Genome engineering with CRISPR/Cas9 for crop improvement

Gopika K Mote[1], Pravin V Jadhav[1], Arpita Mahobia[1], Mangesh P Moharil[1], Vilas Kharche

[1]Biotechnology Centre, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, India
email id :- gopikamote43@gmail.com

ABSTRACT

Targeted genome engineering, commonly referred to as genome editing, a recent development in the life sciences, is one of the best instances of a technology used to investigate the biological phenomenon. Over a decade ago, among the existing genome editing technologies like Zinc Finger Nucleases (ZFNs) and TAL effector nucleases (TALENs), the CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/CRISPR-associated) have gain importance due to its simplicity, accessibility, low cost and flexibility. This chapter is addressing details about genome engineering with the use of CRISPR/Cas9 in crop improvement. It also glimpse on the history of genome engineering, variants of CRISPR/Cas system, components of CRISPR/Cas system, advances in CRISPR/cas technology like base editing, prime editing, CRISPR multiplexing, epigenome editing, challenges in use of CRISPR/Cas9.

Keywords: CRISPR/Cas9, genome engineering, base editing, crop improvement

I. INTRODUCTION

Over the last 70 years, numerous advancements in biology have been made possible by technologies for creating and modifying DNA. The advent of chemical techniques for solid-phase DNA synthesis, which allowed for the detection and investigation of genome organization, marked the beginning of this period. The polymerase chain reaction (PCR) and enzymes (such as polymerases, ligases, and restriction endonucleases) have made it possible to isolate genes and gene fragments as well as to introduce mutations into genes *in vitro*, in cells, and in model animals. One of the most important developments of the past 20 years has been the development of genomic sequencing technology and the quick collection of whole-genome sequencing data for several types and numbers of organisms, including humans. By offering a genome engineering tool based on Watson-Crick base pairing, the RNAguided enzyme Cas9, which comes from the CRISPR/Cas adaptive bacterial immune system, is currently revolutionizing biology. Worldwide laboratories have adopted it quickly due to its simplicity of use and effectiveness. The history of genome engineering, biology of CRISPR/Cas9 and mechanism, advances in CRISPR/Cas9, Engineering plants with CRISPR/Cas9, challenges in CRISPR mediated genome engineering are discussed in details in this chapter.

A. Age of Genome Engineering

Since the discovery of the DNA double helix, scientists have been considering the possibility of making site-specific changes to the genomes of cells and organisms. The earliest approaches of genome editing relied on principle of site-specific recognition of DNA sequences. Cells have endogenous machinery to repair double-strand DNA breaks (DSBs), which would otherwise be fatal [1]. This was discovered through the investigation of natural DNA repair pathways in bacteria and yeast as well as the mechanisms of DNA recombination [2]. As a result, techniques for inducing precise breaks in the DNA at locations where modifications are to be introduced were acknowledged as a useful tactic for targeted genomic engineering. Making modifications to the genetic makeup of many organisms continues to be an area of interest for scientists. Researchers' growing interest in altering or modifying genetic material had led to the development of numerous cutting-edge genome editing tools. With the invention of methods like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas associate nucleases, the era of genome editing has witnessed a lot of advancement.

i. Zinc Finger Nucleases (ZFN)

ZFNs were the first generation of chimerically engineered nuclease-based genome editing tools that were created after the functional Cys2-His2 zinc finger (ZF) domain was discovered. There are 30 amino acid residues in each Cys2-His2 ZF domain, and they are folded up to $\beta\beta\alpha$ configuration [3] The Cys2-His2 ZF proteins attach to DNA by inserting α-helix of the protein into the main groove of the DNA-double helix, according to crystallographic structural studies. Three tandem nucleotides in the DNA can be recognized by each ZF protein. The artificial ZF Cys2-His2 domain is located at the N-terminal area of the generalised ZFN monomer, and a non-

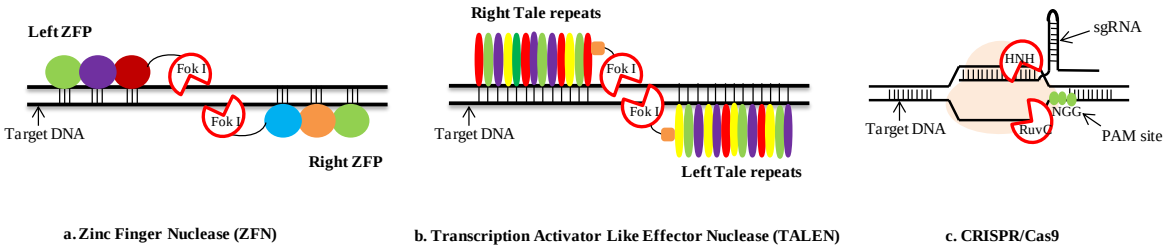
specific FokI DNA cleavage domain is located at the C-terminal region. For ZFN, FokI domain dimerization is crucial for enzymatic activity of ZFN. The modular construction, assembly, and optimisation of zinc fingers against particular target DNA sequences occurs during the design and implementation of ZFNs. Individual ZFs are then linked to target larger sequences. Zinc finger domains have been developed over time to recognise a variety of triplet nucleotides. This made it possible to choose and join zinc fingers in a way that would make it possible to recognise the target sequence of interest [4].Since the first report of zinc fingers in 1996, many species, including plants, have successfully edited using ZFN. Figure 1(a) illustrates about ZFN.

ii. Transcription Activator-Like Effector Nucleases (TALEN)

The development of a novel genome editing system using chimeric nucleases known as TALE nucleases (TALENs) was made possible by the discovery of novel transcription activator-like effector (TALE) proteins that recognise and activate particular plant promoters through a set of tandem repeats [5]. This search for efficient and selective manipulation of target genomic DNA led to the identification of these TALE proteins. TALE proteins are composed of a central domain that binds DNA, a nuclear localization signal, and a domain that activates the transcription of the target gene. Their DNA-binding ability of these protein was first described in 2007 [6], and a year later, two research teams were able to decode the recognition code of the target DNA sequence by TALE proteins [7]. It is demonstrated that the central repeat domain (CRD), which gives DNA binding and host specificity, is a component of the DNA-binding domain in TALE monomers. Each 34-amino acid long tandem repeat in the CRD binds to one nucleotide in the target nucleotide sequence. The CRD is made up of tandem repetitions of 34 amino acid residues. Positions 12 and 13 of the repeat contain two highly variable amino acids known as RVDs, which are in charge of recognizing a specific nucleotide while degenerately binding multiple nucleotides with varying degrees of effectiveness. The final tandem repeat that binds to the nucleotide at the recognition site's 3 end only has 20 amino acid residues, hence the name "half-repeat." While studies have shown that TALE proteins can generally be designed to bind any DNA sequence of interest, they should always bind DNA sequences where the fifth nucleotide base is a thymidine because failure to do so can compromise the effectiveness of TALE transcription factors (TALE-TF), TALE recombinases (TALE-R), and TALENs [8]. Figure 1(b) illustrates about TALEN.

iii. CRISPR/Cas9

The introduction of facile genome engineering in plants and animals utilising RNA-programmable CRISPR-Cas9 has ushered in a breakthrough period for biology. The CRISPR-Cas9 technique is derived from type II CRISPR-Cas systems, which give bacteria adaptive protection to viruses and plasmids. The endonuclease Cas9, which belongs to the CRISPR complex, pairs with DNA target sequences using the tracrRNA:crRNA guide sequence to create a site-specific double-strand break [9]. The Watson-Crick base pairs used to select the target site on the dual tracrRNA:crRNA's 5' side and the duplex RNA structure that binds to Cas9 on its 3' side were retained in the single guide RNA (sgRNA) version of the dual tracrRNA:crRNA. This discovery led to the development of a two-component system that allows the sgRNA programmed Cas9 to target any desired DNA sequence by altering its guide sequence. Numerous natural type II CRISPR-Cas system variants, a DNA cleaving mechanism specific to the CRISPR-Cas9 system, the ability to recognize multiple targets simultaneously, and the ease of CRISPR-Cas9 programming have all led to remarkable advancements in the ability to precisely and effectively target, edit, modify, regulate, and mark genomic loci of a variety of different organisms [9]. Figure 1(c) illustrates about CRISPR/Cas9.



a. Zinc Finger Nuclease (ZFN) b. Transcription Activator Like Effector Nuclease (TALEN) c. CRISPR/Cas9

Figure 1 : Genome engineering platform schematic representation a. Zinc Finger Nuclease(ZFNs)- ZFN consist of DNA recognition domain fused with FokI nuclease catalytic domain. Each zinc finger recognizes 3 nucleotides , 3-4 zinc finger are fused to recognize 9-18 nucelotidein each monomer.On average three to four zinc fingers are fused to recognize 9–12 nucleotides. Fok I nuclease works in dimer, so to create double strand break(DSB) in DNA two ZFN are used. **b. Transcription Activator Like Effector Nucleases (TALENs)**-TALENs consist of TALE repeats fused with catalytically active Fok I nucleases. Each tale repeat recognize a single nucleotide. Fok I nuclease works in dimer, so to create double strand break(DSB) in DNA TALEN works as dimer.

c. CRISPR/Cas 9- 20 bp sgRNA guides the Cas9 to target gene . Cas9 recognizes PAM sequence(NGG-marked in green) and causes a DSB with RuvC and HNH domain.

