), tomato (Tashkandi et al., 2018), cotton (Li et al., 2019; Qin et al., 2020), soybean (Li et al., 2015), cucumber, rapeseed (Braatz et al., 2017), barley (Lawrenson et al., 2015) and cassava (Odiopio et al., 2017; Gomez et al., 2019), for trait improvement.

While the CRISPR/Cas system has been used to boost food security in a variety of crops, is yet to be extensively utilized in cassava, a crop that is considered a potential food reserve. Cassava is among crops that survives in poor soils and can withstand the unfavourable climate change. This makes it a crop importance to curb the problem of food security in the world. The crop is affected by both abiotic and biotic stresses, in which utilization of CRISPR/Cas technology can be utilized to silence or knockout genes which are responsible for their expression. This review provides insight to works that have been done in regards to improving cassava plant.

CELLULAR DNA REPAIR MECHANISMS USING CRISPR/CAS9

CRISPR/Cas systems, particularly CRISPR/Cas9, have been widely used to edit genomes. The CRISPR-Cas9 mechanism induces a Cas9-mediated double-strand break (DSB) in prokaryotes, which is directed by two small RNAs, a CRISPR RNA (crRNA) and a trans-acting crRNA (tracrRNA), or a chimeric single guide RNA (sgRNA). Homologous recombination (HR) and non-homologous end-joining (NEJ) are the two main competing and partially overlapping pathways for repairing DSBs (NHEJ) (Khoury et al., 2018; Jayavaradhan et al., 2019). NHEJ is a flaw-prone repair pathway that can result in frameshift and non-sense mutations by insertion and/or deletion of short DNA sequences at the DSB site, a mechanism widely exploited in recently developed CRISPR-based gene editing

technology (Chhotaray et al., 2018). NHEJ is known as the canonical homology-independent pathway because it only involves the alignment of one to a few complementary bases at most for the re-ligation of two ends (Pardo et al., 2009). HR, on the other hand, is a conservative mechanism that results in the reciprocal exchanging of genetic information between two homologous DNA sequences or, typically, in the unidirectional conversion of genes. The dominance of these two repair mechanisms differs by species, cell type, cell cycle stage, and even end DNA resection, with NHEJ dominant in most somatic cells and HR dominating in yeast, germline, and mammalian embryonic stem cells (Sansbury et al., 2019).

In contrast to single-stranded annealing and breakage-induced replication, which involve short sequence homology, HR requires greater sequence homology when exchanging DNA segments. HR has a high degree of fidelity but a low rate of occurrence (Jasin and Rothstein, 2013). HR requires donor sequences that are homologous for insertion accuracy or substitution at the target site of integration into plant genomes at the DSB site (Shimada, 1978). It only occurs during the S and G2 phases of the cell cycle. HR requires that genetic modification performance be improved by inhibiting enzymes associated with the NHEJ pathway, such as DNA ligase IV (Schmidt et al., 2019). The use of ribonucleoprotein in conjunction with endonucleases can increase the efficiency of HR-mediated repair by a factor of 2 to 6. Injection efficiency is also increased by adjusting the delivery time in complexes that affect the cell cycle (Tang et al., 2019). Controlling endonuclease delivery during the cell cycle can also improve HR performance (Nambiar et al., 2019).

As previously demonstrated in maize and *Arabidopsis*, NHEJ is essential for DSB plant repair (Palareti et al., 2016). NHEJ does not occur naturally during the S/G2 phase of the cell cycle due to the lack of homologous DNA near the DSB, whereas HR does. NHEJ comes in two varieties: (i) Canonical nonhomologous end-joining (C-NHEJ) and (ii) Alternative nonhomologous end-joining (A-NHEJ) pathways (A-NHEJ). The C-NHEJ is ku-dependent, and both are prone to errors. C-NHEJ involves three steps: (1) the ku-protein recognizes and binds the DSBs in a sequence-independent manner; (2) the damaged ends of the DNA are enzymatically processed; and (3) DNA ligase IV ligation at the DSB ends (Shen et al., 2017).

Because NHEJ is prone to mistakes, using it to modify pathways in order to generate targeted knockouts frequently results in deletions or insertions (indels) (Malzahn et al., 2017). In plants, the leading pathway for repairing DNA DSBs is not dependent on a homologous donor (Bernheim et al., 2017). Because NHEJ's non-specificity in the genome reduces its effectiveness in gene targeting, the NHEJ pathway must be inhibited, while the HR pathway for gene editing must be improved,

and spontaneous incorporation of donor molecules and mutagenic off-target effects must be reduced (Li et al., 2017).

NHEJ involves the mechanism of partial end-restriction and promotion of direct ligation of enormous DSBs' free ends, while HR removes breaks and leaves no errors (McFarlane et al., 2018). Because of evolutionary requirements to ensure genome integrity as part of genome complexity, NHEJ in plant somatic cells remains highly subjective (Ye et al., 2018). HR repair involves substitution, chromosome rearrangement, gene disruption, correction, and insertion, while NHEJ repair involves insertions and deletions; thus, biologists must understand each repair pathway and the factors involved in these pathways in order to design experimental designs for genome modification in plants (Devkota, 2018).

For the works that have been done in respect to cassava, the DSBs repair mechanism which has been used commonly in the error-prone NHEJ pathway. For example, Mehta et al., (2018) stated that CRISPR systems failed to confer virus resistance during glasshouse inoculation, resulting in frame-shift mutations. Further analysis showed that there were viral escapes associated with NHEJ method of DNA repair.

DELIVERY OF CRISPR/CAS REAGENTS TO CASSAVA

The delivery of editing reagents to plants and the production of editing events are crucial steps in genome editing in plants. To introduce CRISPR-mediated reagents such as DNA, RNA, and ribonucleoproteins (RNPs) into plant cells, protoplast transfection, *Agrobacterium*-mediated transfer DNA (T-DNA) transformation, or particle bombardment may all be used (Hui-Li et al., 2014). Particle bombardment and *Agrobacterium*-mediated transformation are the two main methods for producing stable edited plants, while protoplast transfection is typically used for transfection expression (McFarlane et al., 2018).

CRISPR/Cas DNA genome editing with stable expression

CRISPR/Cas DNA is delivered into recipient cells via *Agrobacterium*-mediated transformation or particle bombardment and, by selecting for a marker gene, the DNA is integrated into the plant genome and expressed to achieve genome editing (Hui-Li et al., 2014; McFarlane et al., 2018). This strategy has been used in most types of plant genome editing, including cassava. Almost all of the genome editing work done in cassava using the CRISPR system has used the *Agrobacterium*-mediated delivery system for the reagents. The technique of delivery has been perceived to result into transgene mutants and is time-consuming since it involves the

selection stages using the herbicide and/or antibiotics, hence has not been appreciated by the larger public population (Adhikari and Poudel, 2020).

CRISPR constructs and marker genes, on the other hand, have the potential to become incorporated into the genome and trigger side effects such as increased off-target changes, potentially limiting commercial applications. These issues can be avoided by obtaining transgene-free derivatives via genetic segregation via selfing and crossing. Another interesting strategy involves using the suicide genes CMS2 and BARNASE to remove transgene-containing pollen and embryos developed by the T0 plant (He et al., 2018). While genetic segregation is a good way to get transgene-free mutants, it cannot be used on asexually propagated crops like potatoes, bananas (*Musa* species), and cassava (*Manihot esculenta*). Furthermore, a fragment of the DNA build can be integrated into previously unknown locations. In researches involving cassava, these problems can be solved by the use of transient expression methods such as the use of protoplast transfection. The technique is fast as it does not require the selection step and produces transgene free mutants (Bhowmik et al., 2018).

