Functional & Integrative Genomics (2023) 23:119
https://doi.org/10.1007/s10142-023-01036-w

REVIEW

An overview of genome engineering in plants, including its scope, technologies, progress and grand challenges

Muhammad Sufyan[1] · Umar Daraz[2] · Sajjad Hyder[3] · Usman Zulfiqar[4] · Rashid Iqbal[4] · Sayed M. Eldin[5] · Farzana Rafiq[6] · Naveed Mahmood[1] · Khurram Shahzad[7] · Muhammad Uzair[8] · Sajid Fiaz[9] · Iftikhar Ali[10,11]

Received: 27 November 2022 / Revised: 20 March 2023 / Accepted: 21 March 2023

Abstract

Genome editing is a useful, adaptable, and favored technique for both functional genomics and crop enhancement. Over the years, rapidly evolving genome editing technologies, including clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas), transcription activator-like effector nucleases (TALENs), and zinc finger nucleases (ZFNs), have shown broad application prospects in gene function research and improvement of critical agronomic traits in many crops. These technologies have also opened up opportunities for plant breeding. These techniques provide excellent chances for the quick modification of crops and the advancement of plant science in the future. The current review describes various genome editing techniques and how they function, particularly CRISPR/Cas9 systems, which can contribute significantly to the most accurate characterization of genomic rearrangement

✉ Rashid Iqbal
rashid.iqbal@iub.edu.pk

✉ Iftikhar Ali
iftikharali@uswat.edu.pk

Muhammad Sufyan
sufyan11.uaf@ya-hoo.com

Umar Daraz
umar@lzu.edu.cn

Sajjad Hyder
sajjad.hyder@gcwus.edu.pk

Usman Zulfiqar
usman.zulfiqar@iub.edu.pk

Sayed M. Eldin
sayed.eldin22@fue.edu.eg

Farzana Rafiq
Farza-narafiq41.uaf@gmail.com

Naveed Mahmood
naveed1229uaf@gmail.com

Khurram Shahzad
khurram@igsnrr.ac.cn

Muhammad Uzair
sufyan11.uaf@ya-hoo.com

Sajid Fiaz
sfiaz@uoh.edu.pk

[1] College of Biological Sciences, China Agricultural University, Beijing 100083, China

[2] State Key Laboratory of Grassland Agro-Ecosystems, Center for Grassland Microbiome, College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730000, China

[3] Department of Botant, Government College Women University, Sialkot, Pakistan

[4] Department of Agronomy, Faculty of Agriculture and Environment, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

[5] Center of Research, Faculty of Engineering, Future University in Egypt, New Cairo 11835, Egypt

[6] School of Environmental Sciences and Engineering, NCEPU, Beijing, China

[7] Institute of Geographic Sciences and Natural Resources Research, CAS, Beijing, China

[8] National Institute for Genomics and Advanced Biotechnology, Park Road, Islamabad, Pakistan

[9] Department of Plant Breeding and Genetics, The University of Haripur, Haripur 22620, Pakistan

[10] Center for Plant Sciences and Biodiversity, University of Swat, Charbagh 19120, Pakistan

[11] Department of Genetics and Development, Columbia University Irving Medical Center, New York, NY 10032, USA

Extended author information available on the last page of the article

Published online: 06 April 2023

and plant gene functions as well as the enhancement of critical traits in field crops. To accelerate the use of gene-editing technologies for crop enhancement, the speed editing strategy of gene-family members was designed. As it permits genome editing in numerous biological systems, the CRISPR technology provides a valuable edge in this regard that particularly captures the attention of scientists.

Keywords CRISPR/Cas9 · Genome editing · Genetic engineering · Plants · Crop improvement

Introduction

Environmental, agronomic, social, and economic social benefits for farmers and consumers have been promoted by genetically modified plants via *Agrobacterium*-mediated T-DNA insertion, as concerns about the potential risks associated with genetically modified (GM) crops continue to mount, questions surrounding their safety both for the environment and for human consumption have become more prevalent (Craig et al. 2008). However, the process of detecting mutations, identifying T-DNA, and identifying insertions is all time-consuming. Therefore, Voytas (2013) presented programmed sequence-specific nucleases (SSNs) for precise genome editing, which revolutionized genome engineering. In the past few decades, scientists have found many critical SSNs that may be easily modified and reprogrammed to cause double-stranded breaks (DSBs) at the desired chromosomal location. ZFNs, TALENs, and the CRISPR-Cas system are three important genome engineering technologies that have been used for a variety of purposes (Mahfouz et al. 2014).

However, modern plant breeding techniques such as genome editing enable crop health enhancement without the use of a transgene (Zhang et al. 2018), but novel agricultural products are encountering regulatory and social acceptance issues (Araki and Ishii 2015; Schaeffer and Nakata 2015; Gao et al. 2018) In a similar manner, some of the current editing methods are (including zinc finger nucleases (ZFNs), TALENs, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Bogdanove et al. 2018). Several new breeding approaches, such as similarly fast breeding platforms, precise genome editing, and high thrash out genotyping, in combination with genetic engineering, have lately resulted in the development of high-yielding and disease-free agricultural types (Wei et al. 2020). Site-specific nucleases, ZFNs, TALENs, and CRISPR-cas9 are now widely available genome editing techniques (Wei et al. 2020, Zhu et al. 2017), likewise Cas9 with ZFNs being the first absolutely targeting protein reagents. DNA-binding domains called zinc-finger proteins (ZFNs) recognize and bind to a specific sequence of three base pairs (Rai et al. 2019). ZFNs and TALENs are time and labor-intensive compared to CRISPR/Cas9, which may modify single or several genes in plants (Tang et al. 2017; Xie et al. 2015; Ishizaki 2016; Feng et al. 2014; Zong et al. 2017). Furthermore, the application of CRISPR/Cas9 has led to substantial advancements in cotton, rice, wheat, and other crops. These results suggest that this technology could be widely utilized in rapeseed breeding and innovation, promoting directional variation and the evolution of breeding practices (Braatz et al. 2017). The CRISPR/Cas9 nuclease cuts genomic targets that are governed by a single guide RNA (sgRNA), and the resulting double-strand breaks (DSBs) are repaired by an error-prone no homologous end-joining repair mechanism, which can result in indel (insertion-deletion) mutations (Feng et al. 2014; Zhu et al. 2017) As a result, because mutants generated by the CRISPR/Cas9 system do not fundamentally include any exogenous DNA insertions, the CRISPR/Cas9 system may aid agricultural progress via possible escape from GM regulation (Gao et al. 2018; Zhu et al. 2017; Li et al. 2017a, b). Alternatively, mutagenesis products may be subject to existing GM legislation if they are used to deliberately and precisely modify an organism's genetic components in a manner that does not occur naturally (Araki and Ishii 2015). Although CRISPR/Cas9 technologies offer a novel technique to change crops quickly and precisely, such as to increase productivity (Jones 2015). As CRISPR/Cas9 has been employed in crop production, concerns about product protection have grown. This underscores the need for molecular characterization of CRISPR/Cas9 mutants. The majority of molecular investigations of CRISPR/Cas9-induced mutations in plants have relied on the patterns and segregation of the targeted gene alterations in transitory or early systems (Wang et al. 2014; Zhu et al. 2017). While CRISPR nucleases have been shown to be effective in improving plant defenses against DNA or RNA viruses, their effectiveness in enhancing resistance to bacterial diseases remains uncertain. Bacterial pathogens differ from DNA or RNA viruses in that they do not integrate their genetic material into host plant cells but instead remain outside the plant cells. Consequently, genome editing of microbial cells using CRISPR nucleases expressed in plants may be challenging.. Several studies have suggested, however, that pathogenic soil microorganism cells can interchange biological macromolecules as well as small molecules like proteins and microRNAs (Cai et al. 2018; Thakur et al. 2023). Figure 1 evaluates the various genome editing techniques and their subsequent crop enhancement applications, with an emphasis on the genome editing system's advancement and limitations in plants.

Technologies for editing plant genome

Precise plant breeding techniques and potential application of sequence-specific nucleases (SSNs) Sequence-specific nucleases combine a DNA recognition component with a nuclease component. CRISPR/Cas9, TALENs, ZFNs, and mega nucleases are examples of SSNs (Table 1).

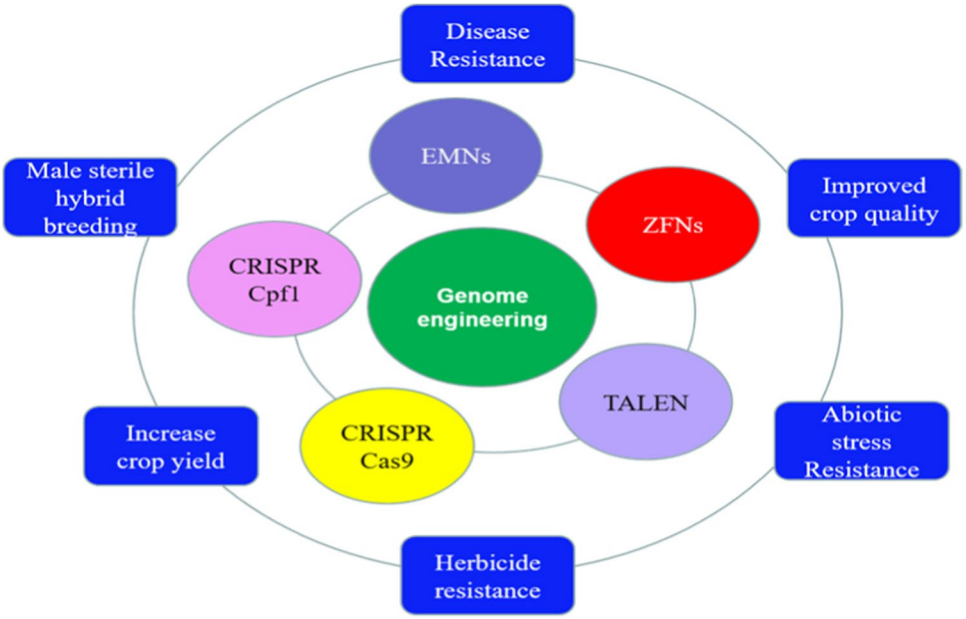

Fig. 1 Overview of the various aspects of genome editing in plants, including its potential applications, challenges, and benefits

Table 1 Comparison between ZFNs, TALENs and CRISPR/Cas on the nuclease platform and clinical trial data (Ahmar et al. 2020)

Characteristics	ZFN	TALEN	CRISPR/Cas9
Full form	Zinc finger nucleases	Transcription activator-like effector nucleases	Clustered regularly interspaced short palindromic repeats
Source	Bacteria, eukaryotes	Bacteria (*Xanthomonas* sp.)	Bacteria (*Streptococcus* sp.)
Double-stranded break pattern	Staggered cut (4break pa′ overhang)	Staggered cut (heterogeneous overhangs)	SpCas9 generates blunt ends; Cpf1 generates Staggered cut (50 overhangs)
Improved/other versions	AZP-SNase	Tev-mTALEN	Cpf1, eSpCas9
Efficiency/inefficiency	The small size of ZFN expression cassettes allow use in a variety of viral vectors	Packing into viral vectors are difficult due to the large size of TALEN	Commonly used Cas9 from S. pyogenes is large, impose packaging problems in viral vectors
Construction	Protein engineering for every single target	Protein engineering for every single target	The 20-Nucleotide sequence of sgRNA
Targeting	Proteinngcleotide sequence of sgRNAe targ	Proteinngcleotide sequence of sgRNAe targ	DNAteinngcleotide sequenghly predictable
Delivery	Two ZFNs around the target sequence are required	Two TALENs around the target sequence are required	sgRNA complementary to the target sequence with Cas9 protein
Multiplexing	Challenging	Challenging	Highly feasible
Feasibility of library construction & transformation for genome-wide screens	Technically challenging	Technically challenging	Highly feasible
Affordability	Resource intensive and time-consuming	Affordable but time-consuming	Highly affordable

Mega nucleases

Mega nucleases (MNs) were a novel class of nucleases that were frequently used in plant genome engineering (D, re a n et al. 2013). Mega nucleases are distinguished from other endonucleases by a large recognition site of around 12–40 bp (base pairs), which makes MNs the most successful delivery route for all vectors, including plant RNA viruses (Mishra and Zhao 2018). Homing endonucleases are the most common name for meganucleases. Non-homologous end-joining (NHEJ) is a technique for repairing double-stranded breaks (DSBs) that enable gene knockout in Arabidopsis and tobacco plants (Zhang et al. 2010). Mega nucleases, on the other hand, are difficult to remodify in conjunction with other genome-targeting approaches because their DNA-binding and catalytic

domains are frequently combined and cannot be separated (Puchta 2005). Earlier work in Arabidopsis, maize, and *Gossypium hirsutum* (cotton) showed that modified mega nucleases might be used to alter genes in plants. However, because controlling mega nucleases looks to be difficult, more research is needed to improve this strategy. As a result, researchers have concentrated on more effective, simple, and accurate gene engineering tools, such as ZFNs, TALENs, and CRISPR.

Zinc-finger nucleases

The advent of zinc-finger nucleases (ZFNs) brought about a significant transformation in genome modification as they were the first protein reagents capable of targeting specific sites. ZFNs function as DNA-binding domains that accurately recognize three base pairs at the intended target location (Rai et al. 2019) Because ZFNs target DSBs, they are highly successful and impactful tools for genome editing (Durai et al. 2005). The initial iteration of genome editing techniques utilizing ZFNs were created through the use of chimeric nucleases, which were made possible through the functionalization of the Cys2-His2 zinc-finger domain (Papworth et al 2006). ZFN contains two domains that are DNA-binding domain and the DNA cleavage domain which are required for its functioning. The DNA-binding domain is made up of 300–600 zinc-finger repeats (Carlson et al. 2012), with each repeat capable of recognizing and interpreting between 9 and 18 base pairs (bp) of DNA sequence.

The DNA cleavage domain is a widely recognized component of the type II restriction endonuclease Fok1, functioning as a nonspecific cleavage domain. This same domain also serves as the DNA cleavage domain in ZFNs (Carroll et al. 2006). Comprised of two monomers that bind to their respective target sequences in reverse between 5 and 6 bp, ZFNs with Fok1 domains are capable of separating DNA from its flanking sequence. The 24-–30-bp segment is identified by a zinc-finger domain, which contains unique or rare targeting locations within the genome (Gaj et al. 2012; Minczuk et al. 2008).

A variety of ZFNs have been developed and tested in a variety of species. Off-target impacts became less common as technology became more specific and efficient. Context-dependent Assembly (CoDA), Oligomerized Pool Engineering (OPEN), and Modular Assembly (MA) are the three most often used technologies for designing ZF domains. Several software packages are available for generating engineered ZFs (ZiFiT), storing a database of ZFs (ZiFDB), and identifying possible ZFN targets in a variety of model organisms (ZFNGenome) (Sander et al. 2007). Zinc-finger nucleases should continue to be a useful technique for editing plants because of their modest size (300 amino acids per zinc finger nuclease monomer) and advances in strategies for redirecting targeting (Sander et al. 2007). Scientists are learning to build and regulate basic and applied genomic targets, which is advancing genome editing.