II. HISTORY OF GENOME ENGINEERING

The distinctive arrangement of brief, partially repeated DNA sequences seen in the prokaryotic genomes is referred to as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat). Prokaryotes employ CRISPR and the protein Cas-9 (Crispr associated proteins) that forms adaptive immunity to protect themselves from viruses and bacteriophages [10].While examining an alkaline phosphatase gene, a team led by Japanese scientist Ishino unintentionally discovered strange repetitive palindromic DNA sequences interrupted by spacers in Escherichia coli. CRISPR was initially discovered in 1987. They failed to establish its biological purpose, nevertheless. The roles of these sequences were unknown when Francisco Mojica discovered comparable sequences in other prokaryotes in 1990 and gave them the name CRISPR. Later in 2007, a CRISPR was experimentally proved as a crucial component of prokaryotes' adaptive defence system against viruses [11]. By inserting short pieces of viral DNA (spacers) into a section of the genome known as the CRISPR array, bacteria become immune during the adaptation process. Thus, spacers act as a genetic repository for past viral infections [12].The three fundamental phases of the CRISPR defence system—adaptation (spacer acquisition), crRNA synthesis (expression), and target interference—protect bacteria from recurrent viral attacks. CRISPR loci are a collection of short repetitive sequences that can be found in prokaryotic chromosomal or plasmid DNA. The Cas gene, which produces the nuclease protein (Cas protein) needed to break or destroy viral nucleic acid, is typically found next to CRISPR. Prior to the discovery of CRISPR/Cas-9, scientists depended on two restriction enzyme-based gene-editing methods: transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFN). The main difficulties for researchers in ZFN and TALEN were the complexity, cost, and time requirements of protein engineering. A long-standing objective of biomedical researchers has been the creation of a reliable and effective technique for gene editing in living cells. Scientists discovered the CRISPR system in prokaryotes and realised that humans, plants, and other orga nisms could benefit from it. In 2012, Doudna, J., and Charpentier, E. found that by using the appropriate template, CRISPR/Cas-9 could be used to edit any desired DNA [13]Since that time, CRISPR/Cas-9 has emerged as the most precise, effective, and widely used way of genome editing tool in all living cells [14]. **Figure 2** gives briefs regarding the milestones of CRISPR/Cas mediated genome engineering.

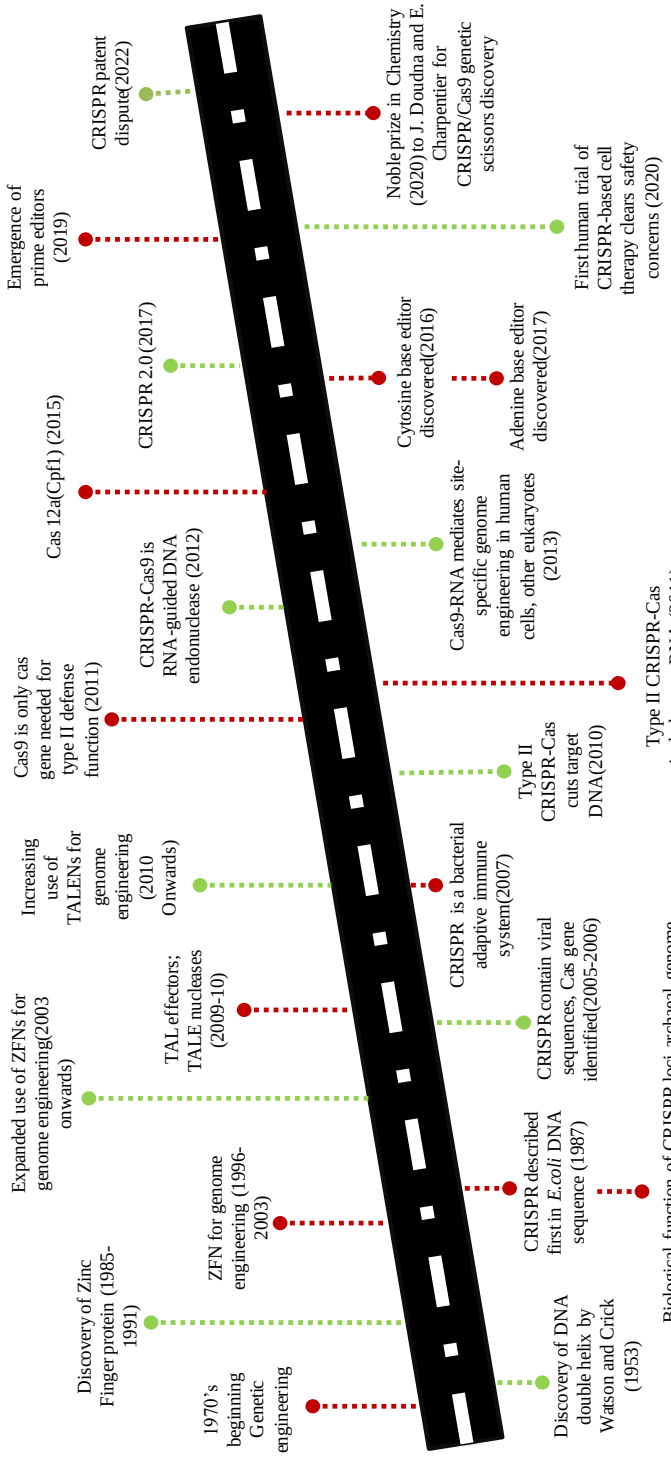


Figure 2: Milestones of CRISPR/Cas mediated genome engineering approach

III. VARIANTS OF CRISPR/CAS9 SYSTEM

CRISPR systems are broadly divided into two groups, each of which includes a variety of CRISPR types. CRISPR systems of types I and III, which are frequently found in Archaea, are included in Class 1. Class 2 CRISPR systems include types II, IV, V, and VI [15]. The type II CRISPR-Cas9 system from *Streptococcus pyogenes* is the most often utilised CRISPR/Cas system for genome targeting, despite the fact that many other CRISPR/Cas systems have also been modified by researchers. Cas9 (spCas9) from *S. pyogenes* is employed in numerous applications due of its minimal NGG PAM sequence requirements. To find Cas9-like effector proteins that might differ in their sizes, PAM needs, and preferred substrates, researchers are still actively investigating various CRISPR systems. Over 10 different CRISPR/Cas proteins have been repurposed for genome editing in the recent years. Particularly interesting among them are some of the more recent discoveries, including the Cpf1 proteins from the bacteria *Lachnospiraceae* and *Acidaminococcus sp*, respectively (AsCpf1 and LbCpf1) [16]. Cpf1 has a single sgRNA need by nature, as opposed to the native Cas9, which needs two distinct short RNAs. Furthermore, unlike Cas9, it produces a 5′ overhang rather than blunt ends when cutting DNA at target locations 3′ downstream of the PAM sequence. The size of naturally occurring Cas9 variants places additional restrictions on their ability to be packaged and delivered into various cell types via lentiviruses or Adeno-Associated Viruses (AAV). For instance, the 1,366 amino acid (aa) SpCas9 protein, which is extensively employed, poses a special therapeutic delivery issue because AAV has a restricted capacity for packaging. Smaller Cas9 variant have a higher therapeutic potential due to small size. The identification of Cas9 proteins from *Neisseria meningitides* (1082 aa Cas9; NmCas9), Staphylococcus aureus (1053 aa Cas9; SaCas9), *Campylobacter jejuni* (984 aa Cas9; CjCas9), and *Campylobacter jejuni* (1084 aa Cas9; CjCas9) are significant advances in the right direction. The trade-off is that the PAM sequences needed for these smaller Cas9 proteins are more intricate. The SaCas9 requires a 5′-NNGRRT-3′ PAM sequence, whereas the CjCas9 requires a 5′-NNNNACAC-3′ PAM sequence [17]. Because of this, although being smaller than SpCas9, these smaller Cas9 proteins have less targeting flexibility and range compared to SpCas9.