Genome editing by transient expression of CRISPR/Cas DNA

Transient gene expression of CRISPR reagents provide an alternative delivery method for transgene-free editing. This approach eliminates canonical selection measures involving herbicides or antibiotics, enabling some of the regenerated plants to be edited without introducing foreign DNA into the genome. This method was first documented in wheat via protoplast transformation (Bhowmik et al., 2018). A CRISPR/Cas9 plasmid was delivered into immature wheat embryos via protoplast transfection, and the resulting plants were regenerated without selection pressure, cutting the time required for tissue culture regeneration by 3 to 4 weeks. The frequency of mutations was comparable to that of the conventional DNA-integration procedure, which employs tissue culture selection pressure. Importantly, transgenes were undetectable in up to 86.8% of T0 mutants. This technique could also be applied to cassava. Because the majority of cassava research using the CRISPR/Cas9 method employs an *Agrobacterium*-mediated delivery system, protoplast transfection may be the best option because it produces transgene-free products and eliminates the tissue culture selection phase.

ADVANTAGES OF CRISPR/CAS9 SYSTEM

Cas9 protein in CRISPR/Cas9 targets the foreign DNA through base pairing mechanism of guide RNAs. This identifies the target sequence upstream the PAM, this is in contrast with ZFNs or TALENs, which targets the DNA

using the protein. Hence, the DNA recognition is more accurate with less off-target effects and lower cytotoxicity (Gaj et al., 2013).

The CRISPR/Cas9 system research community follows an open access policy. In comparison to ZFN's proprietary platform, this has aided in the widespread adoption and use of this technology. <https://chopchop.cbu.uib.no/>, <https://www.benchling.com/crispr/>, and <http://crispor.tefor.net/> are some of the tools available on the platform for selecting gRNA sequences and predicting specificity (Liu et al., 2017; Concordet and Haeussler, 2018; Labun et al., 2019). New researchers have been motivated to embrace the new technologies and contribute to the rapid understanding of the device and its applications as a result of this. Finally, the simplicity with which CRISPR/key Cas9 can be multiplexed is a practical benefit. DSB induction may be used to knock out genes or parallel pathways in an organism by simultaneously editing several genes at multiple sites (Mao et al., 2013). The strategy can also be used to induce deletions or insertions. Multiplexing using CRISPR/Cas9 requires a monomeric Cas9 protein and any different sequence specific gRNA (Li et al., 2012). CRISPR/Cas9 has also been used to modify multiplex genomes, allowing multiple traits to be stacked in an elite array (Wang et al., 2018). This can be accomplished in one of two ways: by assembling multiple sgRNAs into a single vector or by assembling multiple gRNA expression cassettes in separate vector assemblies.

DISADVANTAGES OF CRISPR/CAS SYSTEM

Off-target effects

These can alter a gene's function and cause genomic instability, reducing its capacity and functionality in even the most complex applications. Off-target effects can be reduced using a range of techniques, such as Bowtie alignment, which allows for just three mismatches, and BWA tools, which allow for up to five (Hatem et al., 2011); SgRNA must recognize the specific protospacer adjacent motif (PAM) sequence in order for the CRISPR/Cas9 framework to work. In the absence of the PAM, the Cas9 endonuclease protein does not generate double-stranded breaks in the target DNA region. The SpCas9 variant needs a 5'-NGG-3' PAM just after the 20-nt target sequence, and it only recognizes the NGG PAM site, limiting the CRISPR/Cas9 system's effectiveness. The Agrobacterium-mediated transformation process is time-consuming and labor-intensive, and it causes spontaneous somatic mutations, making it ineffective (Manghwar et al., 2019); and public acceptance is the main setback it faces as far as the agricultural products are concerned. Since foreign DNA can cause side effects in many crops, regulatory authorities have imposed restrictions on modified crops to prevent the

implementation of the CRISPR/Cas9 system (Adhikari and Poudel, 2020).

PROSPECTIVE APPLICATIONS OF CRISPR SYSTEM IN CASSAVA BREEDING

This approach is a promising tool for site-specific genome editing, and it is expected to have a larger impact on plant biology and crop breeding in the future. Genome editing techniques, as opposed to backcrossing in conventional breeding methods, enable elite cultivars to be precisely changed, resulting in cost savings. Since then, the CRISPR/Cas9 method has been used to improve a variety of traits in nearly 20 crop varieties, including yield and biotic and abiotic stress tolerance. It is expected that there will be a food shortage as the world's population grows, resulting in increased demand for food. Furthermore, pathogenic microorganism-induced biotic stresses account for up to 50% of possible yield loss. Many technologies, such as TALENs, ZFNs, and sequence specific nucleases (SSNs), have been developed by the research community to increase crop yield. As a result of predicted climate change, crops like cassava will be needed to withstand harsh weather conditions and grow well in low-fertility soils.

Cassava (*M. esculenta*) is a very important crop which is not only vital to food security in tropics and subtropics, but also a predominant raw material of starch industry (Zhou et al., 2013). Cassava is grown globally for the calories, of which it provides up to 50% (Bredeson et al., 2016) intake of calories for over 800 million people worldwide thus an important staple food (Prochnik et al., 2012). The crop tolerates periods of unpredicted drought (Tomlinson et al., 2018), grows well in poor soils, and can be harvested anytime of the year (Nassar, 2002). The tubers can be retained in the soil for up to two years without rotting (Siritunga and Sayre, 2003). However, there are only few studies on the validity of the CRISPR technique in cassava compared to other crops like rice. Several genome editing projects involving the CRISPR/Cas9 method have recently been completed to increase the yield of cassava, a drought reserve crop, including disease resistance, rapid flowering, herbicide tolerance, and reduced cyanide content in the leaves and roots (Table 1) (Odipio et al., 2017; Hummel et al., 2018; Bull et al., 2018; Mehta et al., 2018; Gomez et al., 2019).

CASSAVA GENOME EDITING WITH CRISPR/CAS9 FOR RESISTANCE TO BIOTIC AND ABIOTIC STRESS

The loss of cassava yield due to disease-causing pathogens is much higher. The two most common diseases affecting cassava crop yield are African cassava mosaic virus (ACMV) and cassava brown streak disease (CBSD). They result in up to 50% crop yield loss

Table 1. Applications of CRISPR/Cas9 in cassava crop breeding.

Target gene	Technology	Method of transformation	Function of the gene	Phenotype (knock-in/knockout)	Repair pathway	References
Phytoene desaturase (MePDS)	CRISPR/Cas9 system	Agrobacterium-mediated delivery	Green pigmentation	Albino (knockout of MePDS)	NHEJ	Odipio et al. (2017)
Viral AC2 gene	CRISPR/Cas9 system	Agrobacterium-mediated delivery	Responsible for the expression of ACMV	Normal (knockout of AC2 gene)	NHEJ	Mehta et al. (2018)
PTST-1 and GBSS	CRISPR/Cas9 system	Agrobacterium-mediated delivery	Integration of flowering locus T of Arabidopsis to accelerate flowering	Accelerated flowering	NHEJ	Bull et al. (2018)
EPSPS	CRISPR/Cas9 system	Agrobacterium-mediated delivery	Activate tolerance to glyphosate (herbicide) in cassava	Normal	NHEJ and HDR	Hummel et al. (2018)
Multiple TFL1-like Floral Repressor	CRISPR/Cas9 system	Agrobacterium-mediated delivery	Floral repression	Activated flowering in cassava	NHEJ	Odipio et al. (2018)
eIF4E isoforms nCBP-1 and nCBP-2	CRISPR/Cas9 system	Agrobacterium-mediated delivery	Suppression of the symptoms of cassava brown streak disease	Normal	NHEJ	Gomez et al. (2019)
MeSSIII	CRISPR/Cas9 system	Agrobacterium-mediated delivery	Regulation of the synthesis of amylopectin glycan	Normal	NHEJ	Zhan et al. (2020)
MeSWEET10a	CRISPR/Cas9 system	Agrobacterium-mediated delivery	CBB susceptibility (S) gene	Visualization of the CBB infection <i>in vivo</i>	HDR	Veley et al. (2021)