Transcriptional activator-like effector nucleases

TALENs (transcription activator-like effector nucleases) are a form of site-directed mutagenesis that operates similarly to ZFNs and were initially discovered in Xanthomonas plant pathogenic bacteria. Adding further information TALENs are extremely selective since they only target one nucleotide at the target site (Boch et al. 2009; Christian et al. 2010). In both angiosperms and bryophytes, TALENs have been successfully employed to modify genomes (Shan et al. 2013; Kopischke et al. 2017). The DNA binding domain of TALENs corresponds to a single TALE protein and a single base pair. In 2014, the recognition preferences of 400 unique RVDs were decoded, making TALENs a valuable tool for researchers studying gene function and gene therapy (Yang et al. 2014). TALEs are also notable for their 34-amino-acid multiplex repetitions, which allow for the effective edition of a single base pair (Zhang et al. 2019). DNA repetitive sequences can affect TALE proteins as well. TALE proteins continually fix the nucleotides of the DNA sequence at the 50-end thymidine base. In the absence of a 50 T, the activities of TALE transcription factors (TALE-TFs) and TALE recombinase (TALE-R) are reduced (Lamb et al. 2013). TALENs are better than ZFNs because they are easier to modulate and have a lower off-target rate. However, TALENs and ZFNs face issues in protein synthesis, validation, and design, which are some of the barriers to their general adoption for standardized use.

CRISPR Cas system

The CRISPR/Cas system is the most recent member that belongs to the SSN family. Many genetically damaging entities, including phages, transposons, and plasmids, have been encountered by archaea and bacteria. Against these environmental stimuli or elements, they have developed a number of defense systems. Several restriction site modification enzymes, for example (BsmI, BcgI, and BfmI etc.), are a defense mechanism that uses variations in host restriction patterns and infectious agents to remove the pathogen A. (Forde and Fitzgerald 1999). Bacteria and archaea have just discovered a new defense mechanism. This defense mechanism is distinct from the CRISPR Cas system (Barrangou and Marraffini 2014), which was previously identified. CRISPR stands for "Clustered Regularly Interspaced Short Palindromic Repeats," a nucleotide sequence family found in bacteria. These DNA sequences are taken from the viral genome. CRISPR sequences are part of a bacterial viral defense system. These sequence repeats are part of many

viruses that have attacked these bacteria, and the bacteria have saved a tiny section of the viral genome called spacers. In 2007, *Streptococcus thermophilus* received a short sequence (spacer) from an infecting phage and integrated it into his CRISPR system for the first time (Sapranauskas et al. 2011). Manipulation of *S. thermophilus*' CRISPR system was used in further research. Researchers removed various spacers to see how they affected the bacterial immune system. Brouns and Oost coined the term "cascade" to describe the CRISPR associated (Cas) protein complex they identified. Cas proteins turn RNA precursor into mature RNA containing spacer sequence (crRNA), which is required for bacteria to defend against viral pathogens (Barrangou and Marraffini 2014). Furthermore, the breakage of the viral genome needed helicase and nuclease activity, which should be part of this cascade. With the passage of time, different Cas proteins were found based on their structure, mode of action, and target (DNA/RNA).

Cas1 and Cas2 Cas1 and Cas2 are CRISPR system Cas proteins that are generally conserved. Both proteins produce a stable complex that is required for spacer acquisition. Cas1 is a metal-dependent nuclease that creates double-stranded DNA. Plant adaptive immunity is based on the Cas1 and Cas2 complexes. This combination creates a Nick in the CRISPR array's double standard DNA near the coding sequence, allowing the spacer to integrate into the array (Nuñez et al. 2014).

Type of CRISPR Cas protein

Cas proteins of type I are most commonly found in archaea and bacteria. They fight viral DNA as part of a riboprotein complex known as a cascade. Cas3 is an example of type I Cas protein. To recognize the target sequence, they need a certain protospacer adjacent motif (PAM) sequence. It simply takes CRISPR RNA (crRNA) to target the invading virus's protospacer (Sinkunas et al. 2011). Cas9 is one of the type II Cas proteins. with many domains that execute distinct activities. Both DNA and RNA can be targeted by Cas proteins of type III. They resemble type I Cas proteins in terms of structure. Repeated associates Mysterious Proteins (RAMPs) are found in type III. A cascade of type I Cas is equivalent to these RAMPs. It has been subdivided into two subtypes: subtype IIIA attacks plasmid DNA and subtype IIIB attacks RNA, although more research is needed. RAMPs are also present in type III CRISPR Cas system, in addition to universal Cas2 protein. This class of nucleases can target both types of genomes at the same time, but more research is needed. Moreover, there are many other types of enzymes that are mentioned in Table 2.

CRISPR-Cas9 The CRISPR/Cas system utilizes a Cas9 protein and an RNA complex composed of a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA) to enable targeted DNA cleavage in this system (tracrRNA). The Cas9 protein cleaves double-stranded DNA upstream of a protospacer-adjacent motif (PAM; e.g., NGG for *Streptococcus pyogenes* Cas9) at regions homologous to the crRNA sequence. To simplify the system, the crRNA and tracrRNA were combined to create a single-guide RNA (sgRNA) for genome editing. However, off-target cleavage remains a concern with the CRISPR/Cas system, as noted by some studies (Fu et al. 2013).

The CRISPR-Cas system uses RNA:DNA interactions to facilitate target site recognition, rather than the protein:DNA interactions utilized by other genome editing technologies such as mega nucleases, zinc-finger nucleases, and TALENs. To redirect Cas9 targets, a modification of 20 nucleotides within the crRNA or gRNA is necessary. These 20 nucleotides direct Cas9 binding and cleavage, and the system is known to tolerate mismatches, with greater tolerance closer to the target sequence's 5′ end (Fu et al. 2013). Recent research indicates that, besides the PAM sequence, the first 8–12 nucleotides are critical for target site recognition (Wu et al. 2019). To minimize off-target effects, several strategies have been developed, including dual-nicking of DNA (Mali et al. 2013), a fusion of catalytically-dead Cas9 with FokI (Tsai et al. 2014), and shortening of gRNA sequences (Tsai et al. 2014; Fu et al. 2013).

Cas9 is an endonuclease that contains two separate nuclease domains: the HNH domain, which cleaves the DNA strand complementary to the target strand, and the RuvC-like domain, which cleaves the DNA strand complementary to the guide RNA sequence (target strand) (Jinek et al. 2012). Double-strand breaks (DSBs) are repaired via non-homologous end joining (NHEJ) or homology-directed repair in the presence of a template. dCas9 is an RNA-guided DNA-binding protein that lacks endonuclease activity due to mutations in both nuclease domains (Asp10 → Ala, His840 → Ala) (Jinek et al. 2012). Specific activities in the genome can be carried out by adding effector domains to dCas9. For example, in Arabidopsis, fusion of the transcriptional activator VP64 with dCas9 resulted in targeted gene activation by modifying flowering time regulation. In Arabidopsis and tobacco, dCas9-VP64 regulated transcriptional activation of endogenous genes, while dCas9-SRDX regulated transcriptional repression (Lowder et al. 2018). Multiplex gene targeting (GT) with several sgRNAs is also possible with these regulatory domains. Base editing enzymes, such as cytidine deaminase linked with dCas9, offer a new dimension to CRISPR/Cas technology by replacing specific bases in the targeted region of DNA and

Table 2 CRISPR/Cas Systems/ classes and their size, target sites, and functions

Class name	Size (bp	PAM	Host	sgRNA size (bp	Cut site	Target	Function	Refs
SpCas9	1368	5368s9onz	*Streptococcus pyogenes*	20	50reptoco	Target dsDNA	The SpCas9 system utilizes a PAM, which is characterized by an NGG consensus sequence that consists of two G:C base pairs	(Jinek et al. 2012) (Gleditzsch et al. 2019)
SpCas9-NG	-	5pCas9-NGch et al. {"citationItems":[{"id":"IT	*S. pyogenes*	-	5. pyogen	Target DNA	The utilization of Cas9-NG has the potential to significantly broaden the scope of genome-editing tools, thereby enabling targeted genome editing, base editing, and genome regulation in plants	(Ren et al. 2019) (Xu et al. 2021)
FnCas9	1629	5629s9 al	*Francisella novicida*	20	50ancisel	Target DNA	FnCas9 recognizes a PAM sequence that contains 5′-NGG-3′, and employs structural insights to engineer a variant that can identify a more permissive PAM sequence containing 5′-YG-3′	(Acharya et al. 2019)
SaCas9	1053	50-NNGRRT-3′	*Staphylococcus aureus*	21	51aphyloc	Target DNA	SaCas9 enables effective in vivo genome editing and identifies a specific PAM sequence of 5′-NNGRRT-3′, where the R denotes a purine base, such as A or G	(Nishimasu et al. 2015)
Nme Cas9	1082	5082Cas9 al.,	*Neisseria meningitidis*	24 and 20	5′ of PAM	Target DNA	The NmeCas9 system necessitates a longer PAM sequence to achieve site-specific cleavage. However, NmCas9 can utilize an sgRNA to direct its activity	(Lee et al. 2016) (Amrani et al. 2018)
St1Cas9	1121	NNAGAAW	*Streptococcus thermophilus*	20	50reptoco	Target DNA	St1Cas9 and SaCas9 are essential components of bacterial positive selection systems. While St1Cas9 functions as a nuclease in human cells	(Kleinstiver et al. 2015) (Agudelo et al. 2020)

Table 2 (continued)

Class name	Size (bp	PAM	Host	sgRNA size (bp	Cut site	Target	Function	Refs
St3Cas9	1049	5049as9o et	*S. thermophilus*	20	50 thermo	Target DNA	St3Cas9 is a protein with multiple domains and a larger size. Within the St3Cas9 system, the Cas9-crRNA complex is capable of inducing dsDNA breaks in vitro at a specific site that possesses a sequence complementary to the crRNA	(Cong et al. 2013) (Arroyo-Olarte et al. 2021)
CjCas9	984	NNNNACAC and NNN-RYAC	*Campylobacter jejuni*	22	52mpyloba	Target DNA	CjCas9 can only cleave a restricted number of sites in the mouse or human genome, but it has been shown to generate mutations at the target site at a considerably high rate	(Kim et al. 2017) (Koo et al. 2018)
Cpf1	-	TTTV	*prevotella* and *Francisella*	20	50rancise	Target DNA	Cpf1 requires a PAM sequence that is rich in T nucleotides located at the 5′-end of the protospacer sequence	(Safari et al. 2019) (Bin Moon et al. 2018)
cpf1 (AsCpf1)	1307 bp	507 bpAsCp	*Acidaminococcus sp.*	24	34idamino	Target DNA	AsCpf1 identifies both the crRNA scaffold and a 5′-TTTN-3′ PAM in a manner that is dependent on the structure and sequence of the target DNA. AsCpf1 features two domains that are appropriately positioned to produce staggered double-stranded DNA breaks	(Manghwar et al. 2019) (Yamano et al. 2016)

Table 2 (continued)

Class name	Size (bp	PAM	Host	sgRNA size (bp	Cut site	Target	Function	Refs
Cas12a	-	Thymine-rich PAM sequences	*Acidaminococcus sp.*		5cidamino	Target DNA	Cas12a, also referred to as Cpf1, is a CRISPR effector of type V-A that relies on RNA guidance to facilitate DNA endonuclease activity. This enzyme is capable of producing double-stranded DNA cuts with staggered ends downstream and distal to PAM sequences that are rich in T nucleotides	(Jeon et al. 2018) (Paul and Montoya 2020)
Cas14	40,014)y		Uncultivated archaea			Target ssDNA	Cas14 proteins are comprised of roughly 400–700 amino acids and possess the ability to cut single-stranded DNA without the need for a specific, restrictive sequence	(Harrington et al. 2018) (Savage 2019)
Cas13	1440	Non-G nucleotide at the 3tionItempacer flanking site (PFS)	Multiple orthologs; *Leptotrichia shaii*	28	-	Target ssRNA	Cas13, also known as C2c2, serves as an effector protein within the type VI CRISPR system. It functions as an RNA-guided ribonuclease, and its nonspecific, trans-acting RNase activity is triggered by the pairing of the crRNA guide to a single-stranded RNA target through base pairing	(Carabias et al. 2021)
xCas9	–	NG, GAA, and GAT	–	–	–	–	xCas9 is a variant of the Cas9 enzyme that can identify a range of PAM sequences, including NG, GAA, and GAT	(Wang et al. 2019) (Zhang et al. 2020)

Table 2 (continued)

Class name	Size (bp	PAM	Host	sgRNA size (bp	Cut site	Target	Function	Refs
AacC2c1	1277	T-rich PAM	*Alicyclobacillus acidoterrestris*	20	50icyclob	Target DNA	AacC2c1 is a CRISPR/Cas endonuclease belonging to the C2c1-a type V-B category. It possesses a bi-lobed architecture comprising an REC lobe and a NUC lobe	(Zhu and Huang 2019)

RNA. In addition, dCas9 can be combined with numerous epigenetic regulatory factors to carry out epigenetic alterations such as DNA acetylation/methylation, post-translational histone modification, ubiquitination, and protein sumoylation and phosphorylation (Shi et al. 2017; Shrestha et al. 2018). Figure 2 illustrates these potential applications of dCas9 in epigenetic modifications.

CRISPR-CpF1 CRISPR/CpF1, a new class of nucleases, was discovered in 2015. CpF1 has several differences from Cas9. Unlike Cas9, it just needs crRNA to guide the CpF1 complex (Tang et al. 2017). CpF1 may be better at multiplexing than Cas9 since it uses less crRNA. CpF1 possesses a "T"-rich PAM, unlike Cas9. CpF1 cuts 18-–23-bp downstream of the recognition site to create staggered ends (Xu et al. 2017).

CRISPR-Cpf1, also known as CRISPR-Cas12a, was introduced to further diversify genome engineering technologies (Fig. 2). Cpf1 is a CRISPR class II endonuclease that belongs to the family of endonucleases (Alok et al. 2020; Zaidi et al. 2017). Because it was identified as Prevotella and Francisella1, it was given the name Cpf1. This technology became popular because it was able to fill the gaps left by prior genome editing tools and can effectively replace CRISPR-Cas9 (Bin Moon et al. 2018; Kim et al. 2021). It is smaller than Cas9 and requires a shorter CRISPR RNA to function properly (Liu et al. 2017). Unlike CRISPR/Cas9, tracrRNA is no longer required to process mature CRISPR RNAs linked with Cpf1 (Zetsche et al. 2015). Furthermore, unlike CRISPR Cas9, which uses a G-rich PAM sequence at the 3′ end, it requires a T-rich PAM sequence at the 5′ end to cleave efficiently, allowing it to target AT-rich areas in the genome. Furthermore, Cpf1 causes staggered incisions that make insertion of a DNA fragment by HDR simple (Gao et al. 2018). CRIPSR-Cpf1 has a lower rate of off-target binding than CRISPR-Cas9, which could be an advantage (Kim et al. 2017; Kleinstiver et al. 2016). CRISPR-Cpf1 has been used for targeted genome editing in a variety of eukaryotes, including plants, according to a number of publications (Kim et al. 2017; Zetsche et al. 2015). It is also been utilized to target numerous genomic targets (Wang et al. 2020) (Table 1).