IV. COMPONENTS OF CRISPR/CAS9

Guide RNA (gRNA) and CRISPR associated (Cas-9) proteins are the two components that plays crucial role in CRISPR/Cas-9 system. The Cas-9 protein, the first Cas protein used in genome editing was isolated from *Streptococcus pyogenes* (SpCas-9). It is a big (1368 amino acids) multi-domain DNA endonuclease responsible for cleaving the target DNA to generate a double stranded break and is called as genetic scissor [18]. Cas-9 consists have two lobe called as REC lobe(Recognition lobe) and the NUC lobe(Nuclease lobe). The REC lobe consists of REC1 and REC2 domains which are used in binding guide RNA, while the NUC lobe consist of RuvC, HNH, and Protospacer Adjacent Motif (PAM) interacting domains. The PAM interacting domain imparts PAM specificity and is in charge of starting binding to target DNA, whereas the RuvC and HNH domains are used to cut each single-stranded DNA [19]. CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) combine to form the two components of guide RNA. The crRNA is of 18-20 base pair having complementary sequence to target DNA sequence and the tracrRNA is having long stretch of loops required for binding to Cas-9 nuclease. In natural system in prokaryotes the guide RNA is utilized to target the viral DNA, whereas in case of genome editing tool, the guide RNA is designed synthetically by combining crRNA and tracrRNA resulting in formation of single guide RNA (sgRNA) practically to target any gene sequence [18].

V. MECHANISMS OF CRISPR/CAS-9 GENOME EDITING

Recognition, cleavage, and repair are the basic divisions of the CRISPR/Cas-9 genome editing mechanism [18]. The sgRNA which is designed complementary to target sequence of the gene of intrest. The sgRNA recognizes the target sequence by complementary base pairing of 5′crRNA. The intended sgRNA controls Cas-9 and identifies the target sequence in the relevant gene through its complementary base pair in the 5crRNA. Without sgRNA, the Cas-9 protein remains dormant. Double-stranded breaks (DSBs) are produced by the Cas-9 nuclease at a location three base pairs upstream of PAM [20].The size of the PAM sequence, which is a short (2–5 base-pair length) conserved DNA sequence located downstream of the cut site, changes depending on the type of bacteria from which the Cas protein belongs. The Cas-9 protein, the most popular nuclease in genome editing tools, recognizes the PAM sequence at 5-NGG-3 (where N can be any nucleotide base).Upon locating a target site with the right PAM, Cas-9 causes local DNA melting, which is followed by the synthesis of an RNA-DNA hybrid. However, the mechanism by which the Cas-9 enzyme melts the target DNA sequence is still unknown. The Cas-9 protein is then activated in order to cut DNA. The HNH domain cleaves the complimentary strand of the target DNA, whereas the RuvC domain cleaves the non-complementary strand to primarily cause blunt-ended DSBs. The host cellular machinery finally fixes the DSB [20].

VI. ADVANCES IN CRISPR/CAS9

A. Base editing

At the target region in the genome, Base editors alter the identity of certain nucleobases without generating double-strand breaks (DSBs) or necessitating DNA repair templates [21]. Current BEs typically comprise of a Cas nickase (nCas) or a Cas nuclease protein (dCas) linked to a nucleoside deaminase enzyme [22]There are two groups of BEs that can be separated based on the two different types of nucleoside deaminases that can be used: cytosine base editors (CBEs) and adenine base editors (ABEs) [22].In CBE, Cytidine deaminase is fused with Cas9 and produces C-to-T transitions by deaminating deoxycytidine to deoxyuridine, a nucleoside with characteristics that are comparable to those of deoxythymidine in terms of base pairing. Adenosine deaminases, which catalyse the oxidative deamination of deoxyadenosine to deoxyinosine, are attached to the Cas9 protein in ABEs. This conversion results in A-to-G transitions because deoxyinosine imitates deoxyguanosine (in that it preferentially base pairs with deoxycytidine). Both uracil and hypoxanthine, the nucleobase of inosine, are foreign bases in DNA that the base excision repair (BER) mechanism often recognises and eliminates. This severely restricts the effectiveness of base editing in DNA by making the fixing of the mutation introduced by BEs dependent on subpar or nonexistent repair prior to DNA replication. When taking into account both DNA strands, CBEs and ABEs have the ability to cause all four base changes (C to T, G to A, A to G, and T to C). Deaminase-type BEs are precise tools for the targeted modification of the identity of single bases in DNA , in contrast to conventional CRISPR/Cas-mediated genome editing, where the editing outcome is largely unpredictable and depends on the cellular DNA repair mechanism used to fix the DSB . Importantly, BEs do not require the presence of any extra exogenous or endogenous protein components or the availability of a nucleic acid template for the nucleotide modification to take place. However, the activity of DNA repair, replication, and recombination processes affects the likelihood that the mutation will fix itself.

B. Prime editing

Prime editing is the most recent advancement in genome editing techniques that aims to: (i) produce more accurate DNA changes (avoid bystander editing); and (ii) produce more versatile editing that can produce all 12 types of point mutations as well as small insertions or deletions without causing DSBs at the target site [23]. Prime editors are made up of a fusion protein with a Cas9 nickase and an RT domain (reverse transcriptase), as well as a prime editing sgRNA (pegRNA) [24]. A primer-binding sequence (PBS) that is complementary to the 3′ end of the nicked DNA strand and an upstream RNA sequence that acts as a reverse transcription template make up the pegRNA, which is a guide RNA with an extension at the 3′ end of the scaffold. In prime editing, the pegRNA thus fulfils two different functions. It does this by first acting as the original sgRNA's guide to the target site of the primary editor, where the displaced strand is cut by the Cas9 nickase activity [24].Second, the PBS anneals with the DNA strand that has been cut, extending the 3′OH end of the DNA through reverse transcription of the RNA template that contains the sequence that will be added by prime editing. The expanded and edited DNA (3′ flap) and the original DNA strand (5′ flap) are two DNA flaps that are formed during cDNA synthesis at the nicked point. The two flaps of the uncut DNA strand hybridise with one another in an equilibrium that is later broken by the DNA repair machinery [24]. A DNA heteroduplex made up of one edited and one unedited strand is created when the unedited 5′ flap is removed. After that, the altered sequence might replace the one in the cellular MMR system [24].

C. CRISPR multiplexing

The scope and efficacy of genetic editing and transcriptional regulation have been greatly increased by multiplexed CRISPR technologies, which allow for the simultaneous expression of several gRNAs or Cas enzymes [25]. DNA cleavage activity in Cas9 and Cas12a is eliminated by changing particular amino acids, resulting in nuclease-null mutants known as dCas9 and dCas12a [26]. Effective transcriptional regulation, including CRISPR-mediated inhibition (CRISPRi) and activation (CRISPRa), is made possible by the fusion of dCas enzymes to effector domains [26]. In order to suppress transcription, dCas enzymes either prohibit RNA polymerase from binding or, if they are directed against open reading frames, interfere with transcription elongation [26]. While dCas enzymes alone are frequently sufficient in bacteria to physically block RNA polymerase and suppress gene expression, in eukaryotes, dCas is typically fused to an effector domain to increase repression by attracting chromatin remodelling proteins [27]. Similar to how RNA polymerase works, CRISPRa effector domains attract endogenous transcriptional activators or RNA polymerase [28]. Multiplexing helps transcriptional control and CRISPR-based gene editing. It is possible to create layered genetic circuits that regulate metabolic pathways or govern cellular behaviour by simultaneously editing, activating, and downregulating numerous target genes by creating multiple gRNAs and a Cas protein *in vivo*[29]. The efficacy of DNA editing and transcriptional control for CRISPRa and CRISPRi gene editing is increased by targeting numerous gRNAs to a single genomic locus [30].