in cassava. So many attempts have been developed to make disease resistant varieties. Gomez et al. (2019) pioneered the work of targeted mutation using Cas9/gRNA. The two isoforms of eIF4E, nCBP-1 and nCBP-2, were edited at the same time, resulting in heritable delayed and suppressed CBSD aerial symptoms, as well as reduced severity and frequency of storage root necrosis. By interference of the ACMV in cassava by the knockout of AC2 gene has led to transgenic lines of cassava offered at least 33 to 48% evolution of the gene resulting into resistance to ACMV. From the results, it was reported by Mehta et al., (2018) that this CRISPR system did not provide effective virus resistance during the glasshouse inoculations.

CRISPR/Cas9-mediated gene insertion and replacement has largely been used to create herbicide-resistant crop varieties. A crucial amino acid in the conserved domain of 5-enolpyruvylshikimate-3-phosphate synthase can be substituted to confer resistance to glyphosate-based herbicides (EPSPS). A process that uses the HR and NHEJ DNA repair pathways was used to improve herbicide resistance in cassava (Hummel et

al., 2018). This method created phenotypically normal glyphosate tolerant cassava and demonstrated the potential of gene editing for further improvement of cassava (Xuan Liu et al., 2017).

Furthermore, Veley et al., (2021) recently described the development of cassava that can be used to visualize the early stages of CBB infection *in vivo*. Plants with scarless insertion of GFP at the 3' end of the CBB susceptibility (S) gene MeSWEET10a were created using CRISPR-mediated homology-directed repair (HDR). At the transcriptional and translational stages, this was successfully visualized.

TRAIT IMPROVEMENT VIA CRISPR/CAS9

By simultaneously developing *MESSIII-1* and *MESSIII-2* mutants isolated from *MESSIII* genes of cassava crop using CRISPR/Cas9 system resulted into cassava with edited genes related to starch synthesis pathway (Zhan Li et al., 2020). This research led to an examination of the role of genes in the regulation of amylopectin glucan

synthesis in cassava. Bull et al., (2018) showed that CRISPR/Cas9-mediated targeted mutagenesis of the two amylose synthesis genes, PTST1 and GBSS, can reduce or remove amylose content in root starch. It was also discovered that incorporating the *Arabidopsis* FLOWERING LOCUS T gene into the genome editing cassette accelerated cassava flowering, which is unusual in glasshouse conditions (Tyagi et al., 2021). By the use of CRISPR/Cas9 mediated disruption of Multiple TFL-like Floral Repressors, Odipio et al., (2018) was also able to achieve activated acceleration of flowering in cassava.

Since the mutants were phenotypically albino during cotyledon-stage somatic embryogenesis, researchers were able to understand the gene's role in the plant using CRISPR/Cas9-mediated genome editing technology to target the phytoene desaturase (MePDS) gene in cassava. This eliminated the need for gene sequencing to establish that a mutation had occurred at the target gene (Odipio et al., 2017). As a result, it served as a valuable forum for testing and optimizing the CRISPR/Cas9 process and other genome editing technologies in cassava.

FUTURE PERSPECTIVES FOR CRISPR/CAS9 IN CASSAVA BREEDING AND RESEARCH

Although CRISPR/Cas9-based genome editing technology has come a long way in recent years, it still faces a number of challenges, including off-target effects, CRISPR/Cas9 delivery methods, side effects on neighboring genes, and regulatory concerns. Even though the CRISPR/Cas9 system is still affected by these issues, it will undoubtedly revolutionize and resolve the majority of them. The CRISPR/Cas9 approach has sparked a surge of interest in genome editing in the scientific community. This quick, dependable, scalable, and low-cost method is expected to be widely used to boost crop performance and address food security in the near future. Cassava is one of these crops.

There have been several attempts to minimize the amount of cyanogenic glycosides in cassava. Attempts have been made to silence the genes responsible for the biosynthesis of cyanogenic glycosides in cassava, including the use of RNAi intervention as reported by Piero, (2013). In the future, this could be done by using the CRISPR/Cas9 method to knockout the cytochrome P450 genes (CYP79D1 and CYP79D2), which encode the enzymes valine monooxygenase I and valine monooxygenase II, which catalyze the dedicated first step in the biosynthesis of cyanogenic glycosides (Mikkelsen and Halkier, 2003).

The system's ability to deliver CRISPR/Cas9 machinery has always been a major obstacle. The latest cassava crop delivery systems are *Agrobacterium*-mediated and protoplast transfection using tissue culture methods, both of which are labor-intensive and time-consuming (Kivrak

et al., 2021). As a result, improved *Agrobacterium*-mediated transformation could extend the delivery system's reach. This will necessitate further advances in genotype-dependent, tissue-free delivery via plant germplines or meristematic cells. Novel delivery systems based on nanotechnology and virus particle-like structures can also improve crop yield (Liu et al., 2009). Carbon nanotubes and polyethylenimine-mediated delivery, for example, have a lot of potential for expanding CRISPR/Cas9's usage because they cause little cellular damage, are low in toxicity, and yield higher transformation efficiencies (Kivrak et al., 2021).

One of the problems that prevents CRISPR/Cas9 from being commonly used is off-target activity. In order to achieve target precision and of the frequency of off-target performance, the Cas9 enzyme has undergone numerous attempts and modifications. Cas9 nuclease fused with FokI, for example, can be inactivated to increase specificity. Inactivation of Cas9 nuclease and double nicking with nickase also improve target activity. The method's specificity can also be improved by changing the PAM to a non-canonical NAG or NGA instead of the regular NGG. The use of a longer protospacer adjacent motif was previously the technique for reducing off-target cleavages. Off-target effects have been reduced by employing a variety of techniques, including Bowtie alignment, which allows for only three mismatches, and BWA tools, which allow for up to five mismatches (Hatem et al., 2011). However, these tools are insufficient to solve the problem of off-targets. Increasing the applications of CRISPR/Cas9 by increasing the specificity of Cas9-linked base editors by extending gRNA sequences, linking with APOBEC1 with Cas9-HF1, and delivering base editors via RNPs (Martin et al., 2019).

CRISPR/SpCas9 has a wide range of potential applications in plant pathogens as a result of its growth. The ability to change the genomes of plant pathogens opens the door to disease resistance phenotypes. Although research is progressing to grow cassava crops resistant to the most common disease causing viruses, such as African cassava mosaic virus and cassava brown streak virus, these studies are still in the early stages. The CRISPR/Cas9 genome editing technique should be used to its full potential in the future for knockout genes, AC2 genes, and nCBP genes that confer plant pathogens (Mehta et al., 2018; Gomez et al., 2019).