Cas variants and other nucleases for efficient plant genome editing

There are three Cas9 variants with differing PAM sequences (Fig. 3): SpCas9-VQR, SpCas9-VRER, and SpCas9-EQR (Kleinstiver et al. 2016). There have also been reports of

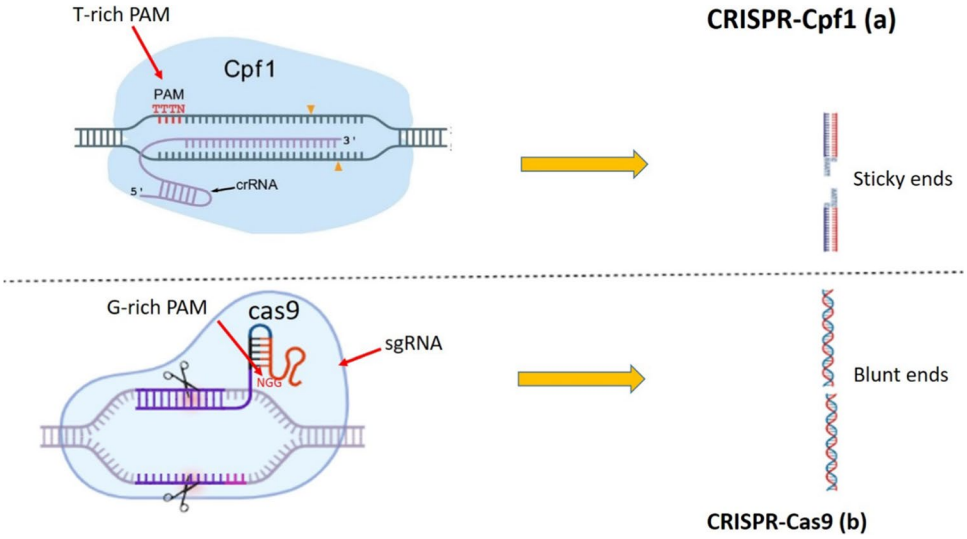

Fig. 2 Comparison of various features of CRISPR-Cpf1 (**a**) and CRISPR-Cas9 (**b**). Source: Adapted from Zaidi et al. (2017) © 2017. Reproduced with the permission of Elsevier

Cas9 orthologs from other species with distinct protospacer adjacent motifs (PAMs) sequences (Cong et al. 2013:, Kim et al. 2017:, Kleinstiver et al. 2015: Xu et al. 2021), including those from Staphylococcus aureus Cas9 (SaCas9), SaCas9-KKH, Streptococcus canis (ScCas9), Streptococcus thermophilus 1 (St1Cas9), and Strepto Additionally, the recently developed SpCas9 variants xCas9 and SpCas9-NG have successfully been tested in mice, rice, and Arabidopsis (Ren et al 2019), and they can identify the non-canonical NGN PAM in human cells (Nishimasu et al. 2015) SpCas9-NG was shown to be more effective in editing at NG PAMs than xCas9 when xCas9 and SpCas9-NG were tested in mammalian cells and plants (Nishimasu et al. 2015: Walton et al. 2020). Moreover, Fig. 4 give us the Schematic representation of the basic steps of plant genome editing. SpRY, a recently created PAM modifier (NRN > NYN) in mammalian cells, was created utilizing a structure-based engineering methodology (Walton et al. 2020) SpRY may or may not function properly in additional model species, though. The potential for precise base replacements and gene knockouts, knockins, and knockins would increase if SpCas9 variants like xCas9, SpCas9-NG, and SpRY could be used in other animals (Table 2).

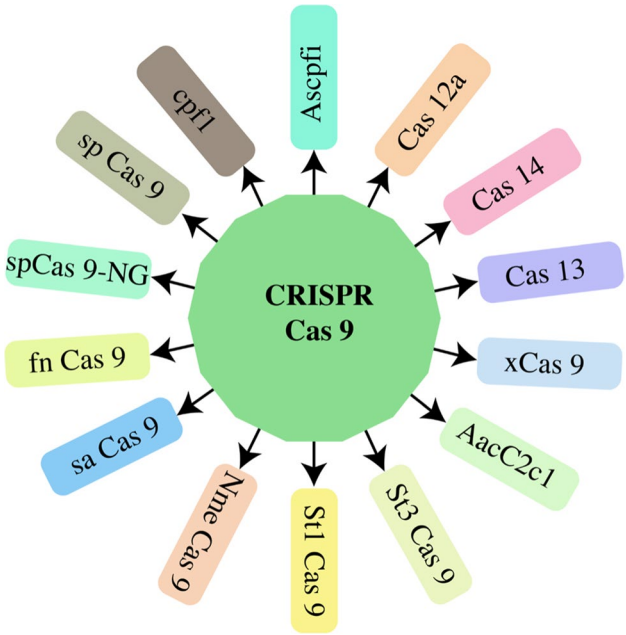

Fig. 3 Different CRISPR/Cas Systems/ classes

Genome editing in plants induces genetic modifications

1) *Targeting double-strand breaks with sequence-specific nucleases*

New techniques in genome engineering, such as the use of sequence-specific nucleases (SSNs) to regulate the site of genomic change, have the potential to hasten breeding processes by altering specific genes in a specific region or introducing new traits in a specific spot. Once thought to be impossible, plants can now have their genomes modified specifically (Paszkowski et al. 1988), SSNs like zinc finger nucleases have made it possible to insert DSBs into DNA at a defined particular place (ZFNs) (Kim et al. 2017) or mega nucleases (Smith J, et al. 2006), the efficiency has skyrocketed (Puchta and Fauser 2013). Both methods have serious drawbacks because of how these SSNs are produced. a major issue is that there are limitations on how precisely the DNA binding site may be designed. Since Boch et al. (2009) and others released the coding for transcription activator-like factors (TALE) (Boch et al. 2009: Moscou and

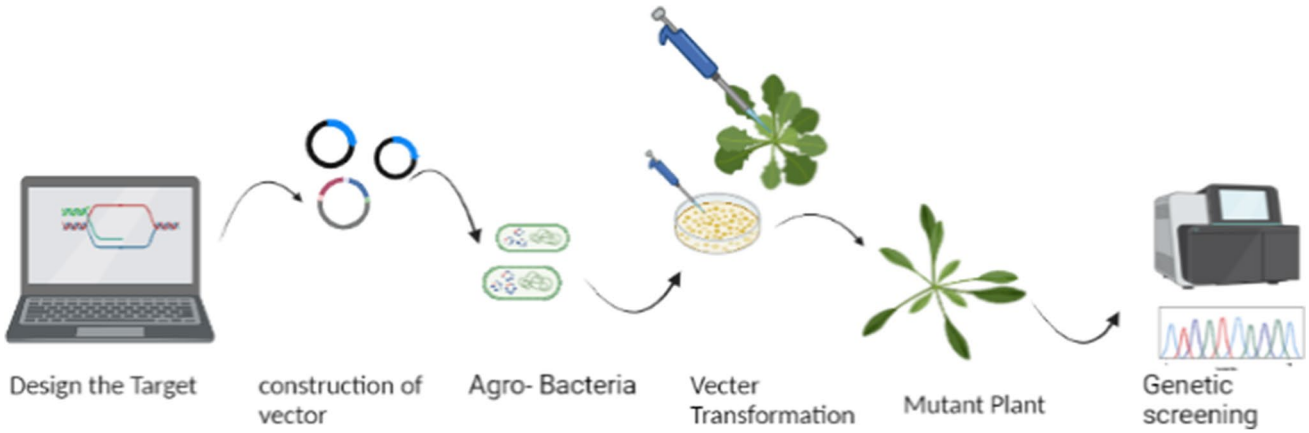

Fig. 4 Schematic representation of the basic steps of plant genome editing

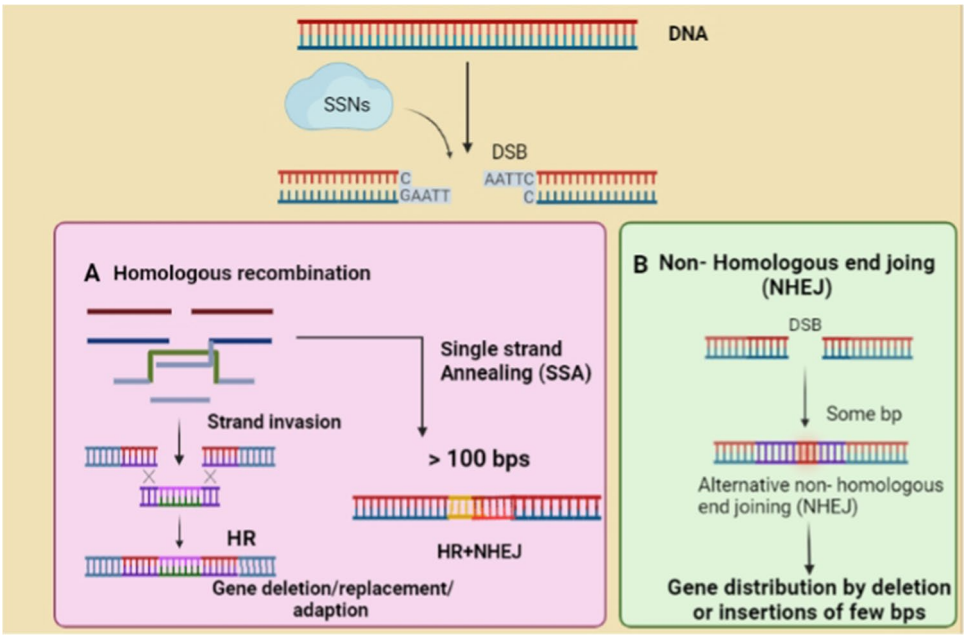

Fig. 5 No homologous end joining (NHEJ) and synthesis-dependent strand annealing (SDSA) products of double-strand-break (DSB) repair. **a** Sequence-specific nuclease introduces a DSB into the target gene. Resection of the DSB in SDSA produces a single-stranded molecule with a 3 end (step 1) that invades the repair template and forms a D-loop (step 2). The sequence alteration to be incorporated into the genome is represented by the blue sections in the repair template, while the grey regions indicate DNA with similarity to the target gene. When the invading strand's 3 ends is lengthened, homology to the second 3 end of the DSB permits the two single strands to anneal and heal the break (steps 3, 4), and products deriving exclusively from homologous recombination (HR) are formed. If the invading strand's three ends cannot identify complementary sequences at the broken target, HR and NHEJ are used to repair the break (steps 5 and 6). The red areas represent insertions or deletions caused by inaccuracy in NHEJ. **b** The break is re-joined by NHEJ after DSB formation, which might introduce insertions or deletions at the break location

Bogdanove 2009), things have altered rapidly. Dan Voytas' lab published the first transcription activator-like effector nucleases (TALENs) in 2010 (Christian et al. 2010), which combined the DNA binding portion of a TALE with an unspecific nuclease. TALENs, like ZFNs, work by fusing a specified DNA binding domain with the nuclease FokI to introduce a DSB at a specific site. The cell's two primary repair processes, non-homologous end joining (NHEJ) and homologous recombination (HR), are activated when a DSB occurs (Fig. 5).

2) *Double-strand-break repair in plants*

When delivered into plant cells, SSN locate and cleave the target DNA, resulting in double-strand breaks (DSBs) that are repaired by natural DNA repair mechanisms like non-homologous end-joining (NHEJ) and homology-directed repair (HDR). NHEJ is the go-to method for fixing DSBs; however, it can introduce indels at the chromosome-joining sites.(Chen et al. 2017; Zhu et al. 2017; Fig. 5). The indels that arise from frameshift mutations are unpredictable in terms of their size and order, and they frequently result in the deletion of entire genes. HDR, on the other hand, is possible if a homologous DNA template is provided. Although HDR-mediated genome editing can result in precise gene replacements, point mutations, DNA insertions and deletions, its effectiveness in plant cells is quite poor (Fig. 5). A tandem homology on both ends of the break is exploited for repair in the comparatively straightforward single strand annealing (SSA) route of homologous recombination (HR), which is separated into the resolution of double holliday junctions (dHJ), generated following strand invasion of the damaged strand. Both a repeating sequence and a DNA fragment from outside the cell can take this form. Gene replacement, addition, or adaptation are frequent results of the pathways (Sprink