D. Epigenome editing

Recent developments in CRISPR/Cas-based epigenome editing technologies have made it possible for scientists to site-specifically programme epigenetic alterations to endogenous DNA and histones as well as to change the structure of native chromatin. Epigenome editing has thereby assisted in identifying the causal links between epigenetic markers and gene expression.Site-specific control over changes to DNA, histones, and chromatin architecture is made possible by the recruitment of epigenome editing effector domains using CRISPR/Cas systems. The epigenome, in a broad sense, is the collection of biological molecules with different sequences, both heritable and not-heritable, that work together to influence the chromatin structure, genome function, and patterns of gene expression [31]. The process of epigenomic regulation involves a complex interplay between proteins that bind to genomic DNA, biochemical alterations of DNA and histones, and structural adjustments that can affect how accessible DNA is to regulatory proteins. Conventional genome editing uses relatively well-studied repair processes to fix the underlying genetic code, which is irreversibly altered [32]. Epigenome editing, in contrast, introduces possibly temporary changes in a dynamic context that is less understood [33]. For instance, a complex histone code is supposedly responsible for controlling how epigenomic disturbances affect gene regulation [34]. DNA methylation dynamics, local and global chromatin structural alterations, and chromatin modifiers all interact with one another inside the nucleus [35]. The mechanistic details and causal roles of many epigenomic changes are still not fully understood, despite the fact that several correlations between these dynamics and gene expression patterns have been inferred through genetic knockouts of chromatin regulators, global epigenomic dysregulation with small molecules, and integrative genomics. Because CRISPR/Cas-based epigenome editing typically depends on turning off the nuclease activity of CRISPR systems that are used for conventional genome editing, advancements in CRISPR/Cas-based genome editing are frequently readily transferable to epigenome editing. Targeting specificity should be improved, as should targeting ranges, and protein sizes should be decreased for more effective delivery. These developments have mainly been made possible by the development of Cas proteins, their associated gRNAs or CRISPR RNAs (crRNAs), and methods to carefully regulate the activity of CRISPR/Cas systems in cells. Nuclease-null Systems using CRISPR/Cas have been modified to serve as platforms for controlling the epigenome at different orders of magnitude. These epigenome editing techniques allow for the biochemical modification of histones and DNA as well as the programmable reorientation of large-scale genomic organization. By altering the recruitment of RNAP II and/or GTFs at particular genomic loci, epigenome editing also enables precise control over RNA production. Effector domain like CRISPR-GO, CasDrop is used in Genome organization; CLOuD9, LADL used in chromatin looping;p300,HDAC3,EZH2,LSD1used in histone modification;DNMT3A-3L,TET1 used in DNA methylation; VPR,SAM,KRAB used in RNAP II/GTF modulation [36]. Figure 3 given below glimpse an overview of genome editing technology.

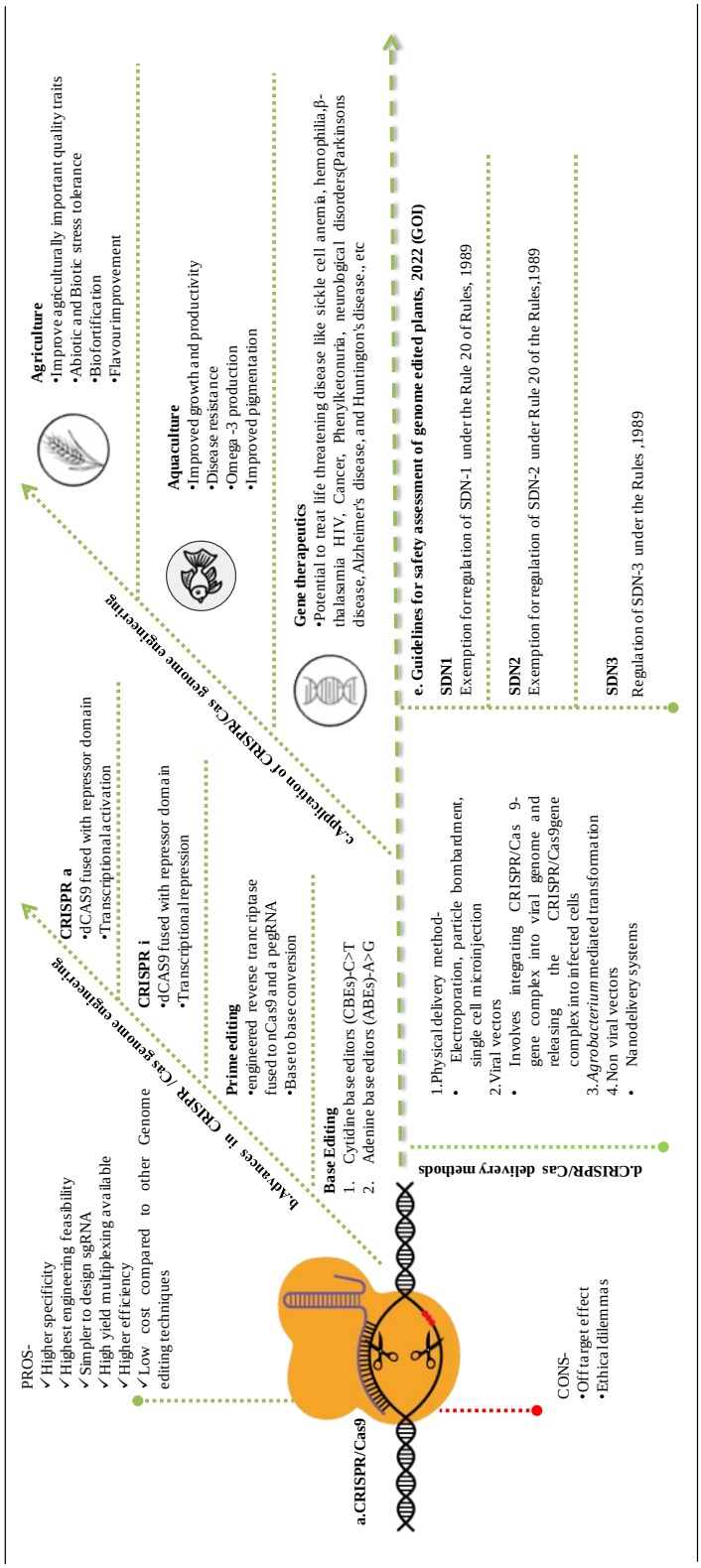


Figure 3: CRISPR/Cas9 genome editing technology an overview: a. pros and cons of CRISPR/Cas9 b. Advances in CRISPR/Cas genome engineering c. Applications of CRISPR/Cas genome engineering d. CRISPR/Cas delivery methods e. Guidelines for safety assessment of genome edited plants,2022 (GOI)

VII. ENGINEERING PLANTS WITH CRISPR/CAS9

Agriculture faces a significant challenge in providing food security for a growing global population in a changing climate. Conventional breeding can only partially address this problem; long-term genetic gain acquired through conventional breeding will not be sufficient. Recent developments in genome editing, such as gene engineering using CRISPR/Cas have created numerous opportunities to speed up plant breeding and close the knowledge gap between conventional breeding and plant molecular biology to study and enhance (complex) traits. When compared to traditional breeding, CRISPR/Cas-mediated genome editing greatly accelerates crop improvement by enabling very accurate and effective targeted modification in most crops. Since CRISPR/Cas was first described as a method for editing the genome of plants, it has been effectively used in nearly 120 crops and model plants, with reports of widespread use for as many as half of them [37]. Site-directed nucleases (SDNs), which can introduce targeted alterations into particular DNA regions of the genome to improve desirable features, are used in mutagenesis approaches to create CRISPR/Cas-edited plants [38]. Site-directed nuclease type I (SDN-1), site-directed nuclease type II (SDN-2), and site-directed nuclease type III (SDN-3) approaches can be distinguished because they provide various editing results.

Plants are prone to a wide range of pathogen infestations (biotic) and are subjected to a variety of climate (abiotic) stressors that negatively impact plant growth and development, decreasing crop yield. Thus, increasing a plant's resilience to these stresses is essential for controlling crop yield and supplying food for the world's expanding population. Traditional disease management and agricultural methods have been used for decades and have proven successful in overcoming these obstacles. However, due to the developing nature of pathogens and the present changing environmental conditions, these conventional techniques have limitations and might not be able to address the new difficulties. The CRISPR/Cas9 technique has been used to increase crop survival under challenging environmental conditions like abiotic stress (drought, salnity, heat., etc), nutritional quality improvement, free from anti-nutritional factors and many more traits are targeted successfully in plants through CRISPR/Cas9 approach. **Table 1.** below summarizing the use of CRIPSR/Cas9 in the scenario of crop improvement.