CONCLUSION AND RECOMMENDATIONS

Food shortages would be exacerbated by the rise in global population to 9.8 billion by 2050, which will be followed by negative climate change. CRISPR/Cas9 has also come at a time when conventional breeding technology is struggling to keep up with the food demand. This possible genome editing has provided the scientific

community with the ability to precisely and quickly insert the desired traits than other conventional breeding techniques due to its versatility, durability, and robustness. In the near future, researchers will be interested in using this genome editing method to boost cassava crop production, quality, cyanogenic glycoside content in leaves and roots, biotic and abiotic stress tolerance, and other traits. Over the last four years, CRISPR/Cas9 has been extensively used in the cassava crop to combat both biotic and abiotic stresses, as well as to develop other essential agronomic traits. CRISPR/Cas9 is mainly used for genome editing and transcriptional control at the moment. CRISPR/Cas9 has also not been used in plants, despite the fact that it has been used for DNA labeling and epigenome editing. CRISPR/Cas9 may be used in plant DNA labeling with fluorescent-labeled Cas9 protein and optimized gRNA in the future, as well as epigenome editing through DNA methylation or histone modifications. The discovery of CRISPR/Cas9 core functions in genome editing has tremendous potential in medicinal plant science and opens up a multitude of new scientific avenues for gene function study. While CRISPR/Cas9 can be used to edit plant genomes, there are still some obstacles to overcome, such as reducing off-target rates, elucidating the mechanism behind this reduction, and optimizing Cas9 work. More research is required to enhance CRISPR/experimental Cas9's application in order to facilitate the potential growth of its basic and applied skill.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

- Abdelrahman M, Al-Sadi AM, Pour-Aboughadareh A, Burritt DJ, Tran LSP (2018). Genome editing using CRISPR/Cas9-targeted mutagenesis: An opportunity for yield improvements of crop plants grown under environmental stresses. *Plant Physiology and Biochemistry* 131:31-36. <https://doi.org/10.1016/j.plaphy.2018.03.012>
- Adhikari P, Poudel M (2020). CRISPR-Cas9 in agriculture: Approaches, applications, future perspectives, and associated challenges. *Malaysian Journal of Halal Research* 3(1):6-16. <https://doi.org/10.2478/mjhr-2020-0002>
- Al-Khayri JM, Jain SM, Johnson DV (2015). Advances in plant breeding strategies: Breeding, biotechnology and molecular tools. *Advances in Plant Breeding Strategies: Breeding, Biotechnology and Molecular Tools* (Vol. 1). <https://doi.org/10.1007/978-3-319-22521-0>
- Andersson M, Turesson H, Olsson N, Fält AS, Ohlsson P, Gonzalez MN, Hofvander P (2018). Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. *Physiologia Plantarum* 164(4):378-384. <https://doi.org/10.1111/ppl.12731>
- Ansari A, Wang C, Wang J, Wang F, Liu P, Gao Y, Zhao K (2017). Engineered dwarf male-sterile rice: A promising genetic tool for facilitating recurrent selection in rice. *Frontiers in Plant Science* 8:1-11. <https://doi.org/10.3389/fpls.2017.02132>
- Arora L, Narula A (2017). Gene editing and crop improvement using CRISPR-cas9 system. *Frontiers in Plant Science* 8:1932. <https://doi.org/10.3389/fpls.2017.01932>
- Barrangou R, Horvath P (2017). A decade of discovery: CRISPR functions and applications. *Nature Microbiology* 2(7):1-9. <https://doi.org/10.1038/nmicrobiol.2017.92>
- Bernheim A, Calvo-Villamañán A, Basier C, Cui L, Rocha EPC, Touchon M, Bikard D (2017). Inhibition of NHEJ repair by type II-A CRISPR-Cas systems in bacteria. *Nature Communications* 8(1):25-28. <https://doi.org/10.1038/s41467-017-02350-1>
- Bhatta BP, Malla S (2020). Improving horticultural crops via crispr/cas9: Current successes and prospects. *Plants* 9(10):1-19. <https://doi.org/10.3390/plants9101360>
- Bhowmik P, Ellison E, Polley B, Bollina V, Kulkarni M, Ghanbarnia K, Kagale S (2018). Targeted mutagenesis in wheat microspores using CRISPR/Cas9. *Scientific Reports* 8(1):1-10. <https://doi.org/10.1038/s41598-018-24690-8>
- Braatz J, Harloff HJ, Mascher M, Stein N, Himmelbach A, Jung C (2017). CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). *Plant Physiology* 174(2):935-942. <https://doi.org/10.1104/pp.17.00426>
- Bredeson JV, Lyons JB, Prochnik SE, Wu GA, Ha CM, Edsinger-Gonzales E, Rokhsar DS (2016). Sequencing wild and cultivated cassava and related species reveals extensive interspecific hybridization and genetic diversity. *Nature Biotechnology* 34(5):562-570. <https://doi.org/10.1038/nbt.3535>
- Bull SE, Seung D, Chanez C, Mehta D, Kuon JE, Truernit E, Vanderschuren H (2018). Accelerated ex situ breeding of GBSS- and PTST1-edited cassava for modified starch. *Science Advances* 4(9):eaat6086 <https://doi.org/10.1126/sciadv.aat6086>
- Carter J, Wiedenheft B (2015). Snapshot: CRISPR-RNA-guided adaptive immune systems. *Cell* 163(1):260-260.e1. <https://doi.org/10.1016/j.cell.2015.09.011>
- Ceballos H, Jaramillo J, Salazar S, Pineda L, Calle F, Setter T (2017). Induction of flowering in cassava through grafting. *Journal of Plant Breeding and Crop Science* 9(2):19-29. <https://doi.org/10.5897/jpbcs2016.0617>
- Char SN, Wei J, Mu Q, Li X, Zhang ZJ, Yu J, Yang B (2020). An Agrobacterium-delivered CRISPR/Cas9 system for targeted mutagenesis in sorghum. *Plant Biotechnology Journal* 18(2):319-321. <https://doi.org/10.1111/pbi.13229>
- Che P, Anand A, Wu E, Sander JD, Simon MK, Zhu W, Jones TJ (2018). Developing a flexible, high-efficiency Agrobacterium-mediated sorghum transformation system with broad application. *Plant Biotechnology Journal* 16(7):1388-1395. <https://doi.org/10.1111/pbi.12879>
- Chiurugwi T, Kemp S, Powell W, Hickey LT (2019). Speed breeding orphan crops. *Theoretical and Applied Genetics* 132(3):607-616. <https://doi.org/10.1007/s00122-018-3202-7>
- Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Voytas DF (2010). Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186(2):756-761. <https://doi.org/10.1534/genetics.110.120717>
- Clarke JL, Zhang P (2013). Plant biotechnology for food security and bioeconomy. *Plant Molecular Biology* 83(1-2):1-3. <https://doi.org/10.1007/s11103-013-0097-1>
- Concordet JP, Haeussler M (2018). CRISPOR: Intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Research* 46(W1):W242-W245. <https://doi.org/10.1093/nar/gky354>
- Devkota S (2018). The road less traveled: Strategies to enhance the frequency of homology-directed repair (HDR) for increased efficiency of CRISPR/Cas-mediated transgenesis. *BMB Reports* 51(9):437-443. <https://doi.org/10.5483/BMBRep.2018.51.9.187>
- Doll NM, Gilles LM, Gérentes MF, Richard C, Just J, Fierle Y, Widiez T (2019). Single and multiple gene knockouts by CRISPR-Cas9 in maize. *Plant Cell Reports* 38(4):487-501. <https://doi.org/10.1007/s00299-019-02378-1>
- Doudna JA, Charpentier E (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science* 346(6213). <https://doi.org/10.1126/science.1258096>
- Fausser F, Schiml S, Puchta H (2014). Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. *Plant Journal* 79(2):348-359. <https://doi.org/10.1111/tpj.12554>