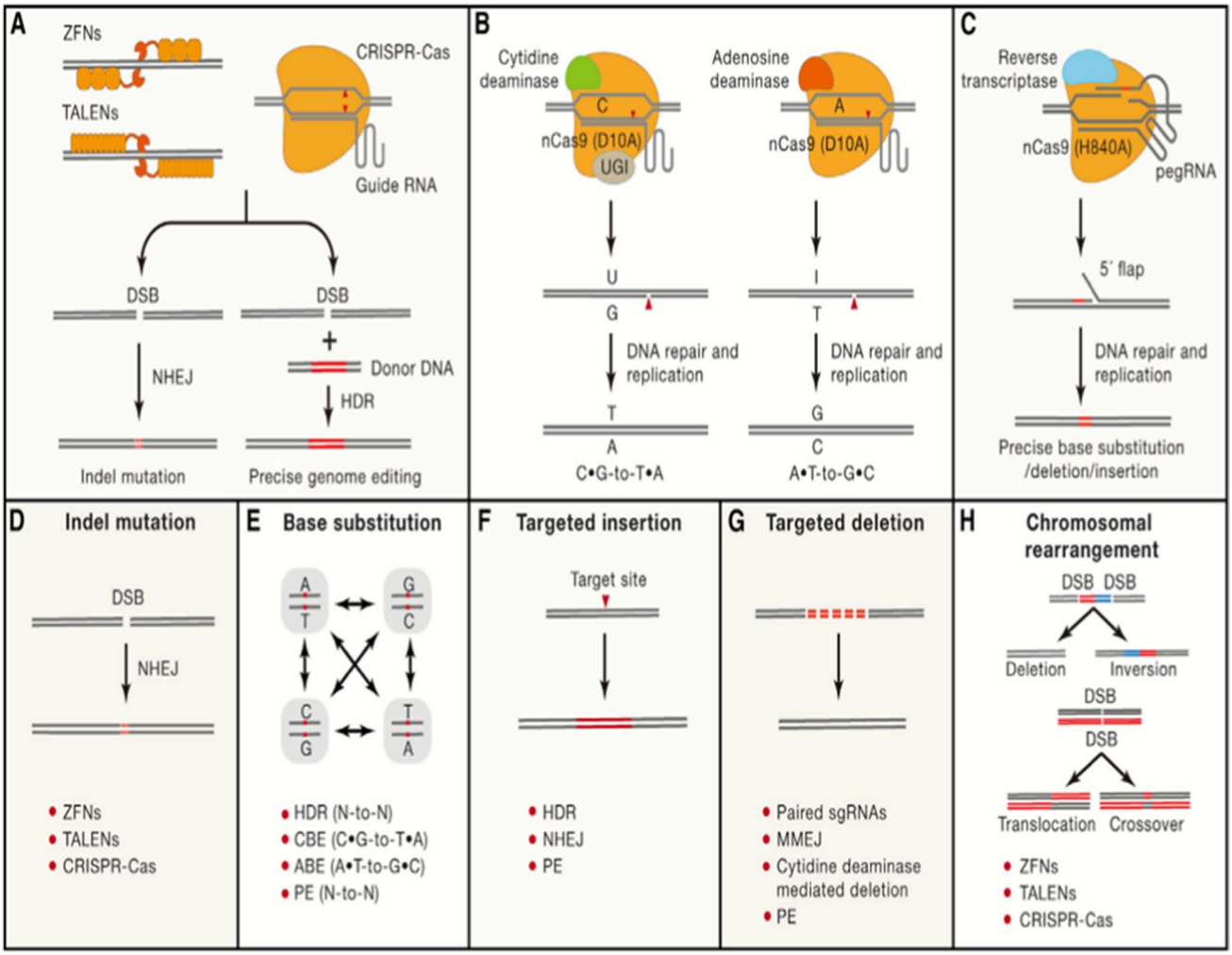

Fig. 6 Plant genome editing results in genetic changes. **A** Schematic representation of the NHEJ and HDR DNA repair processes when sequence-specific nucleases cause DNA double-strand breaks (DSBs) (SSNs). Base editing technology, in (**B**). To create a cytosine base editor (CBE) or an adenine base editor (ABE), respectively, adenosine deaminase or adenosine deaminase is fused with Cas9 nickase (nCas9 (D10A)). A rent chromosomes chromosomal translocations, and inveE, while C-G to T-A base substitutions are produced by the CBE. Inhibitor of uracil DNA glycosylase, or UGI. **C** Top editing software. The reverse transcriptase, prime editing guide RNA, and nCas9 (H840A) combine to form the prime editor (PE) (pegRNA). **D** Small random indels are mutated by ZFNs, TALENs, and the CRISPR-Cas system using the DNA non-homologous end joining (NHEJ) repair pathway. **E** HDR, CBE, ABE, and PE all have the ability to produce base substitutions. **F** HDR, NHEJ, and PE performing targeted insertion editing. **G** Cytidine deaminase-mediated deletion, paired sgRNAs, MMEJ, and PE for targeted deletion editing. **H** Chromosome deletions, inversions, translocations, and crossing are brought about by the simultaneous introduction of pairs of DSBs into chromosomes

et al. 2015) (Fig. 5A). There are two main subfields within the non-homologous end joining (NHEJ) field: the classical (also called canonical) NHEJ, which ligates broken DNA ends together in the absence of homology, and the alternative NHEJ, which ligates broken DNA ends using micro homologies of two nucleotides on the single stranded break ends. In both cases, a gene disruption occurs due to the addition or removal of a few nucleotides (Sprink et al. 2015) (Fig. 5B).

3) *Base editing technology*

Apart from utilizing double-strand break (DSB)-mediated genome editing, the utilization of CRISPR-derived base editors has become increasingly popular for creating targeted single-base modifications in DNA. The two most common types of base editors are cytosine base editors (CBEs) and adenine base editors (ABEs). Deaminases that target single-stranded DNA (ssDNA) and Cas9 (nCas9 D10A), a Cas9 variant with a catalytic impairment, work together to form base editors.. CRISPR-Cas produces R-loops in single-stranded DNA (ssDNA) at their intended sites, and these deaminases catalyze the corresponding C.GTA or ATGC transitions in the ssDNA strand (Gaudelli et al. 2017; Komor et al. 2016; Nishida et al. 2016). Cytidine deaminases such as rAPOBEC1 and PmCDA1 (Fig. 6B, E). Including a uracil DNA glycosylase inhibitor into CBEs improves their base-editing efficacy by modulating the body's natural DNA repair mechanisms (Komor et al. 2016). For plant genomes, CBEs and ABEs have both been optimized (Li et al. 2017a; Zong et al. 2017). Plants have also been successfully treated with other CBEs that utilize the deaminases PmCDA1, hAID, and hAPOBEC3A (Ren et al. 2019; Shimatani et al. 2017; Zong et al. 2017). Plants can make even more extensive use of base editing with the help of dual base editors, which combine the functional domains of CBEs and ABEs to make C.G to T.A and A.T to G.C modifications at the same target site (Li et al. 2017a). Currently available base editors only generate base transitions and cannot create trans versions of DNA bases, or display DNA insertions or deletions (C.G to T.A and A.T to G.C). But, with to a recent technological development known as prime editing, it is now possible to make all 12 types of base substitutions as well as small DNA insertions and deletions in human cells (Anzalone et al. 2019; Fig. 6C).

4) *Prime editing technology*

Prime editors are made up of two parts: a prime editing guide RNA (pegRNA) and a nickase (H840A)-RT fusion protein produced by the Cas9 system. The pegRNA is a sgRNA that has been modified to have three extra bases and a primer binding site (PBS) and an RT template encoding the desired modification (s).

The Cas9 nickase (H840A) finds the target site and nicks the nontarget DNA strand to release ss DNA that mates with the PBS and serves as RT primer. Reverse transcription transfers the pegRNA edit to nontarget DNA.DNA repair integrates the target location after synthesizing the altered DNA flap (Anzalone et al. 2019). Prime editing has been quickly adapted in plant cells, and some cereal crops like rice and maize with prime editing have been successfully regenerated (Zhu et al. 2017). However, the editing efficiency of prime editors is currently much lower than that of base editors at the majority of target sites in plant genomes. Nonetheless, prime editors offer the ability to install various local mutations, including substitutions, insertions, and deletions of dozens of base pairs at targeted DNA sites (Lin et al. 2020). Number of attempts to increase the effectiveness of prime editing by changing the lengths of the PBS and RT template in the pegRNA, using different RTs, processing the pegRNAs with a ribozyme, raising the culture temperature to encourage reverse transcription, using improved promoters for pegRNA expression, and enriching for transformed cells (Xu et al. 2021) have been carried out.

5) *Precise modification using donor template or gene targeting*

Targeted insertion

The precise insertion of DNA, which enables the modification of gene activity, has enabled the stacking of many agricultural features. Plants have a relatively low efficiency for HDR-mediated DNA insertion (Chen et al. 2017; Fig. 6F). As an alternative, the NHEJ pathway can be used to effectively insert DNA into DSB sites if a donor DNA template is made available (Wang et al. 2014). Using the NHEJ pathway to target introns, CRISPR-Cas9 was able to successfully replace and insert genes, as one example of this (Li et al. 2017b; Fig. 6F). Micro homology, or compatible ends, are created by adding short lengths of donor DNA that are homologous to the sequence surrounding the DSB. NHEJ insertions in their intended sites occur more frequently after this operation. Targeted insertion by NHEJ can also be induced by using chemically stabilized double-stranded oligodeoxynucleotide (dsODN) donors with 5′-phosphorylated ends (Lin et al. 2020).

Targeted deletion

Deletion of target DNA is crucial when editing non-coding and regulatory DNA regions. Even minor insertions and deletions (indels) are unlikely to disrupt the function

of these regions. By creating double-stranded breaks (DSBs) using site-specific nucleases (SSNs), it is possible to induce precise deletions at the desired location(Shan et al. 2013; Fig. 6G). For instance, when Cas9 is co-expressed with a pair of sgRNAs, more than 100 kb of DNA might be destroyed between the target sites (Zhou et al. 2018). Targeted deletions can also be generated with a single gRNA by combining Cas9 or Cas12a with T5 exonuclease or by co-expressing an SSN with exonucleases; however, the length of such deletions is a key constraint (Zhang et al. 2020). Because the NHEJ pathway is used for repair, the deleted DNA sequences obtained using these procedures are neither predictable nor exact. Micro homology-mediated end joining (MMEJ), which uses microhomologous microhomologous sequences to align DSB ends before joining (Fig. 6G), can produce precise DNA deletions. This method, however, is limited to producing deletions between pairs of microhomologous sequences. Multinucleated deletions are possible using the recently developed APOBEC-Cas9 fusion-induced deletion systems (AFIDs) (Wang et al. 2020; Fig. 6G). Cas9 generates a DSB at the target DNA regions, and APOBEC converts cytidines on the off-target strand to uridines, which are then removed by uracil DNA glycosylase to provide an abasic (AP) site. The deletion that proceeds from the delaminated cytidine to the DSBs after AP lyase removes the AP site is predictable and exact (Wang et al. 2020).

6) *Chromosome and genome rearrangement*

In addition to modest changes like short insertions/deletions and base substitutions, targeted creation of DSBs employing SSNs also causes massive genome rearrangements including big deletions, chromosomal translocations, and inversions, which can also be brought on by radiation. Large intervening pieces between the digested sites can be deleted by inducing DNA DSBs at two different sites. When SSNs are employed to cause DSBs, It is also possible to do specific chromosomal rearrangements that are helpful for cutting or mending genetic links (Schmidt et al. 2019; Fig. 6H). Deletions and inversions may develop between two DSBs that are simultaneously inserted into the same chromosome (Schmidt et al. 2019; Fig. 6H). Deletions and inversions may develop between two DSBs that are simultaneously inserted into the same chromosome (Schmidt et al. 2019; Shan et al. 2013). According to Schmidt et al. (2019), NHEJ processes, and on occasion MMEJ, are responsible for these rearrangements. Mega base pair (Mbp)-targeted chromosomal inversions have recently been demonstrated to be possible in Arabidopsis thaliana (Schmidt et al. 2019) and maize. Furthermore, the latter showed that this strategy does really make it possible to restore genetic crossings. Crossovers, translocations, and exchanges of sequence information are only some of the inter-chromosomal rearrangements that can occur when two or more DSBs are created on different chromosomes (Schmidt et al. 2019; Fig. 6H). Recently, in the plant *A. thaliana*, reciprocal translocations across heterologous chromosomes were established using CRISPR-Cas9 technology (Beying et al. 2020). It is significant that these translocations were heritable and in the Mbp range. Yet, more effective methods need to be developed to fully tap into the huge potential of targeted chromosomal rearrangements for plant breeding.

7) *Genes targets for genome editing in plant*

Additionally, genome editing has been used in plants to functionally annotate genes that have already been identified and connected to a range of significant activities. As mentioned in the preceding sections, certain genes, such as those linked to stress, desired marker genes, and genes involved in plant architecture, have been targeted.

Target genes for the enhancement of resistance against biotic stresses

Physical and biological stresses cause annual severe yield losses. Engineering crop plants to resist stress is an essential undertaking for dependable and long-lasting yield development. Endophytic fungi, bacteria, nematodes, plant parasites, harmful insects, and plant virus infection are all examples of biotic stressors that plants face. Plant viruses by themselves can reduce output by 10 to 15% globally (van Regenmortel and Mahy 2009). In addition to demonstrating that broad-spectrum Gemini virus resistance can be conferred to *N. benthamiana* plants by targeting the correlated mononucleotide sequence (TYLCV, CLCuKoV, TYLCSV, BCTV-Worland, MeMV, and BCTV-Logan), Ali et al. (2016) demonstrated that the capacity of Gemini viruses to avoid CRISPR/Cas9 reagents is a critical factor to consider, as evidenced by the development of immediate resistance against a number of begomoviruses by targeting correlated non-nucleotide sequences. The ongoing battle between plant hosts and invading viruses can increase the likelihood of such events. Reports show that the CRISPR/Cas9 system is more effective against viruses when non-coding intergenic sequences are targeted, while targeting coding sequences produces virus variants that are able to circumvent the system (Ali, Z. et al. 2016). By targeting the coat protein (CP) or replicas (Rep), Tashkandi et al. (2018) resulted in tomato plants that are resistant to the begomovirus TYLCV, with the most prevalent mutation found in Cas9 targeted sites being a single nucleotide

change. Targeting RNA viruses with CRISPR/Cas9 tools has been challenging because sgRNA-default Cas9's primary target is DNA. Cas9 can be programmed to target RNA, and the type III-B and Type VI-A CRISPR/Cas systems from Leptotrichia shahii (LshCas13a) and Leptotrichia wadei (LwaCas13a) cause cleavage of RNA sequences corresponding to the sgRNA. By reprogramming sgRNA specific for the RNA genome of cucumber mosaic virus (CMV) or tobacco mosaic virus (TMV), Zhang et al. (2018) recently shown the aforementioned strategy could be successfully used (TMV). Using Francisella novicida Cas9 (FnCas9), they were able to produce plants like *Nicotiana benthamiana* and Arabidopsis that had considerably lower viral titers (Zhang et al. 2018). One possible solution to this problem is to develop plants that are resistant to viruses by targeting specific plant genes that are involved in infection rather than the viral RNA. For instance, the eIF4E and eIF(iso)4E genes have been identified as playing essential roles in the infection of Turnip Mosaic Virus (TuMV) in Arabidopsis, among other examples.Using the CRISPR/Cas9 system, this method has been successfully employed in making Arabidopsis plants resistant to the potyvirus TuMV). Two potyviruses, Papaya ringspot mosaic virus-W and Zucchini yellow mosaic virus, as well as an ipomovirus, Cucumber vein yellowing virus, have been developed into fully resistant cucumber (*Cucumis sativus* L.) plants (Chandrasekaran et al. 2016). A number of natural foundations of Potyvirus resistance are brought on by loss-of-function mutations in host initiation factors (Sanfaçon 2015), which lead to broad-spectrum resistance. This makes disrupting eIF4E and eIF(iso)4E-like host factors advantageous. Any host gene that encodes a virus requirement for successful infection spread could be a target, even though translation initiation factors are the top candidates for change in the host genome (Sanfaçon 2015). The system's potential significance for controlling viral infections in crops is highlighted by CRISPR/capacity Cas9's to produce virus resistance. Gene editing has also increased plant tolerance to bacterial and fungal diseases. Numerous crop species have been the subject of intensive research into powdery mildew resistance. Powdery mildew can be effectively controlled by fungicides, but due to the quick evolution of fungal strains resistant to them, the increased costs to growers, and the damaging effects of fungicides on the environment, it is necessary to discover alternate remedies. The most widely used method for breeding resistant cultivars is to target susceptibility genes (S genes), which reduce plants' resistance to powdery mildew (Wang et al. 2014). This implies that broad-spectrum resistance to powdery mildew would result from failed mutations in the MLO alleles. Powdery mildew is a symptom of Blumeria graminis f. sp. tritici (Bgt), one of the most detrimental plant diseases to wheat productivity. In hexaploid wheat, Wang et al. (2014) successfully knocked out all MLO gene homologs using a gene editing approach, conferring long-lasting Bgt resistance on wheat plants. Targeting MLO genes, a different study used CRISPR/Cas9 editing to develop "tomelo" tomato plants that were resistant to powdery mildew. The Erysiphe necator infection, a fungus pathogen that causes powdery mildew, has also been targeted for suppression using MLO7, a susceptibility (S) gene in grapes (Pessina et al. 2016). The CRISPR/Cas9 tools were delivered directly to the Chardonnay grape cultivar protoplasts via ribonucleoproteins (RNPs). Using a similar technique, apple trees resistant to the enterobacterial phytopathogen Erwinia amylovora, which causes fire blight, were created in the same study. For this reason, the genome editing targets for the DIPM-1, DIPM-2, and DIPM-4 genes were chosen (Malnoy et al. 2016). The CRISPR/Cas9 genome editing method has been utilized to establish resistance to blast disease in japonica rice by using sgRNAs to target codons close to the OsERF922 translation initiation codon and produce indels (Wang et al. 2016). This suggests that altering OsERF922 can produce plants with increased resistance without impairing plant development. The researchers also evaluated the mutant lines for several agronomic characteristics such as flag leaf thickness, plant height, panicle length, number of panicles, seed weight, and seed setting rate. However, no significant differences were found in any of these traits compared to the wild-type plants. The use of genome editing techniques in the fight against severe plant diseases appears to be very promising.