Table 1: Use of CRISPR/Cas9 in crop improvement

Crop	Gene targeted	Target function	Improved trait	Editing method	Repair mechanism	Transformation method	Reference
Rice	BADH2	Betaine aldehyde dehydrogenase	Increased fragrance content (2AP)	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[39]
Rice	*RBL1*	CDP-DAG synthase	multipathogen resistance	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[40]
Rice	*HXK1*	Hexokinase gene	Improved Photosynthetic Efficiency and Yield	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[41]
Rice	*SSU-crtI, ZmPsy*	Carotenoid biosynthesis	High carotenoid content	CRISPR/ Cas9	HDR	Particle bombardment	[42]
Rice	*Bsr-d1, Pi21, ERF922*	Up-regulation of SA- and JA-pathway	Blast resistance, Bacterial blight resistance	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[43]
Wheat	*TaAQ, TaDq*	Not fully elucidated	Altered spike morphogenesis and grain threshability	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[44]
Wheat	α-Gladin	α-Gladin	Low-gluten content,reduction in α-Gladin	CRISPR/ Cas9	HDR	Particle bombardment	[45]
Wheat	GW2	Grainweight2/	Increase grain	CRISPR/	NHEJ	Particle	[46]

		RING-type E3ubiquitin ligase	weight and protein content	Cas9		bombardment	
Wheat	IPK1	Inositol Pentakisphosphate 2-Kinase 1	Reduced phytic acid content and improved iron and zinc accumulation	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[47]
Maize	BADH2	Betaine aldehyde dehydrogenase	Popcorn like scent	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[48]
Maize	ALS1, ALS2	Acetolactate synthase (ALS)	sulfonylurea herbicide-resistant	Base editing	NHEJ	*Agrobacterium* mediated transformation	[49]
Banana	eBSV	Ebsv	Resistance to Banana streak Virus	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[50]
Banana	*lycopene epsilon-cyclase*	β-carotene biosynthesis	Increase β-carotene biosynthesis	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[51]
Tomato	G3P, DXS, GGPPS, PDS, ZISO	Biosynthesis of Lycopene, inhibiting the conversion from lycopene to β- and α-carotene	Lycopene Enrichment	CRISPR/ Cas9-Multiplex editing	NHEJ	*Agrobacterium* mediated transformation	[52]
Tomato	SlHyPRP 1	Hybrid Proline Rich Protein 1	Salt tolerace	CRIPSR/ Cas9	NHEJ	PEG-mediated transfection	[52]
Tomato	GABA-TP1, GABA-TP3, CAT9, SSADH	Pyruvate-dependent GABA transaminases 1 and 3, Cationic amino acid transporter, Succinate semialdehyde dehydrogenase	Higher γ-aminobutyric acid levels	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[53]
Soybean	KTI1, KTI3	Kunitz Trypsin Inhibitor	Reduction in trypsin inhibitor activity	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[54]
Soybean	Lox1, Lox2, Lox3	Lipoxygenases	Beany flavor reduction	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[55]
Soybean	BADH2	Betaine aldehyde dehydrogenase	Enhancement of aroma	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[54]
Soybean	FAD2-2	microsomal omega-6 desaturase	Modulation in oleic acid content while	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[56]

			reducing linoleic acid content				
Soybean	AHAS	Acetohydroxy acid synthase	Herbicide tolerance	Cytosine Base editing	NHEJ	*Agrobacterium* mediated transformation	[57]
Brinjal	SmelPO, SmelPO, SmelPO6 genes	Polyphenol oxidases	Reduced levels of flesh browning	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[58]
Potato	SR4	Signal Responsive 4	Accumulation of salicylic acid (SA), *phytophthora infestans* resistant	Cas9 –RNPs	NHEJ	PEG-mediated transfection	[58]
Potato	PPO2	Polyphenol oxidase	Reduced enzymatic browning	Cas9-RNPs	NHEJ	PEG-mediated transfection	[59]
Sugarcane	ALS	Acetolactate synthase	Herbicide tolerance	CRISPR/ Cas9	NHEJ	Biolistic gene transfer	[60]
Cassava	CYP79D1	Cassava cytochrome P450	Lower levels cynide	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[61]
Rapeseed	BnLEC1	Leafy Cotyledon 1	Reduced oil content and C18:1;increase C18:2	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[62]
Rapeseed	BnFAD2	Fatty acid desaturase 2	Increase in oleic acid content	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[63]
Rapeseed	BnFAE 1	Fatty acid elongase 1	Low euric acid content	CRISPR/ Cas9	NHEJ	*Agrobacterium* mediated transformation	[64]
Chickpea	At4CL, AtRVE7	4-coumarate ligase, Reveille 7	Drought stress tolerance	CRISPR/ Cas9	NHEJ	PEG mediated transfection	[65]

VIII. CHALLENGES IN CRISPR/CAS9 MEDIATED GENOME ENGINEERING

A. Off target effect

Off-target effects continue to be a significant obstacle for CRISPR/Cas systems, despite their immense potential in genome engineering [66]. When Cas9 interacts with unintended genomic locations, it causes cleavages that could have unfavourable implications. Since Cas9 is known to tolerate up to three mismatches between sgRNA and genomic DNA the off-target sites are frequently sgRNA-dependent. In this case, *in silico* methods are helpful for searching the entire genome for probable off-target sites and estimating the likelihood of an off-target editing [67]. *In silico* tools, which are typically free and easily accessed online programmes, can be used to forecast CRISPR/Cas9 off-target consequences [68]. The outputs of these methods are typically biased towards sgRNA-dependent off-target effects because the prediction algorithms of these applications are mostly reliant on sgRNA sequences. Off-target prediction using *in silico* technologies requires additional experimental validation since these computational methods typically inadequately account for the complex intranuclear environment, such as the epigenetic and chromatin organization states. According to their output data structure, the off-target prediction software can be divided into two categories. The first group generates information that describes the degree of sgRNA alignment to the presumed off-target locations in the genome. CasOT, Cas-OFFinder, FlashFry, and Crisflash are examples of relevant software. According to [69], CasOT is the first comprehensive tool to predict off-

target sites in user-provided reference genomes. It also offers the ability to customise the PAM sequence and the mismatch number, among other parameters. As a result of Cas-OFFinder's high tolerance for sgRNA length, PAM types, and the quantity of mismatches or bulges, it is used more frequently [70]. The purpose of FlashFry is to quickly characterise thousands of CRISPR target sequences. Using deep learning to forecast off-target cleavage sites, DeepCRISPR is a complete computational platform. To determine genome-wide off-target profiles, it takes into account epigenetic characteristics such as DNA methylation and chromatin opening [71]. Table 2 given below summarizes about the tools available for designing of sgRNA and dectection of potential off target site thereof.

Table. 2. Tools available for designing sgRNA and detection of potential off target sites

Tool name	Purpose	Access link	Reference
GuideMaker	A computational tool to identify target sites and design gRNA sequences that is not limited to any specific CRISPR system or organism.	https://academic.oup.com/gigascience/article/doi/10.1093/gigascience/giac007/6562533	[72]
CROPSR	An Automated Platform for Complex Genome-Wide CRISPR gRNA Design and Validation	https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-022-04593-2	[73]
BE target	A tool to design gRNA for plants	https://www.sciencedirect.com/science/article/pii/S2001037022003269	[74]
BE-Designer and BEAnalyzer	For cytosine and adenine base editors (CBEs and ABEs), target sites are created and mutation ratios are evaluated	https://europepmc.org/article/med/33180295	[75]
PnB Designer	To design prime and base editor gRNA for animals and plants	https://bmcbioinformatics.biomedcentral.com/counter/pdf/10.1186/s12859-021-04034-6.pdf	[76]
crisprRdesign	To forecast sgRNA efficiency and create sgRNA sequences for systems without optimal efficiency scoring techniques at the moment	https://www.jgenomics.com/v08p0062.htm	[77]
CRISPR-Local	A high-throughput local tool for generating genome-wide single-guide RNAs (sgRNAs) in plants and other animals that takes genetic diversity into account	https://academic.oup.com/bioinformatics/article/35/14/2501/5221013	[78]
CRISPR-P 2.0	A web based tool for sgRNA design in plants with minimal off-target	https://www.cell.com/moleculr-plant/pdf/S1674-2052(17)30004-7.pdf	[79]
GuideScan	To design crRNA libraries which can further used to edit coding and non coding genomic regions	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5607865/	[80]
Breaking-Cas	To design SgRNA	http://bioinfogp.cnb.csic.es/tools/breakingcas	[81]
CHOPCHOP v2	To design sgRNA along with their scoring and ranking, supports new generation effectors	https://academic.oup.com/nar/article/44/W1/W272/2499370	[82]
CRISPOR	To design , evaluate and clone guide sequences	http://crispor.tefor.net/	[83]
CRISPRscan	A tool to design highly efficient sgRNA for targeting CRISPR/Cas 9 *in vivo*	https://pubmed.ncbi.nlm.nih.gov/26322839/	[84]
CRISPR Multitargeter	A web tool to find unique and common CRISPR sgRNA targets in	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4351176/	[85]

