CRISPR Cas for bringing desired traits in crops.

Abstract

In the edge of present day agribusiness, confronting the projected surge in global population concomitantly with escalating environmental perturbations, the sustainable production of high quality food has emerged as a substantial challenge. In order to overcome this major undertaking, adaptation of innovative, resilient and science driven agricultural strategies has been an essential step in plant biotechnology. Advanced biotechnological approaches like genome editing with site directed mutagenesis permit to step forward towards new cultivar with desirable traits in economically important crops Latterly, Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspersed short palindromic repeats (CRISPR)/CRISPR associated protein 9 system has revolutionised the plant breeding system with exceptional achievements. On that account, the present review re-evaluates and discusses the information on genome editing technologies specially CRISPR /Cas 9 approach in detailtogether with complete mechanism of CRISPR /Cas, problem and future aspects of the approach aiming to develop desirable agronomic genetic traits.

Introduction

The achievement of plant breeding mainly depends on development of a wide hereditary variation. Plant breeders from decades are using several techniques cross breeding, mutation breeding and transgenic breeding for enhancing quantitative and qualitative traits of the crop. . Cross breeding involves improving trait by crossing genetic material from one variety to another and further choosing outstanding progeny with the preferred trait, followed by backcrossing with the recipient line for subsequent generations to eliminate the linked traits (Mangrauthia et al., 2024). But it takes several years to incorporate desirable alleles and to enhance variability in the plant by the use of cross breeding and moreover, the genetic variability of the crops has been greatly reduced by the large fixed parts of the genome (Swarup et al., 2021). Mutation breeding involves the enhancement of traits by creating random mutations in the plant material by the use of chemical mutagens or physical irradiations (Yali et al., 2022), However,the screening of such a large number of mutants is time taking, laborious and challengingtogether with the burdens for increased crop production

(Sarsu et al., 2023). Transgenic breeding involves transferring of exogenous genes into crop to obtain the enhanced trait and can be useful, but the long and cost regulatory estimation processes and the public concerns in the commercialization of the produced genetically modified crops acts as a barrier for the use of the technology (Marone et al., 2023). Thus, the novelties in the technology with development of new, fast and efficient techniques to create advanced crops with better characteristics are a need of the hour, and are essential to enhance the agricultural yield and accelerate sustainable agricultural development (Patel et al., 2023).

Zinc finger nucleases

The research in the genetics has been revolutionized by the use of genome editing approaches using high throughput sequencing and computational analysis. Several gene editingtechniques leading to site directed mutagenesis has been developed during last few years. Zinc finger nucleases (ZFNs) were the first engineered endonucleases which are site directed and are created by fusion of the DNA-binding domain of the artificial array of the zinc fingers and the non-specific cleavage domain of FokI, which cleave the target DNA by protein-DNA binding (Limbalkaret al., 2025). ZFNs firstly came from the study on FokI restriction enzymes, when it was figured out that the recognition and the cleavage domain of FokI are physically separable and, if the non-specific nuclease domain from FokI are put away from its natural recognition domain, other recognition domains can be incorporated. Using this strategy set of zing fingers were chosen, as zinc fingers naturally occur in eukaryotic sequence specific DNA binding transcription factors (TF) (Zess and Begemann 2021). Each module of these TF is the finger that recognizes principle three base pair of DNA, and successive finger recognize successive triplet in DNA target. So FokI cleavage domain cuts double stranded DNA by assembling different combination of zinc finger on them (Abdelrahmanet al., 2021) (Figure 1).. Despite of its uses, potential of ZFNs technology has not been fulfilled as its nucleases requires protein engineering for recognizing target DNA sequence (Liu et al., 2024). Therefore, due to construction complexity, high but variable off-target rate, high cost and skill, difficulty in customization, its application in the field of genome editing gets restricted (Castro et al., 2021).