Target genes for the enhancement of abiotic stress tolerance in plants

Abiotic stressors are the main factors limiting agricultural productivity, and they will continue to have a detrimental impact as the world heats. Research is challenging because environmental factors have a significant impact on environmental stressors tolerance and are controlled by a range of genes (Ullah et al. 2023; Yijun et al. 2022; Zada et al. 2022). While conventional breeding methods and transgenic systems have both helped generate resilient crop varieties (Ali et al. 2022a, 2022b, 2022c), the complex inheritance of abiotic stress-related traits with increased environmental influences makes the generation of novel cultivars through these methods extremely difficult. On the other hand, induced mutagenesis, which is entirely dependent on chance, is a well-studied method for enhancing the genetics of a range of crop species (Kumawat et al. 2019). The CRISPR/Cas9 system can be used in the forward genetics strategy of changing genes and gene expression to learn more about the genetics of

abiotic stress response and aid in the production of stress-resistant crop varieties.. Despite its widespread use in the field of plant science, very little has been published on the topic of how the CRISPR/Cas9 technique has been put to use in the pursuit of more resilient plants to abiotic stress. Shi et al. (2017) developed a CRISPR/Cas-based method for altering the genetic makeup of corn to boost its resilience and increase its yields in the face of drought.. The protein ARGOS8, which prevents ethylene reactions, was the subject of the investigation. Genome-edited plants with increased ARGOS8 expression were more tolerant to drought (Shi et al. 2017). Another study created truncated gRNAs (tru-gRNAs) using a tissue-specific AtEF1 promoter and Cas9, which led to alterations in inorganic stress-responsive genes including OST2/AHA1, which resulted in enhanced stomatal responses in Arabidopsis. Rice OsRR22 and OsNAC041 have both been identified as salt tolerance targets (Zhang et al. 2019). A recent study found that multiplex genome editing with Acidaminococcus Cas12a (Cpf1) was successful in targeting 25 distinct genomic locations (Paul and Montoya 2020). The strategy outlined above can be helpful for simultaneously treating many genes linked to abiotic stress. With genome editing allowing for the manipulation of solute transport regulators, notably for freshwater, urea, H_2O_2, and silicon, aquaporins are among the most attractive options for abiotic stress improvement (Haq et al. 2022; Atta et al. 2022). These findings suggest that the CRISPR/Cas system can be successfully used for this innovative purpose, and that future work will focus on targeting minor genes of complicated quantitative characteristics connected to abiotic challenges.

Translational and post translational efforts by targeting genes previously annotated with RNAi

Currently, a number of genes that were previously identified using RNAi are being subjected to genome editing to introduce knockout mutations For example, CRISPR/Cas9, has been used to delete ROC gene 5 in rice, which was previously identified through RNAi technology (Feng et al. 2014), Tomatoes with a self-pruning 5G (SP5G) gene, and Mildew Locus O (MLO-7) in grapevine (Pessina et al. 2016). In order to avoid the severe and expensive limits imposed on the commercial production of transgenic cultivars generated using RNAi technology, targeting previously identified genes is crucial for two reasons: first, CRISPR/Cas is still in its infancy and need further research. The US government has already designated crops improved through genome editing that do not contain any foreign DNA as non-transgenic, and it is anticipated that other nations would do the same. Thus, the CRISPR/Cas technology is preferred over RNAi for gene editing in order to release improved crop varieties in an orderly, cost-effective, lucrative, and commercially straightforward manner. The ability to simultaneously target multiple genes with CRISPR/Cas is another capability. CRISPR/Cas multi-targeting constructs contain genes previously identified by RNAi, such as pectin lyase (PL), which is associated with tomato fruit softening. For the comparative analysis of tomato cell wall mutants, PL and two additional pectin-degrading enzymes, polygalacturonase 2a (PG2a) and -galactanase (TBG4), were addressed (Wang et al. 2019). In numerous experimental settings, analyzing mutants is difficult and murky. By simultaneously targeting several genes, CRISPR/Cas gives a solution to this problem. Multiplexed gene targeting is explained in further detail later in this paper.

Genome engineering for enhancing phytoremediation of hazardous metals (loid)

Producing plants for phytoremediation of polluted soils and rivers can be done successfully using engineering. The genes that improve plants' ability to mobilize, stabilize, and/or accumulate metals are advantageous for these plants. Numerous genes have already been found that promote metal tolerance, metal uptake, and hyper accumulation. Most often, transgenic plants with improved phytoremediation abilities are created by introducing a gene that is not native to the plant (Gunarathne and Lee 2019). Oligonucleotide donor sequences can be modified by precise nucleotide exchanges, which may be used to improve plants' resilience to temperature stress. Inserting long sequences via NHEJ or homologous recombination can enable the incorporation of transgenes into specific sites that facilitate high-level transcription without interfering with the functionality of endogenous genes.(Wang et al. 2014). By utilizing this method, it is possible to generate designer plants with hyper-accumulating properties that can remove heavy metals from contaminated soil in diverse environmental conditions(Sanz-Fernandez' et al. 2017; Fig. 7).

Numerous metallicolous plants have been entirely or partially sequenced to date, including Noccaea caerulescens and Arabidopsis juncera. Certain energy crops have also had their genomes examined and modified to increase their resistance to pollution (Estrela and Cate 2016). To better pinpoint the genes responsible for phytoextraction, phytostabilization, phytovolatilization, and phytodegradation of heavy metals, it may be useful to modify the genomic sequences of these plants. Using CRISPR, the desired gene set can be introduced into the plant's DNA

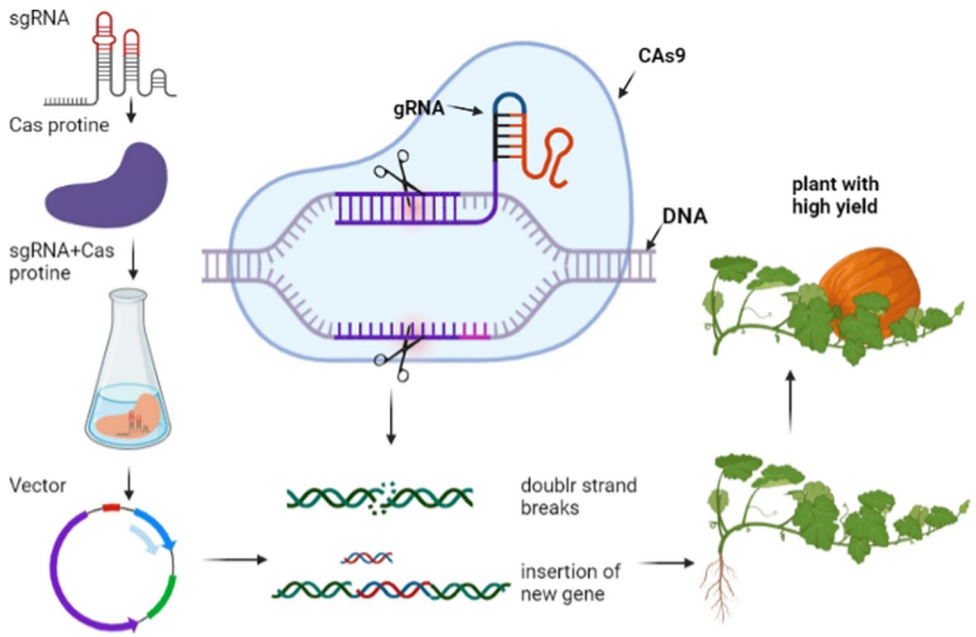

Fig. 7 CRISPR/Cas9 gene editing tool for the purpose of double strand cleavage, sgRNA directs the Cas9 nuclease to the target DNA location upstream of the PAM (protospacer adjacent motif) region. The HNH (His-Asn-His) and RuvC domains of Cas9 nuclease mediate the cleavage function. There are two ways to fix double strand breaks (DSBs) in DNA: HDR (HOMOLOGY-DIRECTED REPAIR) and NJEJ (non-homologous end joining). Genes are knocked in by the HDR and knocked out by the NHEJ. This gene-editing method can be employed to create hyperaccumulators, where the levels of metal transport protein, phytohormones, plant siderophores, and chemotaxis in plant-associated bacteria are enhanced

without the need for a vector. Contrasting with ZFNs and TALENs, this method uses high-throughput gene editing (Jinek et al. 2012; Mali et al. 2013). The availability of sequence data for both monocot and dicot plant genomes, along with computational tools, bioinformatics-based approaches, and the development of codon-optimized versions of Cas9, has opened up new possibilities for the application of CRISPR-Cas9 genome editing in metallicolous plants (Lowder et al. 2018). Increased production of plant growth hormones like auxin, cytokinin, and gibberellic acid, as well as root exudates like metallothionines and phytochelatins, may be the result of CRISPR-mediated gene expression in plants. Several studies dating back to the early 2000s have used CRISPR-Cas9 gene-editing technology to isolate plant and bacterial genes that, when introduced to target plants, exhibited beneficial metal remediation effects.. Arabidopsis and tobacco plants with an active NAS1 gene had increased Mn and Ni absorption and showed greater tolerance to Cu, Cd, Ni, Mn, Fe, Mn, and Zn (Kim et al. 2017). When the genes for metallothionine (MTA1, MT1, and MT2) were overexpressed in tobacco and Arabidopsis plants, the plants were better able to take in Zn, Cd, Cu, and Cd. Many studies have revealed genes in plants and bacteria that function to detoxify and break down both organic and inorganic pollutants (Murtaza et al. 2022, 2023; Sarma and Prasad 2018).

8) *Editing polyploidy genomes—challenges and perspective*

It is challenging and time-consuming to incorporate valuable traits into important crop types using standard breeding techniques, especially in complex polyploid genomes like those of wheat, sugarcane, cotton, and potato (Ali and Mahmood 2015; Ali et al. 2015). Standard breeding practices in polyploid plants make it extremely difficult to introduce a number of desirable traits and change metabolic pathways. On the other hand, genome editing techniques provide important advantages over conventional breeding techniques, such as the capacity to simultaneously target numerous genomic regions or metabolic pathways without leaving a linkage trail. Previously, CRISPR/Cas9 technology has been employed to induce mutations in Arabidospsis (Ali et al. 2022a, 2022b, 2022c) and cotton (Li et al. 2017a, b). This tool has demonstrated high efficacy and specificity in generating DNA-level mutations within the complex allotetraploid cotton genome (Li et al. 2017a, b).

Duncan grapefruit and potato) as well as to develop broad-spectrum resistance to powdery mildew in wheat (Wang et al. 2018). The large genome size and high copy number in polyploid crops make genome editing extremely challenging, especially for site-directed mutagenesis. A polyploid genome makes it challenging to knock out several genes with high homology, but it is possible by establishing

a sequence of allelic variants and segregating them in the following generation to select a desired genotype. Since sugarcane is a polyploid plant, it is difficult to test gene editing methods successfully. Continuous manipulation of all homologs is challenging due to sugarcane's large (10 GB) genome and a range of 8ange of 8ion to select a gene. The number of chromosomes varies even between sugarcane species belonging to the same genus, and the species are interfertile (Jardim-Messeder et al. 2021). In sugarcane, transgene silencing is primarily post-transcriptional in T0 plants and extremely pervasive in primary transformants and is regulated by the plant's growth stage, which is a crucial problem that prevents the practical application of CRISPR-Cas9. Designing sgRNAs that target sugarcane genes is difficult because the sugarcane genome is not well-organized. A key limitation of polyploids is that several mutants are required to examine the various allele types present in polyploids. In crops with annotated genomes, like cotton and wheat, multiple sgRNAs can induce mutagenesis, but this process appears to be more challenging in sugarcane (Jardim-Messeder et al. 2021).

9) *Multi-targeting genome editing approaches*

The CRISPR/Cas9 system's versatility and capacity to simultaneously target multiple genes or sites within a gene make it an attractive tool for both fundamental and applied biological research, allowing for the creation of small or large deletions with numerous potential applications. The expression of several gRNAs is a common practice. One method involves the use of individual promoters for the expression of each gRNA, while another method involves the expression of several gRNAs on a single transcript that is then processed or cleaved to liberate the individual gRNAs (Minkenberg et al. 2017). Effective strategies for CRISPR/Cas9-enabled multiplex genome editing are becoming more available, and they range from similar to (i) altering introns for small interfering RNA expression (ii) T-RNA-mediated multi-targeting genome editing (iii), and Csy4 nuclease-mediated multi-targeting genome editing Drosha-based multi-target genome editing uses a single polymerase II promoter to regulate the production of miRNA (or shRNA)-sgRNA genes that are placed in tandem, one after the other. Pol III promoters are commonly used to generate sgRNAs since they lack distinguishing features such the 5′ cap, 3′ tail, or introns; nonetheless, these promoters are inefficient due to their short length and short life.. Since the 5 cap structure allows for tissue-specific expression and versatility, sgRNAs transcribed by Polymerase II are favored. They do, however, have redundant nuclease activity. A miRNA-based approach can be used to address this issue. It entails producing mature gRNAs and miRNAs using the microprocessor protein complex, which is made up of the RNase III enzyme Drosha and its cofactor, DGCR8 or Pasha (Xie et al. 2018). Despite the fact that it is a very reliable procedure, less plant professionals like it.