	a set of similar sequences		
Off-Spotter	to assist with the design of optimal gRNAs.	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4326336/	[86]
Wu-CRISPR	To design sgRNA	http://crispr.wustl.edu/	[87]
SgRNA designer (CRISPRPick)	Streamline selection of sgRNA	https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design	[88]
E-CRISP	To design crRNA that will target any nucleotide sequence from sigle exon to entire genome	http://www.e-crisp.org/E-CRISP/aboutpage.html	[89]
CRISPRseek	To identify candidate guide RNAs for the Base Editor and Prime Editor of the CRISPR editing system	https://bioconductor.org/packages/release/bioc/html/CRISPRseek.html	[90]
Cas-OFFinder	To find off-target sites of Cas9-RNA guided endonucleases	http://www.rgenome.net/cas-offider/	[70]
CRISPRdirect	To design sgRNA with reduced off-target	https://pubmed.ncbi.nlm.nih.gov/25414360/	[91]
sgRNA scorer 2.0	A guide RNA tool that can predict sgRNA activity across multiple CRISPR system	https://frederick.cancer.gov/resources/repositories/sgrnascorer	[92]
CRISPR-plant	To design and construct specific gRNA for plants	http://omap.org/crispr/	[93]
SgRNAcas9	A tool to design sgRNA and evaluating potential off targets there off	http://www.biootools.com/	[94]

B. Transgene free genome editing

When a target gene has to be modified or inactivated, CRISPR/Cas9-mediated genome editing can introduce tiny InDels or substitutions at the target location. *Agrobacterium (Agrobacterium tumefaciens*)-mediated transformation is typically used in plant genetic engineering to introduce foreign genes into plants and stabilize their integration into the plant genome [95]. Due to constitutive gene expression, integration of the CRISPR/Cas9 cassette might result in unfavourable off-target consequences, plant mortality, and restrictions on conducting functional research pertaining to particular developmental or physiological processes [96]. After genome editing, the CRISPR/Cas9 and sgRNA construct is no longer required and can be removed, leaving modified crop plants devoid of transgenes that are identical to natural variants [97]. In fact, crops that have had their genomes altered are not regulated as GMOs in a few of nations, allowing them to be grown without the usual GMO restriction [98]. Because of this, transgenefree genome-edited plants are often produced by time- and labor-intensive genetic segregation, which can be particularly difficult for crops with large polyploid genomes.

IX. FUTURE PROSPECTS AND CONCLUSION

The CRISPR/Cas9 system is the most cutting-edge technology available today for accelerating crop development by quickly creating agricultural plants that are higher-yielding, better in quality, and resistant to both biotic and abiotic stresses. Additionally, the development of CRISPR/Cas9 technologies such as base editing, multiplexing, and prime editing has had a significant positive impact on plant research. There are still many problems to be resolved, though. Although precise changes are produced by NHEJ-mediated gene repair to silence or change the activity of a particular target gene or genes that are essential for crop-trait-specific applications. The choice of the genes to be targeted for mutations and the sorts of mutations must come first in order to prevent off-target gene editing. By precisely and effectively designing desirable traits into a variety of crop species, the potential of genome editing technology has so far been fully realized. The status of this technology currently allows for numerous applications ideal for enhancing plant features, including nutritional, biotic and abiotic stress tolerance. The creation of novel genome-edited crops and their commercialization in the future will be made possible by continued technical improvement and a better knowledge of the role of unknown genes.

REFERENCES

[1] A. Choulika, A. Perrin, B. Dujon, and J.-F. Nicolas, “Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of Saccharomyces cerevisiae,” Mol. Cell. Biol., 1995.

[2] Y. S. Rong and K. G. Golic, “Gene targeting by homologous recombination in Drosophila,” Science (80-.)., vol. 288, no. 5473, pp. 2013–2018, 2000.
[3] C. O. Pabo, E. Peisach, and R. A. Grant, “Design and selection of novel Cys2His2 zinc finger proteins,” Annu. Rev. Biochem., vol. 70, no. 1, pp. 313–340, 2001.
[4] V. S. Kamburova *et al.*, “Genome editing in plants: an overview of tools and applications,” Int. J. Agron., vol. 2017, 2017.
[5] R. Jankele and P. Svoboda, “TAL effectors: tools for DNA targeting,” Brief. Funct. Genomics, vol. 13, no. 5, pp. 409–419, 2014.
[6] P. Römer, S. Hahn, T. Jordan, T. Strauss, U. Bonas, and T. Lahaye, “Plant pathogen recognition mediated by promoter activation of the pepper Bs3 resistance gene,” Science (80-.)., vol. 318, no. 5850, pp. 645–648, 2007.
[7] J. Boch *et al.*, “Breaking the code of DNA binding specificity of TAL-type III effectors,” Science (80-.)., vol. 326, no. 5959, pp. 1509–1512, 2009.
[8] B. M. Lamb, A. C. Mercer, and C. F. Barbas III, “Directed evolution of the TALE N-terminal domain for recognition of all 5′ bases,” Nucleic Acids Res., vol. 41, no. 21, pp. 9779–9785, 2013.
[9] Jad. Charpentier and J. Doudna, “The new frontier of genome engineering with CRISPR-Cas9,” Science *(80-.)*., vol. 346, no. 6213, pp. 1077–1088, 2014.
[10] F. Hille and E. Charpentier, “CRISPR-Cas: biology, mechanisms and relevance,” Philos. Trans. R. Soc. B Biol. Sci., vol. 371, no. 1707, p. 20150496, 2016.
[11] Y. Ishino, M. Krupovic, and P. Forterre, “History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology,” J. Bacteriol., vol. 200, no. 7, pp. 10–1128, 2018.
[12] A. Ibrahim, M. S. Z. ÖZSÖZ, G. Tirah, and O. Gideon, “Genome engineering using the CRISPR Cas9 system,” J Biomed Pharm Sci, vol. 2, no. 127, p. 2, 2019.
[13] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier, “A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity,” Science (80-.)., vol. 337, no. 6096, pp. 816–821, 2012.
[14] M. Adli, “The CRISPR tool kit for genome editing and beyond. Nat Commun. 2018; 9: 1911.” PUBMED.
[15] E. V Koonin, K. S. Makarova, and F. Zhang, “Diversity, classification and evolution of CRISPR-Cas systems,” Curr. Opin. Microbiol., vol. 37, pp. 67–78, 2017.
[16] I. Fonfara, H. Richter, M. Bratovič, A. Le Rhun, and E. Charpentier, “The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA,” Nature, vol. 532, no. 7600, pp. 517–521, 2016.
[17] E. Kim *et al.*, “In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni,” Nat. Commun., vol. 8, no. 1, p. 14500, 2017.
[18] Y. Mei, Y. Wang, H. Chen, Z. S. Sun, and X.-D. Ju, “Recent progress in CRISPR/Cas9 technology,” J. Genet. Genomics, vol. 43, no. 2, pp. 63–75, 2016.
[19] T. Yamano *et al.*, “Crystal structure of Cpf1 in complex with guide RNA and target DNA,” Cell, vol. 165, no. 4, pp. 949–962, 2016.
[20] S. A. Ceasar, V. Rajan, S. V Prykhozhij, J. N. Berman, and S. Ignacimuthu, “Insert, remove or replace: A highly advanced genome editing system using CRISPR/Cas9,” Biochim. Biophys. Acta (BBA)-Molecular Cell Res., vol. 1863, no. 9, pp. 2333–2344, 2016.
[21] A. C. Komor, A. H. Badran, and D. R. Liu, “CRISPR-based technologies for the manipulation of eukaryotic genomes,” Cell, vol. 168, no. 1, pp. 20–36, 2017.
[22] N. M. Gaudelli *et al.*, “Programmable base editing of A• T to G• C in genomic DNA without DNA cleavage,” Nature, vol. 551, no. 7681, pp. 464–471, 2017.
[23] A. V Anzalone *et al.*, “Search-and-replace genome editing without double-strand breaks or donor DNA,” Nature, vol. 576, no. 7785, pp. 149–157, 2019.
[24] J. Tan, J. Forner, D. Karcher, and R. Bock, “DNA base editing in nuclear and organellar genomes,” Trends Genet., 2022.
[25] N. S. McCarty, A. E. Graham, L. Studená, and R. Ledesma-Amaro, “Multiplexed CRISPR technologies for gene editing and transcriptional regulation,” Nat. Commun., vol. 11, no. 1, p. 1281, 2020.
[26] L. S. Qi *et al.*, “Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression,” Cell, vol. 152, no. 5, pp. 1173–1183, 2013.
[27] S. Zhang and C. A. Voigt, “Engineered dCas9 with reduced toxicity in bacteria: implications for genetic circuit design,” Nucleic Acids Res., vol. 46, no. 20, pp. 11115–11125, 2018.
[28] L. A. Gilbert *et al.*, “CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes,” Cell, vol. 154, no. 2, pp. 442–451, 2013.
[29] J. Lian, M. HamediRad, S. Hu, and H. Zhao, “Combinatorial metabolic engineering using an orthogonal tri-functional CRISPR system,” Nat. Commun., vol. 8, no. 1, p. 1688, 2017.
[30] N. S. McCarty, W. M. Shaw, T. Ellis, and R. Ledesma-Amaro, “Rapid assembly of gRNA arrays via modular cloning in yeast,” ACS Synth. Biol., vol. 8, no. 4, pp. 906–910, 2019.
[31] C. M. Rivera and B. Ren, “Mapping human epigenomes,” Cell, vol. 155, no. 1, pp. 39–55, 2013.
[32] R. Ceccaldi, B. Rondinelli, and A. D. D’Andrea, “Repair pathway choices and consequences at the double-strand break,” Trends Cell Biol., vol. 26, no. 1, pp. 52–64, 2016.
[33] C. D. Allis and T. Jenuwein, “The molecular hallmarks of epigenetic control,” Nat. Rev. Genet., vol. 17, no. 8, pp. 487–500, 2016.
[34] B. D. Strahl and C. D. Allis, “The language of covalent histone modifications,” Nature, vol. 403, no. 6765, pp. 41–45, 2000.
[35] M. J. Rowley and V. G. Corces, “Organizational principles of 3D genome architecture,” Nat. Rev. Genet., vol. 19, no. 12, pp. 789–800, 2018.
[36] J. H. Goell and I. B. Hilton, “CRISPR/Cas-based epigenome editing: advances, applications, and clinical utility,” Trends Biotechnol., vol. 39, no. 7, pp. 678–691, 2021.
[37] T. Cardi *et al.*, “CRISPR/Cas-mediated plant genome editing: outstanding challenges a decade after implementation,” Trends Plant Sci., 2023.
[38] J. Metje-Sprink, J. Menz, D. Modrzejewski, and T. Sprink, “DNA-free genome editing: past, present and future,” Front. Plant Sci., vol. 9, p. 1957, 2019.
[39] S. Ashokkumar *et al.*, “Creation of novel alleles of fragrance gene OsBADH2 in rice through CRISPR/Cas9 mediated gene editing,” PloS oneiii, vol. 15, no. 8, p. e0237018, 2020.
[40] G. Sha *et al.*, “Genome editing of a rice CDP-DAG synthase confers multipathogen resistance,” Nature, pp. 1–7, 2023.
[41] S. Zheng *et al.*, “Improving the rice photosynthetic efficiency and yield by editing OsHXK1 via CRISPR/Cas9 system,” Int. J. Mol.