- Gaj T, Gersbach CA, Barbas CF (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology* 31(7):397-405. <https://doi.org/10.1016/j.tibtech.2013.04.004>
- Gao J, Wang G, Ma S, Xie X, Wu X, Zhang X, Xia Q (2015). CRISPR/Cas9-mediated targeted mutagenesis in *Nicotiana tabacum*. *Plant Molecular Biology* 87(1-2):99-110. <https://doi.org/10.1007/s1103-014-0263-0>
- Garrett RA, Vestergaard G, Shah SA (2011). Archaeal CRISPR-based immune systems: Exchangeable functional modules. *Trends in Microbiology* 19(11):549-556. <https://doi.org/10.1016/j.tim.2011.08.002>
- Georges F, Ray H (2017). Genome editing of crops: A renewed opportunity for food security. *GM Crops and Food* 8(1):1-12. <https://doi.org/10.1080/21645698.2016.1270489>
- Hogare R, Williamson-Benavides B, Ramírez-Torres F, Dhingra A (2020). CRISPR-associated nucleases: the Dawn of a new age of efficient crop improvement. *Transgenic Research* 29:1-35. <https://doi.org/10.1007/s11248-019-00181-y>
- Ghosh S, Watson A, Gonzalez-Navarro OE, Ramirez-Gonzalez RH, Yanes L, Mendoza-Suárez, M, Hickey LT (2018a). Speed breeding in growth chambers and glasshouses for crop breeding and model plant research. *Nature Protocols* 13(12):2944-2963. <https://doi.org/10.1038/s41596-018-0072-z>
- Ghosh S, Watson A, Gonzalez-Navarro OE, Ramirez-Gonzalez RH, Yanes L, Mendoza-Suárez, M, Hickey LT (2018b). Speed breeding in growth chambers and glasshouses for crop breeding and model plant research. *Nature Protocols* 13(12):2944-2963. <https://doi.org/10.1101/369512>
- Godfray HCJ, Beddington JR, Crute IR, Haddad L, Lawrence D, Muir JF, Toulmin C (2010). Food security: The challenge of feeding 9 billion people. *Science* 327(5967):812-818. <https://doi.org/10.1126/science.1185383>
- Gomez MA, Lin ZD, Moll T, Chauhan RD, Hayden L, Renninger K, Bart RS (2019). Simultaneous CRISPR/Cas9-mediated editing of cassava eIF4E isoforms nCBP-1 and nCBP-2 reduces cassava brown streak disease symptom severity and incidence. *Plant Biotechnology Journal* 17(2):421-434. <https://doi.org/10.1111/pbi.12987>
- Gupta RM, Musunuru K (2014). The emergence of genome-editing technology. *The Journal of Clinical Investigation* 124(10):4154-4161. <https://doi.org/10.1172/JCI72992.transcription>
- Haque E, Taniguchi H, Hassan MM, Bhowmik P, Karim M R, Śmiech M, Islam T (2018). Application of CRISPR/Cas9 genome editing technology for the improvement of crops cultivated in tropical climates: Recent progress, prospects, and challenges. *Frontiers in Plant Science* 9:617. <https://doi.org/10.3389/fpls.2018.00617>
- Hatem A, Bozdağ D, Çatalyürek ÜV (2011). Benchmarking short sequence mapping tools. *Proceedings - 2011 IEEE International Conference on Bioinformatics and Biomedicine, BIBM 2011*, 109-113. <https://doi.org/10.1109/BIBM.2011.83>
- He Y, Zhu M, Wang L, Wu J, Wang Q, Wang R, Zhao Y (2018). Programmed Self-Elimination of the CRISPR/Cas9 Construct Greatly Accelerates the Isolation of Edited and Transgene-Free Rice Plants. *Molecular Plant* 11(9):1210-1213. <https://doi.org/10.1016/j.molp.2018.05.005>
- Hoffmann MD, Aschenbrenner S, Grosse S, Rapti K, Domenger C, Fakhiri J, Niopek D (2019). Cell-specific CRISPR-Cas9 activation by microRNA-dependent expression of anti-CRISPR proteins. *Nucleic Acids Research* 47(13):e75. <https://doi.org/10.1093/nar/gkz271>
- Hui-Li X, D L, Wang Z, Zhang H, Han C, Liu B, Chen Q (2014). A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *Plant Biology* 1(1):3-13. <https://doi.org/10.1111/j.1439-0418.1914.tb01110.x>
- Hummel AW, Chauhan RD, Cermak T, Mutka AM, Vijayaraghavan A, Boyher A, Taylor NJ (2018). Allele exchange at the EPSPS locus confers glyphosate tolerance in cassava. *Plant Biotechnology Journal* 16(7):1275-1282. <https://doi.org/10.1111/pbi.12868>
- Hussain B, Lucas SJ, Budak H (2018). CRISPR/Cas9 in plants: at play in the genome and at work for crop improvement. *Briefings in Functional Genomics* 17(5):319-328. <https://doi.org/10.1093/bfpg/ely016>
- Ishino Y, Shinagawa H, Makino K, Amemura M, Nakamura A (1987). Nucleotide sequence of the *iap* gene, responsible for alkaline phosphatase isoenzyme conversion in *Escherichia coli*, and identification of the gene product. *Journal of Bacteriology* 169(12):5429-5433. <https://doi.org/10.1128/jb.169.12.5429-5433.1987>
- Ishino, Yoshizumi, Krupovic, M, Forterre P (2018). History of CRISPR-Cas from Encounter with a Mysterious. *Journal of Bacteriology* 200(7):e00580-17.
- Jansen R, Van Embden JDA, Gaastra W, Schouls LM (2002). Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular Microbiology* 43(6):1565-1575. <https://doi.org/10.1046/j.1365-2958.2002.02839.x>
- Jasin M, Rothstein R (2013). Repair of strand breaks by homologous recombination. *Cold Spring Harbor Perspectives in Biology* 5(11):1-18. <https://doi.org/10.1101/cshperspect.a012740>
- Jayavaradhan R, Pillis DM, Goodman M, Zhang F, Zhang Y, Andreassen PR, Malik P (2019). CRISPR-Cas9 fusion to dominant-negative 53BP1 enhances HDR and inhibits NHEJ specifically at Cas9 target sites. *Nature Communications* 10(1):1-13. <https://doi.org/10.1038/s41467-019-10735-7>
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012). A Programmable Dual-RNA - Guided Cas9 Nuclease. *Science* 337(6096):816-821.
- Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Doudna JA (2014). Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* 343(6176):2-18. <https://doi.org/10.1126/science.1247997>
- Khoury LYE, Campbell JM, Clark KJ (2018). The transition of zebrafish functional genetics from random mutagenesis to targeted integration. *Molecular-Genetic and Statistical Techniques for Behavioral and Neural Research*. Elsevier Inc. <https://doi.org/10.1016/B978-0-12-804078-2.00017-9>
- Kivrak E, Pauzaitė T, Copeland NA, Hardy JG, Kara P, Firlak M, Ozsoz M (2021). Detection of CRISPR-Cas9-Mediated Mutations Using a Carbon Nanotube-Modified Electrochemical Genosensor. *Biosensors* 11(1):17. <https://doi.org/10.3390/bios11010017>
- Labun K, Montague TG, Krause M, Torres Cleuren YN, Tjeldnes H, Valen E (2019). CHOPCHOP v3: Expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Research* 47(W1):W171-W174. <https://doi.org/10.1093/nar/gkz365>
- Lawrenson T, Shorinola O, Stacey N, Li C, Østergaard L, Patron N, Harwood W (2015). Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. *Genome Biology* 16(1):1-13. <https://doi.org/10.1186/s13059-015-0826-7>
- Lee K, Zhang Y, Kleinstiver BP, Guo JA, Aryee MJ, Miller J, Wang K (2019). Activities and specificities of CRISPR/Cas9 and Cas12a nucleases for targeted mutagenesis in maize. *Plant Biotechnology Journal* 17(2):362-372. <https://doi.org/10.1111/pbi.12982>
- Li C, Chen C, Chen H, Wang S, Chen X, Cui Y (2018). Verification of DNA motifs in *Arabidopsis* using CRISPR/Cas9-mediated mutagenesis. *Plant Biotechnology Journal* 16(8):1446-1451. <https://doi.org/10.1111/pbi.12886>
- Li G, Zhang X, Zhong C, Mo J, Quan R, Yang J, Wu Z (2017). Small molecules enhance CRISPR/Cas9-mediated homology-directed genome editing in primary cells. *Scientific Reports* 7(1):1-11. <https://doi.org/10.1038/s41598-017-09306-x>
- Li J, Manghwar H, Sun L, Wang P, Wang G, Sheng H, Zhang X (2019). Whole genome sequencing reveals rare off-target mutations and considerable inherent genetic or/and somaclonal variations in CRISPR/Cas9-edited cotton plants. *Plant Biotechnology Journal*, 17(5):858-868. <https://doi.org/10.1111/pbi.13020>
- Li Q, Sapkota M, van der Knaap E (2020). Perspectives of CRISPR/Cas-mediated cis-engineering in horticulture: unlocking the neglected potential for crop improvement. *Horticulture Research* 7(1). <https://doi.org/10.1038/s41438-020-0258-8>
- Li S, Xia L (2020). Precise gene replacement in plants through CRISPR/Cas genome editing technology: current status and future perspectives. *ABIOTECH* 1(1):58-73. <https://doi.org/10.1007/s42994-019-00009-7>
- Li T, Liu B, Spalding MH, Weeks DP, Yang B (2012). High-efficiency TALEN-based gene editing produces disease-resistant rice. *Nature Biotechnology* 30(5):390-392. <https://doi.org/10.1038/nbt.2199>