10) *Precision genome editing approaches-challenges and perspective*

Due to its error-prone nature, NHEJ, the major repair pathway for DSBs, generally results in the loss of gene function.. Contrarily, HR results in sequence alterations or substitutions (Rouet et al. 1994: Puchta and Fauser 2014). Gain-of-function point mutations, which can theoretically be produced by HR, are helpful for genetic research and crucial for understanding how genes work. Numerous significant agronomic features, such as herbicide resistance conferred by the acetolactate synthase (ALS) gene, are the result of point mutations in the coding areas of genes (Voytas and Gao 2014). CRISPR-Cas9 gene replacement has been demonstrated in rice and maize; although there are still concerns (Svitashev et al. 2016), the extremely poor effectiveness of HR precludes its wider application in plant cells. It is also necessary to provide plant cells with a DNA repair template for HR to take place, and the amount of template sent to a cell can have a major impact on HR's efficacy. CRISPR-Cas9 reagents and a DNA repair template were given by DNA replicons, leading to a greater than tenfold increase in GT frequencies in tobacco, tomatoes, potatoes, hexaploid wheat, and rice (deconstructed geminiviruses) (Butler and Tector 2017). Plants that have had their genomes modified by DNA replicons may be free of GT agents since geminiviruses do not integrate into their host plants' genomes. In addition, because DNA replicons employed in them have a wide range of hosts, many additional plants, including some significant crops, can be accurately edited using this method. Utilizing NHEJ can lead to gene substitution in plant cells. An effective intron-mediated site-specific gene substitution technique employing CRISPR-Cas9 is one recent example (Li et al. 2017b). Using this method, we were able to introduce a pair of sgRNAs targeting adjacent introns of an exon, together with a donor DNA template that had the sgRNA target sequence split between its two ends, resulting in a 2% frequency of exon substitutions in the regenerated plants. Introns are tolerant of modest alterations as long as the splicing sites are unaltered, therefore even though indels are regularly created in introns at the junctions between the donor template and the endogenous gene, the final spliced gene product is usually acceptable. Additionally, the site-specific gene replacements reliably passed down through inheritance and conferred the anticipated phenotypes (Li et al. 2017b). Consequently, this method may be a good substitute for producing gene replacements in genes that contain introns.

CRISPR-Cas9-based base editing is a novel method for altering single DNA bases at genomic target sites in animal,

yeast, and bacterial cells without generating double-strand breaks (DSBs) or requiring the addition of a donor DNA template (Komor et al. 2016:, Nishida et al. 2016; Yang et al. 2014). The most efficient form of the base editor employs a Cas9 nickase (Cas9-D10A) fused to a cytidine deaminase that can convert C to T (or G to A) and a uracil glycosylase inhibitor (UGI) to block base excision repair of the base change (Komor et al. 2016:, Nishida et al. 2016). Despite their limitations, modern base editors provide a game-changing approach of accurately altering individual nucleotides inside a genome by switching them from C to T (or G to A). Site-specific C to T conversions are available from a number of plant species thanks to base editors that feature codon optimization for plants (Lu and Zhu 2017: Zong et al. 2017: Ren et al. 2019: Shimatani et al. 2017). It seems that plant cells may have a little wider deamination window for base editing than animal cells (Zong et al. 2017). Furthermore, hardly any indel mutations were discovered in the altered plants (Zong et al. 2017), indicating that this method is quite specialized to plants.

To enhance characteristics and study gene function in plants, base editing offers a potent technique for producing point mutations. Currently, deaminase converts not only the target C but also any other Cs inside the deamination window; however, advances in the field may one day allow for the deamination window to be as small as a single base pair (Zong et al. 2017). In fact, cytidine deaminase's window has recently been modified to shrink from 5 to 1–2 nucleotides (Kim et al. 2017). Cas9 variants with a variety of PAM requirements have been employed to increase the range of base editing (Shimatani et al. 2017). To overcome the constraints imposed by certain PAM sequences, it could be feasible to use various cytidine deaminases in conjunction with diverse Cas9 orthologs or Cpf1.. Using methods such as phage-assisted continuous evolution, we have been able to alter the fusion enzyme (Carlson et al. 2012), Therefore, it is not out of the question that one day we will have cutting-edge technologies that can modify those three extra bases.. The aforementioned progresses should greatly expand the range of point mutations, eventually allowing the targeting of any single nucleotide in the genome.

Off-target effect and strategies to avoid off-targeting

The Cas9 nuclease's ability to cleave DNA presents a significant obstacle to the use of the CRISPR system. Using computational, crystallographic, and single-molecule methods, the off-target effect of Cas9 has been investigated (Klein et al. 2018; Chen et al. 2017; Singh et al. 2016). It is known that PAM recognition triggers DNA denaturation at the PAM proximal position, enabling the attachment of the target site and the crRNA to arrange the R-loop. The ability of Cas9 to cleave targets with a small number of mismatches is what causes the off-target impact (Herai 2019; Newton et al. 2019). Some methods have been developed to anticipate off-target binds based on the permitted and accepted level of mismatches for DNA cleavage. For instance, a highly specific sequence free of mismatches is necessary for DNA cleavage in at least 7–9 PAM-proximal bases; in contrast, Cas9 binding but not cleavage is acceptable with 4 bases mismatched in pam-distal position (Singh et al. 2016; Dagdas et al. 2017). To lessen the off-target effect, a few strategies have been put forth. In general, on-target binding is stabilized whereas off-target stability is destabilized. One experimental method takes use of Cas9's short half-life by using self-regulatory mechanisms that lower expression in transcription and translation or an inducible Cas9 gene (Davis et al. 2015; Shen et al. 2019). The gRNA backbone can also be chemically altered as an alternative strategy. Partially replacing RNA nucleotides with their DNA counterparts, or including 2′-O-methyl-3′-phosphonoacetate at specific gRNA sites, has been shown to significantly decrease off-target cleavage (Yin et al. 2018; Ryan et al. 2018). Another technique for decreasing off-target binds and interactions is to limit the concentration of effector complex components, such as Cas9 and gRNR (Tsai et al. 2015; Li et al. 2019a, b). One experimental approach to accomplishing this is to directly introduce Cas9 protein and gRNA to mammalian cells along with a premade RNP complex and in vitro premixed Cas9 protein. RNP is a short-lived, degradable compound that has been shown to aid researchers in achieving greater specificity (Kim et al. 2014). However, it is yet unknown whether bacteria are capable of electroporation and delivery of active RNP complexes (Peters et al. 2015). Another method relies on the use of two separate Cas9 nickases, each of which can cut one of the DNA strands in order to create two checkpoints for DNA cleavage. Due to the rarity of two contiguous off-target sites in the entire genome, this method significantly reduces the off-target effect (Cho et al. 2018). Another method of improving the likelihood of on-target binding is by Cas9 protein modification. It is for this reason that other variants of Cas9 have been developed, such as SpCas9-HF1 (high-fidelity SpCas9) (Kleinstiver et al. 2016), eSpCas9 (enhanced-specificity SpCas9) (Slaymaker et al. 2016), and HypaCas9 (high-fidelity SpCas9 with improved homing ability) (hyper-accurate SpCas9) (Chen et al. 2017). The SpCas9-HF1 is designed to decrease off-target DNA contacts without affecting on-target activity. The novel protein rendered almost all off-target sequences undetectable when sgRNA was targeted to non-repetitive target sequences. Much less off-target activity was observed, even for repetitive target sites, when compared to wild-type SpCas9 (Kleinstiver et al. 2016). This eSpCas9 variant was evaluated using an independent whole-genome off-target assay, which revealed an increase in selectivity as well as significant on-target activity (Slaymaker et al. 2016). HypaCas9 displays genome-wide

specificity that is comparable to or even higher than that of SpCas9-HF1 and eSpCas9 (Chen et al. 2017).

Tools available for designing sgRNA and detection of off-target sites

Reduced Cas9 off-target effects and confirmation that the chosen target site is located inside the gene's coding region depend on the identification of acceptable target sites. There are various bioinformatics tools available that make it easier to choose appropriate target locations and create sgRNA in accordance, fully eliminating the need for the aforementioned off-target effects reduction techniques. GC content on the target site and restriction sites inside the selected target are two additional indicators that can be tracked. Some tools include CCTop (Stemmer et al. 2015), Cas-OFFinder (Bae et al. 2014), Breaking-Cas (Oliveros et al. 2016), ZiFiT (Sander et al. 2007), CRISPR Direct (Naito et al. 2015), E-CRISP website (Heigwer et al. 2014), CRISPRSeek (00), fly CRISPR Optimal Target Finder (Zhu et al. 2017), CHOPCHOP, CRISPR-Multitarget (Montague et al. 2014), sgRNAcas9 (Prykhozhij et al. 2015), CRISPR-P (Xie et al. 2014), SSFinder (Liu et al. 2017), and GT-Scan (Upadhyay and Sharma 2014).

Germline-specific gene expression

Although both rice and Arabidopsis were employed in many early CRISPR/Cas studies, rice was significantly more successful at passing on mutations to succeeding generations than was Arabidopsis. First transgenic rice generations bred in experiments with CRISPR/Cas knocking out Pds retained the albino phenotype (Shan et al. 2013). Most mutations in Arabidopsis occurred in somatic cells, and while 1-bp deletions and chimeric mutations were common, they required multiple generations of breeding to produce homozygous mutants (Feng et al. 2014).

This is most likely connected to the fact that different cell types have different capacity for DSB repair. Researchers used cell or tissue-specific promoters, including germline-specific gene expression, to enhance the production of gRNA and Cas nuclease. The sample of these promoters is shown in Table 2. One of the problems with germline-specific promoters is that no expression can be seen in vegetative tissues, making it impossible to determine whether sgRNA is being produced correctly at an early stage. If T0 plants' intended sites do not change, T2 or later generations may be needed. Yet, in refractory species, equivalent promoters can enhance gene editing frequency, and there is lot of space for technical development.

Challenges for efficient plant transformation

Despite its effectiveness, CRISPR/Cas has yet to be widely used in crop enhancement and translational research (Agarwal et al. 2020; Wang et al. 2020). One of the challenges that must be overcome is the effective delivery of transformation vectors into the appropriate host cells, as well as the subsequent successful regeneration of plants. Plant transformation encompasses two distinct phases: transitory transformation and permanent transformation. Edited plants with heritable mutations are created through stable transformation, from which it is possible to extract the nuclease-incorporated transgene to create plants without it. The two most common transformation techniques are *Agrobacterium*-mediated and biolistic transformation, but they are ineffective for many crops because of the following problems: (1) decreased proportions of plants with stable transformations; (2) prolonged periods of tissue culture (3) injury to tissues brought on by biomorphic metamorphosis; (4) the restriction of *Agrobacterium*-mediated transformation to a small number of genotypes in a species; (5) browning and necrosis of tissues brought on by Agrobacterium; (6) induction of somatic mutations; (7) challenges in using *Agrobacterium* to transform monocot species; and (8) the requirement for Agrobacterium-mediated transformation to transfer little quantities of DNA for effective HDR. Additionally, it is not always possible to regenerate or transform cells, thus careful Tissue and cell culture conditions must be optimized with appropriate growth regulators (Altpeter et al. 2016). More effective, user-friendly, labor-saving, and simplified transformation solutions are therefore needed. We will look at some of the more potent transformation strategies in the section that follows.

Future prospects

CRISPR/Cas genome editing has come to be seen as a game-changer in recent years because of its enormous potential to generate desired genome modifications and for a variety of diagnostic applications. In recent years, CRISPR/Cas genome editing has emerged as a game-changer due to its enormous potential to produce desired genome modifications and for a variety of diagnostic applications. CRISPR/Cas is a powerful genome editing method, and its use has increased as bioinformatics tools linked with it have advanced. It is necessary to forget how difficult it is to educate the public about the CRISPR/Cas mediated technology of crop modification because doing so is crucial to ensuring agriculture's long-term existence. This highlights the need for guidelines that distinguish between gene-edited plants containing foreign DNA and those that do not, exempting the latter from regulation and facilitating their easy application. Genome-edited plants with mutations identical to those found in natural variation will be released commercially with less

restriction than transgene-free plants with unique mutations, especially in nations with unfavorable regulations.

Effective genome editing methods seem to hold promise in this regard. The distance between the lab and the field is constantly widening as a result of the absence of clear regulatory policies. As gene editing can result in unintentional alterations to the genome, and since it can be difficult to tell the difference between the effects of "conventional" alterations and those of genome editing, more control of the technique is unnecessary. Another viewpoint holds that in order to identify the possible danger, the legislator should pay attention to the distinctive features of the final product rather than the manufacturing process. As this technology is applied to the study of human reproduction and genetics, it must take into account appropriate risk assessment and management, regulation of the gene drive issue, and necessary safety measures. Aside from these difficulties, significant improvements to transformation procedures are required because they have been found to be a barrier to effectively examining new developments and are not yet optimized for different crop species.

CRISPR/Cas genome editing is currently exclusively employed in highly specialized molecular genetics laboratories that are primarily concerned with fundamental biological challenges. On the other hand, crop breeders still have a ways to go before adopting new technological developments in crop development programs. Pay-per-use public and private facilities that provide service for construct manufacturing, transformation, and evaluation of genome-edited plants will revolutionize the transfer of laboratory discoveries to the field by supplying crop breeders with the most effective genome editing tools. Understanding the gaps in knowledge and the difficulties in adapting technology to new situations will be facilitated by the data presented here, both of which are essential to the effective implementation of genome editing techniques in agricultural research and development.

Conclusion

Genome-editing techniques can accurately alter any live organism's genome. Insertions, deletions, and replacements of specific DNA regions from the genome are possible. Scientific research has always targeted these changes to human needs. A wide range of prospects for plant breeding are made possible by the development of genome editing technology in plants. Genome editing's effective, focused, and targeted mutagenesis has created the groundwork for a number of next-generation breeding techniques that will transform agriculture in the future. Genome editing allows smart crop development. These fast and reliable plant breeding techniques give outcomes comparable to traditional breeding. Most popular and effective are ZFNs, TALENs, and CRISPR-Cas9. Discovering the CRISPR/Cas9 system, an RNA-guided and simple technology, was a major step forward in the field of genome editing. This technology is fascinating since it is the simplest, cheapest, and most efficient way to alter genes at the moment. Moreover, The recent addition of CRISPR-Cpf1, a variant of the CRISPR-Cas system, has greatly broadened the applicability of genome editing tools by resolving several of its shortcomings. In order to assure the general use of genome editing in agriculture, it must be integrated with other technologies such as high-throughput phenotyping, genomic selection, and speed breeding. However, next-generation breeding based on genome editing is not likely to totally replace traditional methods, though. This interdisciplinary approach will transform plant breeding, allowing us to sustain a second Green Revolution and meet the increasing food demands of a rapidly growing global population in a changing environment.

Future direction and challenges

Several laboratories have been investing time and resources on multiplex CRISPR/Cas genome editing in recent years, and this technique has been successfully applied to editing genes in a variety of organisms' genomes. Several genes can be edited at once, but there are risks involved, such as off-target effects and the loss of vast stretches of DNA. Hence, multiplex CRISPR/Cas genome editing is not a robust and cutting-edge method, as it merely involves the addition of one or more gRNAs to the constructs. More off-target effects and the elimination of lengthy DNA segments will result in more severe results. This means that therapeutic treatments utilizing multiplex CRISPR/Cas genome editing and precision breeding are still in their infancy. It may take much longer to pick and discover CRISPR/Cas mutations following genome editing, even for gene function research. When using multiplex CRISPR/Cas genome editing technology, the targeted sites should not be placed on the same chromosome in order to limit any side effects. This will help lower the frequency of deletions of lengthy DNA fragments.