Sci., vol. 22, no. 17, p. 9554, 2021.
[42] O. X. Dong *et al.*, "Marker-free carotenoid-enriched rice generated through targeted gene insertion using CRISPR-Cas9," Nat. Commun., vol. 11, no. 1, p. 1178, 2020.
[43] Y. Zhou *et al.*, "Engineering of rice varieties with enhanced resistances to both blast and bacterial blight diseases via CRISPR/Cas9," Plant Biotechnol. J., vol. 20, no. 5, pp. 876–885, 2022.
[44] H. Liu *et al.*, "CRISPR/Cas9 editing of wheat TaQ genes alters spike morphogenesis and grain threshability," J. Genet. Genomics, vol. 47, no. 9, pp. 563–575, 2020.
[45] S. Sánchez-León *et al.*, "Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9," Plant Biotechnol. J., vol. 16, no. 4, pp. 902–910, 2018.
[46] Y. Zhang *et al.*, "Analysis of the functions of Ta GW 2 homoeologs in wheat grain weight and protein content traits," Plant J., vol. 94, no. 5, pp. 857–866, 2018.
[47] S. Ibrahim, B. Saleem, N. Rehman, S. A. Zafar, M. K. Naeem, and M. R. Khan, "CRISPR/Cas9 mediated disruption of Inositol Pentakisphosphate 2-Kinase 1 (TaIPK1) reduces phytic acid and improves iron and zinc accumulation in wheat grains," J. Adv. Res., vol. 37, pp. 33–41, 2022.
[48] Y. Wang *et al.*, "Creation of aromatic maize by CRISPR/Cas," J. Integr. Plant Biol., vol. 63, no. 9, pp. 1664–1670, 2021.
[49] Y. Li *et al.*, "Precise base editing of non-allelic acetolactate synthase genes confers sulfonylurea herbicide resistance in maize," Crop J., vol. 8, no. 3, pp. 449–456, 2020.
[50] J. N. Tripathi, V. O. Ntui, M. Ron, S. K. Muiruri, A. Britt, and L. Tripathi, "CRISPR/Cas9 editing of endogenous banana streak virus in the B genome of Musa spp. overcomes a major challenge in banana breeding," Commun. Biol., vol. 2, no. 1, p. 46, 2019.
[51] N. Kaur *et al.*, "CRISPR/Cas9 directed editing of lycopene epsilon-cyclase modulates metabolic flux for β-carotene biosynthesis in banana fruit," Metab. Eng., vol. 59, pp. 76–86, 2020.
[52] X. Li *et al.*, "Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing," Front. Plant Sci., vol. 9, p. 559, 2018.
[53] R. Li *et al.*, "Multiplexed CRISPR/Cas9-mediated metabolic engineering of γ-aminobutyric acid levels in Solanum lycopersicum," Plant Biotechnol. J., vol. 16, no. 2, pp. 415–427, 2018.
[54] Z. Wang *et al.*, "Development of new mutant alleles and markers for KTI1 and KTI3 via CRISPR/Cas9-mediated mutagenesis to reduce trypsin inhibitor content and activity in soybean seeds," Front. Plant Sci., vol. 14, p. 1111680, 2023.
[55] J. Wang *et al.*, "Generation of seed lipoxygenase-free soybean using CRISPR-Cas9," Crop J., vol. 8, no. 3, pp. 432–439, 2020.
[56] N. Al Amin *et al.*, "CRISPR-Cas9 mediated targeted disruption of FAD2–2 microsomal omega-6 desaturase in soybean (Glycine max. L)," BMC Biotechnol., vol. 19, no. 1, pp. 1–10, 2019.
[57] A. Bao *et al.*, "Z, Shan, Z," Yang, S. Yuan, C. Zhang, X. Zhang, B. Liu, F. Komg, Xia Li, X. Zhou, LSP Tran D. Cao, 2019.
[58] A. Maioli *et al.*, "Simultaneous CRISPR/Cas9 editing of three PPO genes reduces fruit flesh browning in Solanum melongena L.," Front. Plant Sci., vol. 11, p. 607161, 2020.
[59] M. N. González *et al.*, "Reduced enzymatic browning in potato tubers by specific editing of a polyphenol oxidase gene via ribonucleoprotein complexes delivery of the CRISPR/Cas9 system," Front. Plant Sci., vol. 10, p. 1649, 2020.
[60] M. T. Oz, A. Altpeter, R. Karan, A. Merotto, and F. Altpeter, "CRISPR/Cas9-mediated multi-allelic gene targeting in sugarcane confers herbicide tolerance," Front. genome Ed., vol. 3, p. 673566, 2021.
[61] B. S. Juma, A. Mukami, C. Mweu, M. P. Ngugi, and W. Mbinda, "Targeted mutagenesis of the CYP79D1 gene via CRISPR/Cas9-mediated genome editing results in lower levels of cyanide in cassava," Front. Plant Sci., vol. 13, p. 1009860, 2022.
[62] G. Yan *et al.*, "DELLA proteins BnaA6. RGA and BnaC7. RGA negatively regulate fatty acid biosynthesis by interacting with BnaLEC1s in Brassica napus," Plant Biotechnol. J., vol. 19, no. 10, pp. 2011–2026, 2021.
[63] H. Liu *et al.*, "CRISPR/Cas9-mediated editing of double loci of BnFAD2 increased the seed oleic acid content of rapeseed (Brassica napus L.)," Front. Plant Sci., vol. 13, p. 1034215, 2022.
[64] Y. Liu *et al.*, "CRISPR/Cas9-targeted mutagenesis of BnaFAE1 genes confers low-erucic acid in Brassica napus," Front. Plant Sci., vol. 13, p. 848723, 2022.
[65] S. Badhan, A. S. Ball, and N. Mantri, "First report of CRISPR/Cas9 mediated DNA-free editing of 4CL and RVE7 genes in chickpea protoplasts," Int. J. Mol. Sci., vol. 22, no. 1, p. 396, 2021.
[66] M. Pacesa *et al.*, "Structural basis for Cas9 off-target activity," *Cell*, vol. 185, no. 22, pp. 4067–4081, 2022.
[67] M. Naeem, S. Majeed, M. Z. Hoque, and I. Ahmad, "Latest developed strategies to minimize the off-target effects in CRISPR-Cas-mediated genome editing," Cells, vol. 9, no. 7, p. 1608, 2020.
[68] X. R. Bao, Y. Pan, C. M. Lee, T. H. Davis, and G. Bao, "Tools for experimental and computational analyses of off-target editing by programmable nucleases," Nat. Protoc., vol. 16, no. 1, pp. 10–26, 2021.
[69] A. Xiao *et al.*, "CasOT: a genome-wide Cas9/gRNA off-target searching tool," Bioinformatics, vol. 30, no. 8, pp. 1180–1182, 2014.
[70] S. Bae, J. Park, and J. S. Kim, "Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics [Internet]." Oxford University Press, 2014.
[71] G. Chuai *et al.*, "DeepCRISPR: optimized CRISPR guide RNA design by deep learning," Genome Biol., vol. 19, pp. 1–18, 2018.
[72] R. Poudel, L. T. Rodriguez, C. R. Reisch, and A. R. Rivers, "GuideMaker: Software to design CRISPR-Cas guide RNA pools in non-model genomes," Gigascience, vol. 11, p. giac007, 2022.
[73] H. Müller Paul, D. D. Istanto, J. Heldenbrand, and M. E. Hudson, "CROPSR: an automated platform for complex genome-wide CRISPR gRNA design and validation," BMC Bioinformatics, vol. 23, no. 1, pp. 1–19, 2022.
[74] X. Xie *et al.*, "BEtarget: a versatile web-based tool to design guide RNAs for base editing in plants," Comput. Struct. Biotechnol. J., vol. 20, pp. 4009–4014, 2022.
[75] G.-H. Hwang and S. Bae, "Web-based base editing toolkits: BE-designer and BE-analyzer," *Comput. Methods Synth. Biol.*, pp. 81–88, 2021.
[76] S. M. Siegner, M. E. Karasu, M. S. Schröder, Z. Kontarakis, and J. E. Corn, "PnB Designer: a web application to design prime and base editor guide RNAs for animals and plants," BMC Bioinformatics, vol. 22, pp. 1–12, 2021.
[77] D. Beeber and F. J. J. Chain, "crispRdesignR: A versatile guide RNA design package in R for CRISPR/cas9 applications," J. genomics, vol. 8, p. 62, 2020.
[78] J. Sun *et al.*, "CRISPR-Local: a local single-guide RNA (sgRNA) design tool for non-reference plant genomes," Bioinformatics, vol. 35, no. 14, pp. 2501–2503, 2019.