- Li, Zhan, Wang, Y, Lu X, Li, R, Liu J, Fu S, Yao Y (2020). Construction and Verification of CRISPR/Cas9 Gene Editing Vector for Cassava *MeSSIII* Gene. *Molecular Plant Breeding* 11(17):1-8. <https://doi.org/10.5376/mpb.2020.11.0017>
- Li, Zhongsen, Liu, Z. Bin, Xing, A., Moon, B. P., Koellhoffer, J. P., Huang, L, Cigan, A. M. (2015). Cas9-guide RNA directed genome editing in soybean. *Plant Physiology* 169(2):960-970. <https://doi.org/10.1104/pp.15.00783>
- Liu H, Ding Y, Zhou Y, Jin W, Xie K, Chen LL (2017). CRISPR-P 2.0: An Improved CRISPR-Cas9 Tool for Genome Editing in Plants. *Molecular Plant* 10(3):530-532. <https://doi.org/10.1016/j.molp.2017.01.003>
- Liu Q, Chen B, Wang Q, Shi X, Xiao Z, Lin J, Fang X (2009). Carbon nanotubes as molecular transporters for walled plant cells. *Nano Letters* 9(3):1007-1010. <https://doi.org/10.1021/nl803083u>
- Liu, Xuan, Wu, S, Xu J, Sui C, Wei J (2017). Application of CRISPR/Cas9 in plant biology. *Acta Pharmaceutica Sinica B* 7(3):292-302. <https://doi.org/10.1016/j.apsb.2017.01.002>
- Liu, Xuejun, Xie C, Si, H, Yang J (2017). CRISPR/Cas9-mediated genome editing in plants. *Methods* 121-122:94-102. <https://doi.org/10.1016/j.ymeth.2017.03.009>
- Xiaonan M, Zhang X, Liu H, Li Z (2020). Highly efficient DNA-free plant genome editing using virally delivered CRISPR-Cas9. *Nature Plants* 6(7):773-779. <https://doi.org/10.1038/s41477-020-0704-5>
- Xingliang M, Mau M, Sharbel TF (2018). Genome Editing for Global Food Security. *Trends in Biotechnology* 36(2):123-127. <https://doi.org/10.1016/j.tibtech.2017.08.004>
- Xingliang M, Zhu Q, Chen Y, Liu YG. (2016). CRISPR/Cas9 Platforms for Genome Editing in Plants: Developments and Applications. *Molecular Plant* 9(7):961-974. <https://doi.org/10.1016/j.molp.2016.04.009>
- Macovei A, Sevilla NR, Cantos C, Jonson GB, Slamet-Loedin I, Čermák T, Chadha-Mohanty P (2018). Novel alleles of rice *elf4G* generated by CRISPR/Cas9-targeted mutagenesis confer resistance to Rice tungro spherical virus. *Plant Biotechnology Journal* 16(11):1918-1927. <https://doi.org/10.1111/pbi.12927>
- Makarova KS, Haft DH, Barrangou R, Brouns SJJ, Charpentier E, Horvath P, Koonin EV (2011). Evolution and classification of the CRISPR-Cas systems. *Nature Reviews Microbiology* 9(6):467-477. <https://doi.org/10.1038/nrmicro2577>
- Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Koonin EV (2015). An updated evolutionary classification of CRISPR-Cas systems. *Nature Reviews Microbiology* 13(11):722-736. <https://doi.org/10.1038/nrmicro3569>
- Malzahn A, Lowder L, Qi Y (2017). Plant genome editing with TALEN and CRISPR. *Cell and Bioscience* 7(1):1-18. <https://doi.org/10.1186/s13578-017-0148-4>
- Manghwar H, Lindsey K, Zhang X, Jin S (2019). CRISPR/Cas System: Recent Advances and Future Prospects for Genome Editing. *Trends in Plant Science* 24(12):1102-1125. <https://doi.org/10.1016/j.tplants.2019.09.006>
- Mao Y, Zhang H, Xu N, Zhang B, Gou F, Zhu JK (2013). Application of the CRISPR-Cas system for efficient genome engineering in plants. *Molecular Plant* 6(6):2008-2011. <https://doi.org/10.1093/mp/sst121>
- Martin AS, Salamango DJ, Serebrenik AA, Shaban NM, Brown WL, Harris RS (2019). A panel of eGFP reporters for single base editing by APOBEC-Cas9 editosome complexes. *Scientific Reports* 9(1):1-8. <https://doi.org/10.1038/s41598-018-36739-9>
- McFarlane GR, Whitelaw CBA, Lillico SG (2018). CRISPR-Based Gene Drives for Pest Control. *Trends in Biotechnology* 36(2):130-133. <https://doi.org/10.1016/j.tibtech.2017.10.001>
- Mehta D, Stürchler A, Hirsch-Hoffmann M, Gruissem W, Vanderschuren H (2018). CRISPR-Cas9 interference in cassava linked to the evolution of editing-resistant geminiviruses. *BioRxiv* pp. 1-10. <https://doi.org/10.1101/314542>
- Mekler V, Kuznedelov K, Severinov K (2020). Quantification of the affinities of CRISPR-Cas9 nucleases for cognate protospacer adjacent motif (PAM) sequences. *Journal of Biological Chemistry* 295(19):6509-6517. <https://doi.org/10.1074/jbc.RA119.012239>
- Mikkelsen MD, Halkier BA (2003). Metabolic engineering of valine- and isoleucine-derived glucosinolates in *Arabidopsis* expressing CYP79D2 from cassava. *Plant Physiology* 131(2):773-779. <https://doi.org/10.1104/pp.013425>