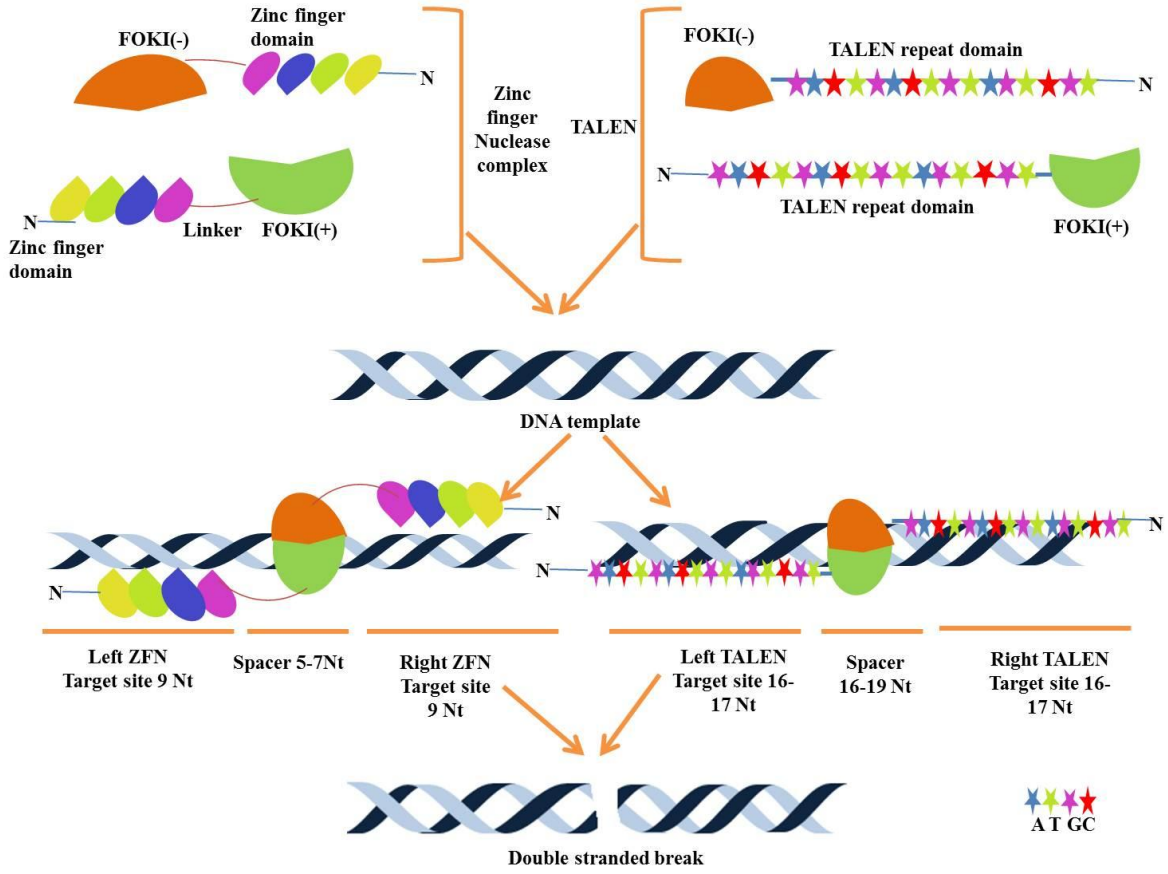

Figure 1: Comparative mechanism of Zinc finger nucleases and TALENS. ZFN-Zinc finger nuclease

Transcription activator-like effector nucleases (TALENs)

TALENs system arose from studies of bacterial plant pathogen of the genus *Xanthomonas*, these bacteria produce proteins that they secrete into plant host cell. In the host cells these protein binds upstream of the host gene and regulates its transcription in a way that promotes the bacterial infection (Teperet al., 2023). TALENs are chimeric enzymes formed by binding of transcription activator like (TALE) protein fused with catalytic domain of the FokI endonuclease to cleave the target DNA. The specificity in recognizing the target DNA is due to the central repeat region of 33 to 35 amino acid tandem repeats, which differ at two amino acids which determines at which nucleotide it will bind. By combining 12 to 31 of these repeats, TALENs can be customized to bind to specific DNA template.Two sets of TALEN system must dimerize to cleave the target DNA resulting into double stranded break. The cells repair the DNA by NHEJ mechanism leading to frame shift mutation (Bhardwaj and Nain 2021) (Figure 1). Although the efficiency of the TALENs is high as compared to the ZFNs as it involve no re-engineering of proteins (Mishra et al., 2023), but production of

novel TALE arrays can be long and relatively costly, due to large size of TALEN, along with a pair of proteins to identify the antiparallel DNA strand and create DSB, restricts its use as the gene editing technology (Bhagtaney and Sundarrajan 2023). To overcome the shortcomings of these techniques, a new technique known as CRISPR came into existence.