[79] H. Liu, Y. Ding, Y. Zhou, W. Jin, K. Xie, and L.-L. Chen, "CRISPR-P 2.0: an improved CRISPR-Cas9 tool for genome editing in plants," Mol. Plant, vol. 10, no. 3, pp. 530–532, 2017.
[80] A. R. Perez *et al.*, "GuideScan software for improved single and paired CRISPR guide RNA design," Nat. Biotechnol., vol. 35, no. 4, pp. 347–349, 2017.
[81] J. C. Oliveros *et al.*, "Breaking-Cas—interactive design of guide RNAs for CRISPR-Cas experiments for ENSEMBL genomes," Nucleic Acids Res., vol. 44, no. W1, pp. W267–W271, 2016.
[82] K. Labun, T. G. Montague, J. A. Gagnon, S. B. Thyme, and E. Valen, "CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering," Nucleic Acids Res., vol. 44, no. W1, pp. W272–W276, 2016.
[83] M. Haeussler *et al.*, "Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR," Genome Biol., vol. 17, pp. 1–12, 2016.
[84] M. A. Moreno-Mateos *et al.*, "CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo," Nat. Methods, vol. 12, no. 10, pp. 982–988, 2015.
[85] S. V Prykhozhij, V. Rajan, D. Gaston, and J. N. Berman, "Correction: CRISPR MultiTargeter: a web tool to find common and unique CRISPR single guide RNA targets in a set of similar sequences," PLoS One, vol. 10, no. 9, p. e0138634, 2015.
[86] I. Rigoutsos and V. Pliatsika, "'Off-Spotter': very fast and exhaustive enumeration of genomic lookalikes for designing CRISPR/Cas guide RNAs," 2015.
[87] N. Wong, W. Liu, and X. Wang, "WU-CRISPR: characteristics of functional guide RNAs for the CRISPR/Cas9 system," Genome Biol., vol. 16, no. 1, pp. 1–8, 2015.
[88] J. G. Doench *et al.*, "Rational design of highly active sgRNAs for CRISPR-Cas9–mediated gene inactivation," Nat. Biotechnol., vol. 32, no. 12, pp. 1262–1267, 2014.
[89] F. Heigwer, G. Kerr, and M. Boutros, "E-CRISP: fast CRISPR target site identification," Nat. Methods, vol. 11, no. 2, pp. 122–123, 2014.
[90] L. J. Zhu, B. R. Holmes, N. Aronin, and M. H. Brodsky, "CRISPRseek: a bioconductor package to identify target-specific guide RNAs for CRISPR-Cas9 genome-editing systems," PLoS One, vol. 9, no. 9, p. e108424, 2014.
[91] Y. Naito, "hino K, bono h, ui-Tei K. crisPrdirect: software for designing CRISPR," Cas Guid. RNA with Reduc. offtarget sites. Bioinforma., vol. 31, pp. 1120–1123, 2015.
[92] R. Chari, N. C. Yeo, A. Chavez, and G. M. Church, "sgRNA Scorer 2.0: a species-independent model to predict CRISPR/Cas9 activity," ACS Synth. Biol., vol. 6, no. 5, pp. 902–904, 2017.
[93] K. Xie, J. Zhang, and Y. Yang, "Genome-wide prediction of highly specific guide RNA spacers for CRISPR–Cas9-mediated genome editing in model plants and major crops," Mol. Plant, vol. 7, no. 5, pp. 923–926, 2014.
[94] S. Xie, B. Shen, C. Zhang, X. Huang, and Y. Zhang, "sgRNAcas9: a software package for designing CRISPR sgRNA and evaluating potential off-target cleavage sites," PLoS One, vol. 9, no. 6, p. e100448, 2014.
[95] S. B. Gelvin, "Agrobacterium-mediated plant transformation: the biology behind the 'gene-jockeying' tool," Microbiol. Mol. Biol. Rev., vol. 67, no. 1, pp. 16–37, 2003.
[96] D. Modrzejewski *et al.*, "Which factors affect the occurrence of off-target effects caused by the use of CRISPR/Cas: a systematic review in plants," Front. Plant Sci., vol. 11, p. 574959, 2020.
[97] D. Chilcoat, Z.-B. Liu, and J. Sander, "Use of CRISPR/Cas9 for crop improvement in maize and soybean," Prog. Mol. Biol. Transl. Sci., vol. 149, pp. 27–46, 2017.
[98] C. Turnbull, M. Lillemo, and T. A. K. Hvoslef-Eide, "Global regulation of genetically modified crops amid the gene edited crop boom–a review," Front. Plant Sci., vol. 12, p. 630396, 2021.