- Molina R, Sofos N, Montoya G (2020). Structural basis of CRISPR-Cas Type III prokaryotic defence systems. *Current Opinion in Structural Biology* 65:119-129. <https://doi.org/10.1016/j.sbi.2020.06.010>
- Musunuru K (2017). The hope and hype of CRISPR-Cas9 genome editing: A review. *JAMA Cardiology* 2(8):914-919. <https://doi.org/10.1001/jamacardio.2017.1713>
- Nakayama T, Fish MB, Fisher M, Oomen-Hajagos J, Thomsen GH, Grainger RM (2013). Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in *Xenopus tropicalis*. *Genesis* 51(12):835-843. <https://doi.org/10.1002/dvg.22720>
- Nambiar TS, Billon P, Diedenhofen G, Hayward SB, Tagliatela A, Cai K, Ciccia A (2019). Stimulation of CRISPR-mediated homology-directed repair by an engineered RAD18 variant. *Nature Communications* 10(1):1-13. <https://doi.org/10.1038/s41467-019-11105-z>
- Nassar NMA (2002). Cassava, *Manihot esculenta* Crantz, genetic resources: Origin of the crop, its evolution and relationships with wild relatives. *Genetics and Molecular Research* 1(4):298-305. <https://doi.org/0045> [pii]
- Nocker S Van, Gardiner SE (2014). Breeding better cultivars, faster: Applications of new technologies for the rapid deployment of superior horticultural tree crops. *Horticulture Research* 1(1):1-8. <https://doi.org/10.1038/hortres.2014.22>
- O'Connor DJ, Wright GC, Dieters MJ, George DL, Hunter MN, Tatnell JR, Fleischfresser DB (2013). Development and Application of Speed Breeding Technologies in a Commercial Peanut Breeding Program. *Peanut Science* 40(2):107-114. <https://doi.org/10.3146/ps12-12.1>
- Odipio J, Alicai T, Ingelbrecht I, Nusinow DA, Bart R, Taylor NJ (2017). Efficient CRISPR/cas9 genome editing of phytoene desaturase in cassava. *Frontiers in Plant Science* 8:1780. <https://doi.org/10.3389/fpls.2017.01780>
- Odipio J, Alicai T, Nusinow D, Bart R, Taylor N (2018). CRISPR/Cas9-mediated Disruption of Multiple TFL1-like Floral Repressors Activates Flowering in Cassava. In *In Vitro Cellular and Developmental Biology-Animal* 54:S47-S47. 233 Spring St, New York, NY 10013 Usa: Springer.
- Okada A, Arndell T, Borisjuk N, Sharma N, Watson-Haigh NS, Tucker EJ, Whitford R (2019). CRISPR/Cas9-mediated knockout of *Ms1* enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. *Plant Biotechnology Journal* 17(10):1905-1913. <https://doi.org/10.1111/pbi.13106>
- Palareti G, Legnani C, Cosmi B, Antonucci E, Erba N, Poli D, Tosetto A (2016). Comparison between different D-Dimer cutoff values to assess the individual risk of recurrent venous thromboembolism: Analysis of results obtained in the DULCIS study. *International Journal of Laboratory Hematology* 38(1):42-49. <https://doi.org/10.1111/ijlh.12426>
- Pardo B, Gómez-González B, Aguilera A (2009). DNA double-strand break repair: How to fix a broken relationship. *Cellular and Molecular Life Sciences* 66(6):1039-1056. <https://doi.org/10.1007/s00018-009-8740-3>
- Piero Peisach E, Carl P, Robert G (2001). Design And Selection Of novel Cys2his2 Zinc Finger Proteins. *Annual Review of Biochemistry* 70(1):291-321.
- Peng R, Lin G, Li J (2016). Potential pitfalls of CRISPR/Cas9-mediated genome editing. *FEBS Journal* 283(7):1218-1231. <https://doi.org/10.1111/febs.13586>
- MN (2013). Regeneration and RNAi-mediated downregulation of cyanoglycoside biosynthesis in cassava 167. Retrieved from <https://ir-library.ku.ac.ke/handle/123456789/9082>
- Pinilla-Redondo R, Mayo-Muñoz D, Russel J, Garrett RA, Randau L, Sørensen SJ, Shah SA (2020). Type IV CRISPR-Cas systems are highly diverse and involved in competition between plasmids. *Nucleic Acids Research* 48(4):2000-2012. <https://doi.org/10.1093/nar/gkz1197>
- Prochnik S, Marri PR, Desany B, Rabinowicz PD, Kodira C, Mohiuddin M, Rounsley S (2012). The Cassava Genome: Current Progress, Future Directions. *Tropical Plant Biology* 5(1):88-94. <https://doi.org/10.1007/s12042-011-9088-z>
- Qin L, Li J, Wang Q, Xu Z, Sun L, Alarici M, Jin S (2020). High-efficient and precise base editing of C•G to T•A in the allotetraploid cotton