Acknowledgements The authors are thankful to HEC Islamabad for supporting this study.

Author contribution MS, UD, IA and RI designed the study, IA, FR, NM, KS, MU, SH, UZ, SME, and SF analyzed the data and edited the manuscript. All authors approved the final version.

Data availability All the data is available in the manuscript.

Declarations

Ethics approval and consent to participate The authors declare that all the permissions or licenses were obtained to collect the data and that all study complies with relevant institutional, national, and international guidelines and legislation for research ethics.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

References

Acharya S, Mishra A, Paul D, Ansari AH, Azhar M, Kumar M, Rauthan R, Sharma N, Aich M, Sinha D, Sharma S (2019) Francisella novicida Cas9 interrogates genomic DNA with very high specificity and can be used for mammalian genome editing. Proc Natl Acad Sci 116(42):20959–20968

Agarwal G, Kudapa H, Ramalingam A, Choudhary D, Sinha P, Garg V, Singh VK, Patil GB, Pandey MK, Nguyen HT, Guo B (2020) Epigenetics and epigenomics: underlying mechanisms, relevance, and implications in crop improvement. Funct Integr Genomics 20:739–761

Agudelo D, Carter S, Velimirovic M, Duringer A, Rivest JF, Levesque S, Loehr J, Mouchiroud M, Cyr D, Waters PJ, Laplante M (2020) Versatile and robust genome editing with Streptococcus thermophilus CRISPR1-Cas9. Genome Res 30(1):107–117

Ahmar S, Saeed S, Khan MH, Ullah Khan S, Mora-Poblete F, Kamran M, Faheem A, Maqsood A, Rauf M, Saleem S, Hong WJ (2020) A revolution toward gene-editing technology and its application to crop improvement. Int J Mol Sci 21(16):5665

Ali I, Mahmood T (2015) Identification and analysis of regulatory elements in the germin and germin-like proteins family promoters in rice. Turk J Bot 39(3):389–400

Ali I, Sardar Z, Rasheed A, Mahmood T (2015) Molecular characterization of the puroindoline-a and b alleles in synthetic hexaploid wheats and in silico functional and structural insights into Pina-D1. J Theor Biol 376:1–7

Ali I, Sher H, Ali A, Hussain S, Ullah Z (2022a) Simplified floral dip transformation method of Arabidopsis thaliana. J Microbiol Methods 197:106492

Ali I, Sher H, Ullah Z, Ali A, Iqbal J, Yang WC (2022b) The poly (A) polymerase PAPS1 mediates pollen maturation by regulating sperm cell differentiation in plants. Plant Direct 6(5):e397

Ali I, Salah KB, Sher H, Ali H, Ullah Z, Ali A, Alam N, Shah SA, Iqbal J, Ilyas M, Al-Quwaie DA (2022c) Drought stress enhances the efficiency of floral dip method of Agrobacterium-mediated transformation in Arabidopsis thaliana. Braz J Biol 84:e259326. https://doi.org/10.1590/1519-6984.259326

Amrani N, Gao XD, Liu P, Edraki A, Mir A, Ibraheim R, Gupta A, Sasaki KE, Wu T, Donohoue PD, Settle AH (2018) NmeCas9 is an intrinsically high-fidelity genome-editing platform. Genome Biol 19(1):1–25

Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, Chen PJ, Wilson C, Newby GA, Raguram A, Liu DR (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576(7785):149–157

Araki M, Ishii T (2015) Towards social acceptance of plant breeding by genome editing. Trends Plant Sci 20(3):145–149

Arroyo-Olarte RD, Bravo Rodríguez R, Morales-Ríos E (2021) Genome editing in bacteria: CRISPR-Cas and beyond. Microorganisms 9(4):844

Atta MI, Zehra SS, Dai DQ, Ali H, Naveed K, Ali I, Sarwar M, Ali B, Iqbal R, Bawazeer S, Abdel-Hameed UK (2022) Amassing of heavy metals in soils, vegetables and crop plants irrigated with wastewater: health risk assessment of heavy metals in Dera Ghazi Khan, Punjab, Pakistan. Front Plant Sci 13:1080635. https://doi.org/10.3389/fpls.2022.1080635

Bae S, Park J, Kim JS (2014) Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics 30(10):1473–1475

Barrangou R, Marraffini LA (2014) CRISPR-Cas systems: prokaryotes upgrade to adaptive immunity. Mol Cell 54(2):234–244

Beying N, Schmidt C, Pacher M, Houben A, Puchta H (2020) CRISPR–Cas9-mediated induction of heritable chromosomal translocations in Arabidopsis. Nat Plants 6(6):638–645

Bin Moon SU, Lee JM, Kang JG, Lee NE, Ha DI, Kim DY, Kim SH, Yoo K, Kim D, Ko JH, Kim YS (2018) Highly efficient genome editing by CRISPR-Cpf1 using CRISPR RNA with a uridinylate-rich 3′-overhang. Nat Commun 9(1):3651

Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A, Bonas U (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. Science 326(5959):1509–1512

Bogdanove AJ, Bohm A, Miller JC, Morgan RD, Stoddard BL (2018) Engineering altered protein–DNA recognition specificity. Nucleic Acids Res 46(10):4845–4871

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 Physiol 174(2):935–942

Butler JR, Tector AJ (2017) CRISPR genome-editing: a medical revolution. J Thorac Cardiovasc Surg 153(2):488–491

Cai Q, Qiao L, Wang M, He B, Lin FM, Palmquist J, Huang SD, Jin H (2018) Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. Science 360(6393):1126–1129

Carabias A, Fuglsang A, Temperini P, Pape T, Sofos N, Stella S, Erlendsson S, Montoya G (2021) Structure of the mini-RNA-guided endonuclease CRISPR-Cas12j3. Nat Commun 12(1):4476

Carlson DF, Fahrenkrug SC, Hackett PB (2012) Targeting DNA with fingers and TALENs. Molecular Therapy-Nucleic Acids 1(1):e3

Carroll D, Morton JJ, Beumer KJ, Segal DJ (2006) Design, construction and in vitro testing of zinc finger nucleases. Nat Protoc 1(3):1329–1341

Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M, Sherman A, Arazi T, Gal-On A (2016) Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. Mol Plant Pathol 17(7):1140–1153

Chen JS, Dagdas YS, Kleinstiver BP, Welch MM, Sousa AA, Harrington LB, Sternberg SH, Joung JK, Yildiz A, Doudna JA (2017) Enhanced proofreading governs CRISPR–Cas9 targeting accuracy. Nature 550(7676):407–410

Cho S, Choe D, Lee E, Kim SC, Palsson B, Cho BK (2018) High-level dCas9 expression induces abnormal cell morphology in Escherichia coli. ACS Synth Biol 7(4):1085–1094

Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Bogdanove AJ, Voytas DF (2010) Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 186(2):757–761

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339(6121):819–823

Craig W, Tepfer M, Degrassi G, Ripandelli D (2008) An overview of general features of risk assessments of genetically modified crops. Euphytica 164:853–880

Dagdas YS, Chen JS, Sternberg SH, Doudna JA, Yildiz A (2017) A conformational checkpoint between DNA binding and cleavage by CRISPR-Cas9. Science Advances 3(8):eaao0027

Davis KM, Pattanayak V, Thompson DB, Zuris JA, Liu DR (2015) Small molecule–triggered Cas9 protein with improved genome-editing specificity. Nat Chem Biol 11(5):316–318

Durai S, Mani M, Kandavelou K, Wu J, Porteus MH, Chandrasegaran S (2005) Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. Nucleic Acids Res 33(18):5978–5990

Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang DL, Wang Z, Zhang Z, Zheng R, Yang L, Zeng L (2014) Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/

Cas-induced gene modifications in Arabidopsis. Proc Natl Acad Sci 111(12):4632–4637
Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK, Sander JD (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. Nat Biotechnol 31(9):822–826
Gaj T, Guo J, Kato Y, Sirk SJ, Barbas CF III (2012) Targeted gene knockout by direct delivery of zinc-finger nuclease proteins. Nat Methods 9(8):805–807
Gao W, Xu WT, Huang KL, Guo MZ, Luo YB (2018) Risk analysis for genome editing-derived food safety in China. Food Control 84:128–137
Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR (2017) Programmable base editing of A• T to G• C in genomic DNA without DNA cleavage. Nature 551(7681):464–471
Gleditzsch D, Pausch P, Müller-Esparza H, Özcan A, Guo X, Bange G, Randau L (2019) PAM identification by CRISPR-Cas effector complexes: diversified mechanisms and structures. RNA Biol 16(4):504–517
Gunarathne AN, Lee KH (2019) Environmental and managerial information for cleaner production strategies: an environmental management development perspective. J Clean Prod 237:117849
Haq I, Binjawhar DN, Ullah Z, Ali A, Sher H, Ali I (2022) Wild vicia species possess a drought tolerance system for faba bean improvement. Genes 13(10):1877
Harrington LB, Burstein D, Chen JS, Paez-Espino D, Ma E, Witte IP, Cofsky JC, Kyrpides NC, Banfield JF, Doudna JA (2018) Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. Science 362(6416):839–842
Heigwer F, Kerr G, Boutros M (2014) E-CRISP: fast CRISPR target site identification. Nat Methods 11(2):122–123
Herai RH (2019) Avoiding the Off-Target Effects of CRISPR/Cas9 System Is Still a Challenging Accomplishment for Genetic Transformation. Gene 700:176–178
Ishizaki T (2016) CRISPR/Cas9 in rice can induce new mutations in later generations, leading to chimerism and unpredicted segregation of the targeted mutation. Mol Breeding 36(12):165
Jardim-Messeder D, Felix-Cordeiro T, Barzilai L, de Souza-Vieira Y, Galhego V, Bastos GA, Valente-Almeida G, Aiube YR, Faria-Reis A, Corrêa RL, Sachetto-Martins G (2021) Genome-wide analysis of general phenylpropanoid and monolignol-specific metabolism genes in sugarcane. Funct Integr Genomics 21:73–99
Jeon Y, Choi YH, Jang Y, Yu J, Goo J, Lee G, Jeong YK, Lee SH, Kim IS, Kim JS, Jeong C (2018) Direct observation of DNA target searching and cleavage by CRISPR-Cas12a. Nat Commun 9(1):2777
Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. Science 337(6096):816–821
Jones HD (2015) Regulatory uncertainty over genome editing. Nat Plants 1(1):1–3
Kim S, Kim D, Cho SW, Kim J, Kim JS (2014) Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res 24(6):1012–1019
Kim E, Koo T, Park SW, Kim D, Kim K, Cho HY, Song DW, Lee KJ, Jung MH, Kim S, Kim JH (2017) In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. Nat Commun 8(1):14500
Kim D, Hager M, Brant E, Budak H (2021) Efficient genome editing in wheat using Cas9 and Cpf1 (AsCpf1 and LbCpf1) nucleases. Funct Integr Genomics 21:355–366
Klein M, Eslami-Mossallam B, Arroyo DG, Depken M (2018) Hybridization kinetics explains CRISPR-Cas off-targeting rules. Cell Rep 22(6):1413–1423
Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales AP, Li Z, Peterson RT, Yeh JR, Aryee MJ (2015) Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 523(7561):481–485
Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z, Joung JK (2016) High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. Nature 529(7587):490–495
Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533(7603):420–424. https://doi.org/10.1038/nature17946
Koo T, Lu-Nguyen NB, Malerba A, Kim E, Kim D, Cappellari O, Cho HY, Dickson G, Popplewell L, Kim JS (2018) Functional rescue of dystrophin deficiency in mice caused by frameshift mutations using Campylobacter jejuni Cas9. Mol Ther 26(6):1529–1538
Kopischke S, Schüßler E, Althoff F, Zachgo S (2017) TALEN-mediated genome-editing approaches in the liverwort Marchantia polymorpha yield high efficiencies for targeted mutagenesis. Plant Methods 13:1–1
Kumawat S, Rana N, Bansal R, Vishwakarma G, Mehetre ST, Das BK, Kumar M, Yadav SK, Sonah H, Sharma TR, Deshmukh R (2019) Expanding avenue of fast neutron mediated mutagenesis for crop improvement. Plants 8(6):164
Lamb BM, Mercer AC, Barbas CF III (2013) Directed evolution of the TALE N-terminal domain for recognition of all 5′ bases. Nucleic Acids Res 41(21):9779–9785
Lee CM, Cradick TJ, Bao G (2016) The Neisseria meningitidis CRISPR-Cas9 system enables specific genome editing in mammalian cells. Mol Ther 24(3):645–654
Li C, Unver T, Zhang B (2017a) A high-efficiency CRISPR/Cas9 system for targeted mutagenesis in Cotton (Gossypium hirsutum L). Scientific reports. 7(1):1
Li X, Zhou W, Ren Y, Tian X, Lv T, Wang Z, Fang J, Chu C, Yang J, Bu Q (2017b) High-efficiency breeding of early-maturing rice cultivars via CRISPR/Cas9-mediated genome editing. J Genet Genomics 44(3):175–178
Li D, Zhou H, Zeng X (2019a) Battling CRISPR-Cas9 off-target genome editing. Cell Biol Toxicol 35:403–406
Li J, Manghwar H, Sun L, Wang P, Wang G, Sheng H, Zhang J, Liu H, Qin L, Rui H, Li B (2019b) Whole genome sequencing reveals rare off-target mutations and considerable inherent genetic or/and somaclonal variations in CRISPR/Cas9-edited cotton plants. Plant Biotechnol J 17(5):858–868
Lin J, Zhang Z, Zhang S, Chen J, Wong KC (2020) CRISPR-net: a recurrent convolutional network quantifies CRISPR off-target activities with mismatches and Indels. Adv Sci 7(13):1903562
Liu L, Chen P, Wang M, Li X, Wang J, Yin M, Wang Y (2017) C2c1-sgRNA complex structure reveals RNA-guided DNA cleavage mechanism. Mol Cell 65(2):310–322
Lowder LG, Zhou J, Zhang Y, Malzahn A, Zhong Z, Hsieh TF, Voytas DF, Zhang Y, Qi Y (2018) Robust transcriptional activation in plants using multiplexed CRISPR-Act2. 0 and mTALE-act systems. Mol Plant 11(2):245–256
Mahfouz MM, Piatek A, Stewart CN Jr (2014) Genome engineering via TALENs and CRISPR/Cas9 systems: challenges and perspectives. Plant Biotechnol J 12(8):1006–1014
Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM (2013) RNA-guided human genome engineering via Cas9. Science 339(6121):823–826
Malnoy M, Viola R, Jung MH, Koo OJ, Kim S, Kim JS, Velasco R, Nagamangala Kanchiswamy C (2016) DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front Plant Sci 7:1904