- (*Gossypium hirsutum*) genome using a modified CRISPR/Cas9 system. *Plant Biotechnology Journal* 18(1):45-56. <https://doi.org/10.1111/pbi.13168>
- Ray DK, Mueller ND, West PC, Foley JA (2013). Yield Trends Are Insufficient to Double Global Crop Production by 2050. *PLoS ONE* 8(6):e66428. <https://doi.org/10.1371/journal.pone.0066428>
- Razzaq A, Saleem F, Kanwal M, Mustafa G, Yousaf S, Arshad HMI, KhanJoyia FA (2019). Modern trends in plant genome editing: An inclusive review of the CRISPR/Cas9 Toolbox. *International Journal of Molecular Sciences* 20(16). <https://doi.org/10.3390/ijms20164045>
- Ricroch A (2019). Global developments of genome editing in agriculture. *Transgenic Research* 28(2):45-52. <https://doi.org/10.1007/s11248-019-00133-6>
- Rouillon C, Zhou M, Zhang J, Politis A, Beilsten-Edmands V, Cannone G, White MF (2013). Structure of the CRISPR interference complex CSM reveals key similarities with cascade. *Molecular Cell* 52(1):124-134. <https://doi.org/10.1016/j.molcel.2013.08.020>
- Sansbury BM, Hewes AM, Kmiec EB (2019). Understanding the diversity of genetic outcomes from CRISPR-Cas generated homology-directed repair. *Communications Biology* 2(1):1-10. <https://doi.org/10.1038/s42003-019-0705-y>
- Savary S, Willcoquet L, Pethybridge SJ, Esker P, McRoberts N, Nelson A (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology and Evolution* 3(3):430-439. <https://doi.org/10.1038/s41559-018-0793-y>
- Savic N, Ringnalda F, Bargsten K, Li Y, Berk C, Hall J, Schwank G (2017). Covalent linkage of the DNA repair template to the CRISPR/Cas9 complex enhances homology-directed repair. *BioRxiv* <https://doi.org/10.1101/218149>
- Schenke D, Cai D (2020). Applications of CRISPR/Cas to Improve Crop Disease Resistance: Beyond Inactivation of Susceptibility Factors. *Iscience* 23(9):101478. <https://doi.org/10.1016/j.isci.2020.101478>
- Schmidt C, Pacher M, Puchta H (2019). DNA break repair in plants and its application for genome engineering. *Methods in Molecular Biology* 1864:237-266. https://doi.org/10.1007/978-1-4939-8778-8_17
- Sharma S, Kaur R, Singh A (2017). Recent advances in CRISPR/Cas mediated genome editing for crop improvement. *Plant Biotechnology Reports* 11(4):193-207. <https://doi.org/10.1007/s11816-017-0446-7>
- Shen H, Strunks GD, Klemann BJPM, Hooykaas PJJ, de Pater S (2017). CRISPR/Cas9-induced double-strand break repair in *Arabidopsis* nonhomologous end-joining mutants. *G3: Genes, Genomes, Genetics* 7(1):193-202. <https://doi.org/10.1534/g3.116.035204>
- Shimada T (1978). Plant regeneration from the callus induced from wheat embryo. *The Japanese Journal of Genetics* 53(5):371-374. <https://doi.org/10.1266/jgg.53.371>
- Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, Koonin EV (2015). Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems. *Molecular Cell* 60(3):385-397. <https://doi.org/10.1016/j.molcel.2015.10.008>
- Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Koonin EV (2017a). Diversity and evolution of class 2 CRISPR-Cas systems. *Nature Reviews Microbiology* 15(3):169-182. <https://doi.org/10.1038/nrmicro.2016.184>
- Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Koonin EV (2017b). Diversity and evolution of class 2 CRISPR-Cas systems. *Nature Reviews Microbiology* 15(3):169-182. <https://doi.org/10.1038/nrmicro.2016.184>
- Siritunga D, Sayre RT (2003). Generation of cyanogen-free transgenic cassava. *Planta* 217(3):367-373. <https://doi.org/10.1007/s00425-003-1005-8>
- Stinson BM, Moreno AT, Walter JC, Loparo JJ (2020). A Mechanism to Minimize Errors during Non-homologous End Joining. *Molecular Cell* 77(5):1080-1091. <https://doi.org/10.1146/annurev-genet-110410-132435>
- Tang XD, Gao F, Liu MJ, Fan QL, Chen DK, Ma WT (2019). Methods for enhancing clustered regularly interspaced short palindromic repeats/Cas9-mediated homology-directed repair efficiency. *Frontiers in Genetics* 10:551. <https://doi.org/10.3389/fgene.2019.00551>
- Tashkandi M, Ali Z, Aljedaani F, Shami A, Mahfouz MM (2018). Engineering resistance against Tomato yellow leaf curl virus via the CRISPR/Cas9 system in tomato. *Plant Signaling and Behavior* 13(10):1-7. <https://doi.org/10.1080/15592324.2018.1525996>
- Tomlinson KR, Bailey AM, Alicai T, Seal S, Foster GD (2018). Cassava brown streak disease: historical timeline, current knowledge and future prospects. *Molecular Plant Pathology* 19(5):1282-1294. <https://doi.org/10.1111/mpp.12613>
- Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Joung JK (2015). GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology* 33(2):187-198. <https://doi.org/10.1038/nbt.3117>
- Tyagi S, Kumar R, Kumar V, Won SY, Shukla P (2021). Engineering disease resistant plants through CRISPR-Cas9 technology. *GM Crops and Food* 12(1):125-144. <https://doi.org/10.1080/21645698.2020.1831729>
- Ueta R, Abe C, Watanabe T, Sugano SS, Ishihara R, Ezura H, Osakabe K (2017). Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. *Scientific Reports* 7(1):1-8. <https://doi.org/10.1038/s41598-017-00501-4>
- Veley KM, Okwuonu I, Jensen G, Yoder M, Taylor NJ, Meyers BC, Bart RS (2021). Gene tagging via CRISPR-mediated homology-directed repair in cassava. *G3 Genes|Genomes|Genetics* 11(4):jkab028 <https://doi.org/10.1093/g3journal/jkab028>
- Walsh RM, Hochedlinger K (2013). A variant CRISPR-Cas9 system adds versatility to genome engineering. *Proceedings of the National Academy of Sciences of the United States of America* 110(39):15514-15515. <https://doi.org/10.1073/pnas.1314697110>
- Wang M, Wang S, Liang Z, Shi W, Gao C, Xia G (2018). From Genetic Stock to Genome Editing: Gene Exploitation in Wheat. *Trends in Biotechnology* 36(2):160-72. <https://doi.org/10.1016/j.tibtech.2017.10.002>
- Wang W, Pan Q, He F, Akhunova A, Chao S, Trick H, Akhunov E (2018). Transgenerational CRISPR-Cas9 Activity Facilitates Multiplex Gene Editing in Allopolyploid Wheat. *The CRISPR Journal* 1(1):65-74. <https://doi.org/10.1089/crispr.2017.0010>
- Watters K (2018). The CRISPR Revolution: Potential Impacts on Global Health Security. Retrieved from <http://ebot.gmu.edu/handle/1920/11338>
- Williams BO, Warman ML (2017). CRISPR/CAS9 Technologies. *Journal of Bone and Mineral Research* 32(5):883-888. <https://doi.org/10.1002/jbmr.3086>
- Chhotaray C, Tan Y, Mugweru J, Islam MM, Hameed HA, Wang S, Lu Z, Wang C, Li X, Tan S, Liu J (2018). Advances in the development of molecular genetic tools for *Mycobacterium tuberculosis*. *Journal of Genetics and Genomics* 45(6):281-297. <https://doi.org/10.1016/j.jgg.2018.06.003>
- Ye L, Wang C, Hong L, Sun N, Chen D, Chen S, Han F (2018). Programmable DNA repair with CRISPRa/i enhanced homology-directed repair efficiency with a single Cas9. *Cell Discovery* 4(1):1-12. <https://doi.org/10.1038/s41421-018-0049-7>
- Yin K, Han T, Liu G, Chen T, Wang Y, Yu AYL, Liu Y (2015). A geminivirus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing. *Scientific Reports* 5:1-10. <https://doi.org/10.1038/srep14926>
- Young K, Gasior SL, Jones S, Wang L, Navarro P, Vickroy B, Barrangou R (2019). The repurposing of type I-E CRISPR-Cascade for gene activation in plants. *Communications Biology* 2(1):1-7. <https://doi.org/10.1038/s42003-019-0637-6>
- Zaidi SSeA, Mukhtar MS, Mansoor S (2018). Genome Editing: Targeting Susceptibility Genes for Plant Disease Resistance. *Trends in Biotechnology* 36(9):898-906. <https://doi.org/10.1016/j.tibtech.2018.04.005>
- Zhang Q, Xing HL, Wang ZP, Zhang HY, Yang F, Wang XC, Chen QJ (2018). Potential high-frequency off-target mutagenesis induced by CRISPR/Cas9 in *Arabidopsis* and its prevention. *Plant Molecular Biology* 96(4-5):445-456. <https://doi.org/10.1007/s11103-018-0709-x>
- Zhang ZT, Jiménez-Bonilla P, Seo SO, Lu T, Jin YS, Blaschek HP, Wang Y (2018). Bacterial genome editing with CRISPR-Cas9: Taking *Clostridium beijerinckii* as an example. *Methods in Molecular Biology* 1772:297-325. https://doi.org/10.1007/978-1-4939-7795-6_17
- Zhou H, Liu B, Weeks DP, Spalding MH, Yang B (2014). Large

- chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Research* 42(17):10903-10914. <https://doi.org/10.1093/nar/gku806>
- ZHOU W, MA Q, ZHANG P, WANG L, WANG H, XU J, YANG J (2013). Key Scientific Questions and Recent Advances in Cassava Molecular Breeding. *Scientia Sinica Vitae* 43(12):1082-1089. <https://doi.org/10.1360/052013-320>
- Zuo Z, Zolekar A, Babu K, Lin VJ, Hayatshahi HS, Rajan R, Liu J (2019). Structural and functional insights into the bona fide catalytic state of *Streptococcus pyogenes* Cas9 HNH nuclease domain. *Elife* 8:e46500.