Manghwar H, Lindsey K, Zhang X, Jin S (2019) CRISPR/Cas system: recent advances and future prospects for genome editing. Trends Plant Sci 24(12):1102–1125

Minczuk M, Papworth MA, Miller JC, Murphy MP, Klug A (2008) Development of a single-chain, quasi-dimeric zinc-finger nuclease for the selective degradation of mutated human mitochondrial DNA. Nucleic Acids Res 36(12):3926–3938

Minkenberg B, Wheatley M, Yang Y (2017) CRISPR/Cas9-enabled multiplex genome editing and its application. Prog Mol Biol Transl Sci 149:111–132

Mishra R, Zhao K (2018) Genome editing technologies and their applications in crop improvement. Plant Biotechnology Reports 12:57–68

Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E (2014) CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic Acids Res 42(W1):W401–W407

Murtaza G, Ahmed Z, Dai DQ, Iqbal R, Bawazeer S, Usman M, Rizwan M, Iqbal J, Akram MI, Althubiani AS, Tariq A (2022) A review of mechanism and adsorption capacities of biochar-based engineered composites for removing aquatic pollutants from contaminated water. Front Environ Sci 10:2155

Murtaza G, Ahmed Z, Eldin SM, Ali I, Usman M, Iqbal R, Rizwan M, Abdel-Hameed UK, Haider AA, Tariq A (2023) Biochar as a green sorbent for remediation of polluted soils and associated toxicity risks: a critical review. Separations 10(3):197

Naito Y, Hino K, Bono H, Ui-Tei K (2015) CRISPRdirect: software for designing CRISPR/Cas guide RNA with reduced off-target sites. Bioinformatics 31(7):1120–1123

Newton MD, Taylor BJ, Driessen RP, Roos L, Cvetesic N, Allyjaun S, Lenhard B, Cuomo ME, Rueda DS (2019) DNA stretching induces Cas9 off-target activity. Nat Struct Mol Biol 26(3):185–192

Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, Tabata M, Mochizuki M, Miyabe A, Araki M, Hara KY, Shimatani Z (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. Science 353(6305):aaf8729

Nishimasu H, Cong L, Yan WX, Ran FA, Zetsche B, Li Y, Kurabayashi A, Ishitani R, Zhang F, Nureki O (2015) Crystal structure of Staphylococcus aureus Cas9. Cell 162(5):1113–1126

Nuñez JK, Kranzusch PJ, Noeske J, Wright AV, Davies CW, Doudna JA (2014) Cas1–Cas2 complex formation mediates spacer acquisition during CRISPR–Cas adaptive immunity. Nat Struct Mol Biol 21(6):528–534

Oliveros JC, Franch M, Tabas-Madrid D, San-León D, Montoliu L, Cubas P, Pazos F (2016) Breaking-Cas—interactive design of guide RNAs for CRISPR-Cas experiments for ENSEMBL genomes. Nucleic Acids Res 44(W1):W267–W271

Papworth M, Kolasinska P, Minczuk M (2006) Designer zinc-finger proteins and their applications. Gene 366(1):27–38

Paul B, Montoya G (2020) CRISPR-Cas12a: Functional overview and applications. Biomedical Journal 43(1):8–17

Paul B, Montoya G (2020) CRISPR-Cas12a: functional overview and applications. Biom J 43(1):8–17

Pessina S, Lenzi L, Perazzolli M, Campa M, Dalla Costa L, Urso S, Valè G, Salamini F, Velasco R, Malnoy M (2016) Knockdown of MLO genes reduces susceptibility to powdery mildew in grapevine. Hortic Res 3

Peters JM, Silvis MR, Zhao D, Hawkins JS, Gross CA, Qi LS (2015) Bacterial CRISPR: accomplishments and prospects. Curr Opin Microbiol 27:121–126

Rai KM, Ghose K, Rai A, Singh H, Srivastava R, Mendu V (2019) Genome engineering tools in plant synthetic biology. In: Singh SP, Pandey A, Du G, Kumar S (eds) Current developments in biotechnology and bioengineering. Elsevier, Amsterdam, pp 47–73.https://doi.org/10.1016/B978-0-444-64085-7.00003-4

Prykhozhij SV, Rajan V, Gaston D, Berman JN (2015) CRISPR multitargeter: a web tool to find common and unique CRISPR single guide RNA targets in a set of similar sequences. PLoS One 10(3):e0119372

Puchta, (2005) The Repair of Double-Strand Breaks in Plants: Mechanisms and Consequences for Genome Evolution. J Exp Bot 56(409):1–14

Ren B, Liu L, Li S, Kuang Y, Wang J, Zhang D, Zhou X, Lin H, Zhou H (2019) Cas9-NG greatly expands the targeting scope of the genome-editing toolkit by recognizing NG and other atypical PAMs in rice. Mol Plant 12(7):1015–1026

Ryan DE, Taussig D, Steinfeld I, Phadnis SM, Lunstad BD, Singh M, Vuong X, Okochi KD, McCaffrey R, Olesiak M, Roy S (2018) Improving CRISPR–Cas specificity with chemical modifications in single-guide RNAs. Nucleic Acids Res 46(2):792–803

Safari F, Zare K, Negahdaripour M, Barekati-Mowahed M, Ghasemi Y (2019) CRISPR Cpf1 proteins: structure, function and implications for genome editing. Cell Biosci 9:1–21

Sander JD, Zaback P, Joung JK, Voytas DF, Dobbs D (2007) Zinc finger Targeter (ZiFiT): an engineered zinc finger/target site design tool. Nucleic Acids Res 35(suppl_2):W599–W605

Sanfaçon H (2015) Plant translation factors and virus resistance. Viruses 7(7):3392–3419

Sapranauskas R, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V (2011) The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. Nucleic Acids Res 39(21):9275–9282

Savage DF (2019) Cas14: big advances from small CRISPR proteins. Biochemistry 58(8):1024–1025

Schaeffer SM, Nakata PA (2015) CRISPR/Cas9-mediated genome editing and gene replacement in plants: transitioning from lab to field. Plant Sci 240:130–142

Schmidt C, Pacher M, Puchta H (2019) Efficient induction of heritable inversions in plant genomes using the CRISPR/Cas system. Plant J 98(4):577–589

Shan Q, Wang Y, Chen K, Liang Z, Li J, Zhang Y, Zhang K, Liu J, Voytas DF, Zheng X, Zhang Y (2013) Rapid and efficient gene modification in rice and Brachypodium using TALENs. Mol Plant 6(4):1365–1368

Shen CC, Hsu MN, Chang CW, Lin MW, Hwu JR, Tu Y, Hu YC (2019) Synthetic switch to minimize CRISPR off-target effects by self-restricting Cas9 transcription and translation. Nucleic Acids Res 47(3):e13

Shi DQ, Ali I, Tang J, Yang WC (2017) New insights into 5hmC DNA modification: generation, distribution and function. Front Genet 8:100

Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H, Teramura H, Yamamoto T, Komatsu H, Miura K, Ezura H (2017) Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. Nat Biotechnol 35(5):441–443

Shrestha A, Khan A, Dey N (2018) Cis–trans engineering: advances and perspectives on customized transcriptional regulation in plants. Mol Plant 11(7):886–898

Singh D, Sternberg SH, Fei J, Doudna JA, Ha T (2016) Real-time observation of DNA recognition and rejection by the RNA-guided endonuclease Cas9. Nat Commun 7(1):12778

Sinkunas T, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V (2011) Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system. EMBO J 30(7):1335–1342

Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX, Zhang F (2016) Rationally engineered Cas9 nucleases with improved specificity. Science 351(6268):84–88

Sprink T, Metje J, Hartung F (2015) Plant genome editing by novel tools: TALEN and other sequence specific nucleases. Curr Opin Biotechnol 32:47–53

Stemmer M, Thumberger T, del Sol KM, Wittbrodt J, Mateo JL (2015) CCTop: an intuitive, flexible and reliable CRISPR/Cas9 target prediction tool. PLoS One 10(4):e0124633

Svitashev S, Schwartz C, Lenderts B, Young JK, Mark Cigan A (2016) Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. Nat Commun 7(1):13274

Tang X, Lowder LG, Zhang T, Malzahn AA, Zheng X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q, Kirkland ER (2017) A CRISPR–Cpf1 system for efficient genome editing and transcriptional repression in plants. Nature Plants 3(3):1–5

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 Signal Behav 13(10):e1525996

Thakur N, Nigam M, Mann NA, Gupta S, Hussain CM, Shukla SK, Shah AA, Casini R, Elansary HO, Khan SA (2023) Host-mediated gene engineering and microbiome-based technology optimization for sustainable agriculture and environment. Funct Integr Genomics 23(1):57

Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D, Goodwin MJ, Aryee MJ, Joung JK (2014) Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. Nat Biotechnol 32(6):569–576

Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, Aryee MJ (2015) GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat Biotechnol 33(2):187–197

Ullah A, Ali I, Noor J, Zeng F, Bawazeer S, Eldin SM, Asghar MA, Javed HH, Saleem K, Ullah S, Ali H (2023) Exogenous γ-aminobutyric acid (GABA) mitigated salinity-induced impairments in mungbean plants by regulating their nitrogen metabolism and antioxidant potential. Front Plant Sci 13:1081188

Voytas DF (2013) Plant genome engineering with sequence-specific nucleases. Annu Rev Plant Biol 64:327–350

Walton RT, Christie KA, Whittaker MN, Kleinstiver BP (2020) Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. Science 368(6488):290–296

Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, Qiu JL (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat Biotechnol 32(9):947–951

Wang L, Wang L, Tan Q, Fan Q, Zhu H, Hong Z, Zhang Z, Duanmu D (2016) Efficient inactivation of symbiotic nitrogen fixation related genes in Lotus japonicus using CRISPR-Cas9. Front Plant Sci 7:1333

Wang X, Tu M, Wang D, Liu J, Li Y, Li Z, Wang Y, Wang X (2018) CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. Plant Biotechnol J 16(4):844–855

Wang J, Meng X, Hu X, Sun T, Li J, Wang K, Yu H (2019) xCas9 expands the scope of genome editing with reduced efficiency in rice. Plant Biotechnol J 17(4):709

Wang X, Lv S, Liu T, Wei J, Qu S, Lu Y, Zhang J, Oo S, Zhang B, Pan X, Liu H (2020) CRISPR/Cas9 genome editing shows the important role of AZC_2928 gene in nitrogen-fixing bacteria of plants. Funct Integr Genomics 20:657–668

Wei T, Cheng Q, Min YL, Olson EN, Siegwart DJ (2020) Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. Nat Commun 11(1):3232

Wu W, Yang Y, Lei H (2019) Progress in the application of CRISPR: from gene to base editing. Med Res Rev 39(2):665–683

Xie K, Minkenberg B, Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci 112(11):3570–3575

Xie H, Tang L, He X, Liu X, Zhou C, Liu J, Ge X, Li J, Liu C, Zhao J, Qu J (2018) SaCas9 requires 5′-NNGRRT-3′ PAM for sufficient cleavage and possesses higher cleavage activity than SpCas9 or FnCpf1 in human cells. Biotechnol J 13(4):1700561

Xu R, Qin R, Li H, Li D, Li L, Wei P, Yang J (2017) Generation of targeted mutant rice using a CRISPR-Cpf1 system. Plant Biotechnol J 15(6):713–717

Xu Z, Kuang Y, Ren B, Yan D, Yan F, Spetz C, Sun W, Wang G, Zhou X, Zhou H (2021) SpRY greatly expands the genome editing scope in rice with highly flexible PAM recognition. Genome Biol 22(1):1–5

Yamano T, Nishimasu H, Zetsche B, Hirano H, Slaymaker IM, Li Y, Fedorova I, Nakane T, Makarova KS, Koonin EV, Ishitani R (2016) Crystal structure of Cpf1 in complex with guide RNA and target DNA. Cell 165(4):949–962

Yang J, Zhang Y, Yuan P, Zhou Y, Cai C, Ren Q, Wen D, Chu C, Qi H, Wei W (2014) Complete decoding of TAL effectors for DNA recognition. Cell Res 24(5):628–631

Yijun G, Zhiming X, Jianing G, Qian Z, Rasheed A, Hussain MI, Ali I, Shuheng Z, Hassan MU, Hashem M, Mostafa YS (2022) The intervention of classical and molecular breeding approaches to enhance flooding stress tolerance in soybean–an review. Front Plant Sci 13:108536

Yin H, Song CQ, Suresh S, Kwan SY, Wu Q, Walsh S, Ding J, Bogorad RL, Zhu LJ, Wolfe SA, Koteliansky V (2018) Partial DNA-guided Cas9 enables genome editing with reduced off-target activity. Nat Chem Biol 14(3):311–316

Zada A, Ali A, Binjawhar DN, Abdel-Hameed UK, Shah AH, Gill SM, Hussain I, Abbas Z, Ullah Z, Sher H, Ali I (2022) Molecular and physiological evaluation of bread wheat (Triticum aestivum L.) genotypes for stay green under drought stress. Genes 13(12):2261

Zaidi SS, Mahfouz MM, Mansoor S (2017) CRISPR-Cpf1: a new tool for plant genome editing. Trends Plant Sci 22(7):550–553

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A, Koonin EV (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163(3):759–771

Zhang F, Maeder ML, Unger-Wallace E, Hoshaw JP, Reyon D, Christian M, Li X, Pierick CJ, Dobbs D, Peterson T, Joung JK (2010) High frequency targeted mutagenesis in Arabidopsis thaliana using zinc finger nucleases. Proc Natl Acad Sci 107(26):12028–12033

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 Mol Biol 96:445–456

Zhang HX, Zhang Y, Yin H (2019) Genome editing with mRNA encoding ZFN, TALEN, and Cas9. Mol Ther 27(4):735–746

Zhang C, Kang G, Liu X, Zhao S, Yuan S, Li L, Yang Y, Wang F, Zhang X, Yang J (2020) Genome engineering in plant using an efficient CRISPR-xCas9 toolset with an expanded PAM compatibility. Frontiers in Genome Editing 2:618385

Zhou Z, Tan H, Li Q, Chen J, Gao S, Wang Y, Chen W, Zhang L (2018) CRISPR/Cas9-mediated efficient targeted mutagenesis of RAS in Salvia miltiorrhiza. Phytochemistry 148:63–70

Zhu Y, Huang Z (2019) Recent advances in structural studies of the CRISPR-Cas-mediated genome editing tools. Natl Sci Rev 6(3):438–451

Zhu C, Bortesi L, Baysal C, Twyman RM, Fischer R, Capell T, Schillberg S, Christou P (2017) Characteristics of genome editing mutations in cereal crops. Trends Plant Sci 22(1):38–52

Zong Y, Wang Y, Li C, Zhang R, Chen K, Ran Y, Qiu JL, Wang D, Gao C (2017) Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. Nat Biotechnol 35(5):438–440