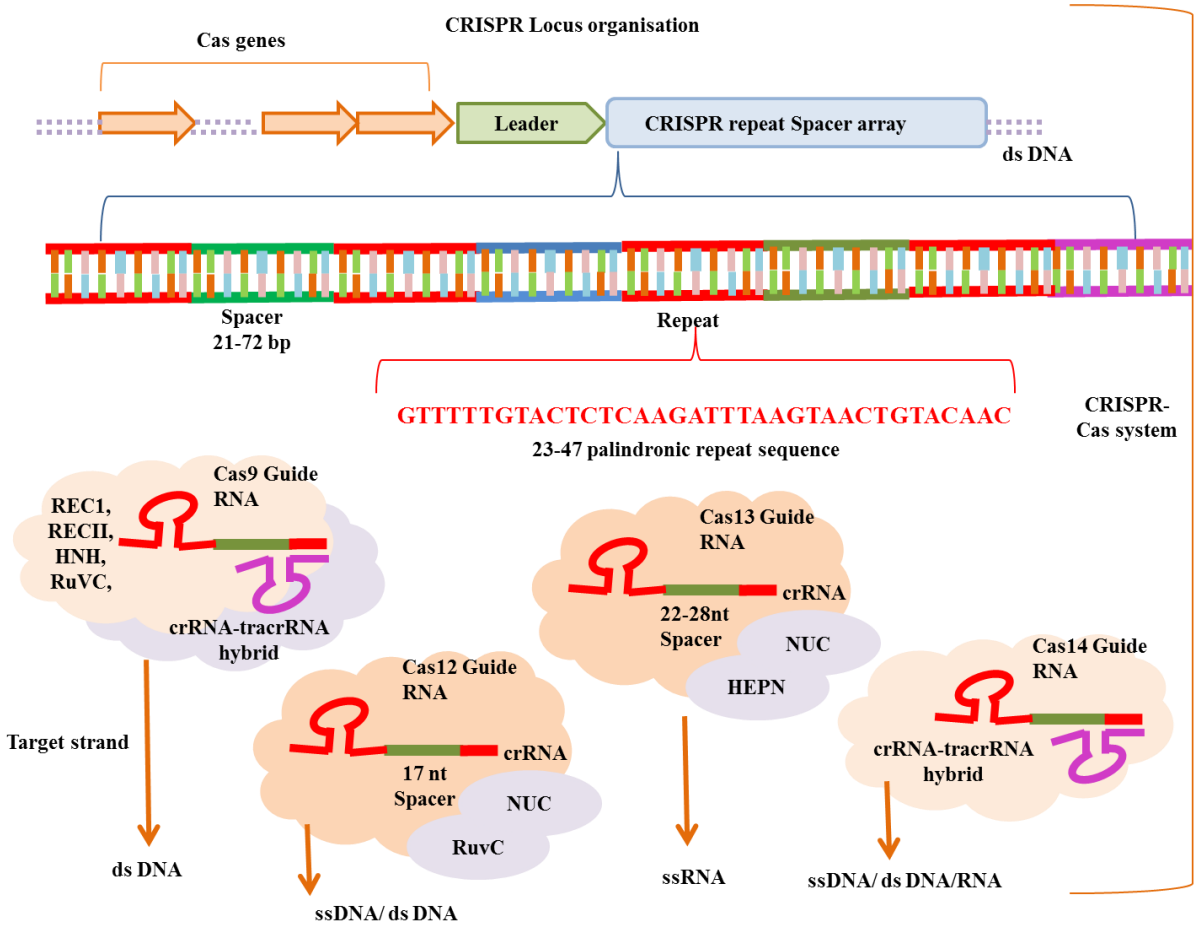

Figure2: CRISPR- Cas system- CRISPR locus organisation and complex of different Cas proteins like 9, 12, 13 and 14 forming the guide RNA . NUC- Nuclease lobe, HNH-Ribonuclease domain and RuVC-Ribonuclease, REC1 and REC 2-Recognition lobe-HEPN- Higher eukaryotes and prokaryotes nucleotide binding domain.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) proteins are extensively found in bacterial and archaeabacterial genomes as adaptive immune systems (Ganger et al., 2023).The first CRISPR/ Cas system known to cleave the target DNA was the type II system which belonged to *Streptococcus pyogenes* (Varble and Marraffini 2022).

The CRISPR system mainly consists of two components Cas 9 endonuclease and guide RNA. The guide RNA guides the Cas 9 endonuclease to create double stranded breaks in the targeted DNA (Aksoyet al., 2022). This is the crucial step as after the cleavage of DNA the DNA repair mechanism comes in activity and start repairing the DNA either by NHEJ orHDR resulting into either deletion or insertion mutations (Nambiaret al., 2022)(Figure 2).

In recent years, CRISPR/Cas9 genome editing has emerged as a promising alternative due to its precise editing capabilities (Cheng et al., 2025). This innovative tool enables targeted modification of DNA sequences. The ground-breaking technology is poised to revolutionize biotechnological research by facilitating the manipulation of target genes associated with plant metabolism, immunity, and stress resistance, thus facilitating the development of improved crops (Kumar et al., 2023).This technology has been observed to be better than the traditional breeding technologies(Kumar et al., 2020).the system precisely edits the genome of the plants with great efficiency, thus making it a suitable technology for use (Chib et al., 2020).However the technology still possesses certain limitations like off targeting as the CRISPR/Cas system is still under modifications to bring maximum application advantages to the researchers (Ali et al., 2023).

Mechanism of action in CRISPR

CRISPR is originally found in bacteria and archae as an adaptive immune system to protect from the invading virus(Garcia-Robledoet al., 2020).CRISPR systems have been classified into two; Class 1 and Class 2, further categorized into three types each. Class 1 (Types I, III, and IV) utilize multiple Cas proteins for RNA guided cleavage, while Class 2 (Types II, V, and VI) rely on only one endonuclease protein for the DNA cleavage (Hillary and Ceasar 2023). The most commonly used system is type IICRISPR/Cas 9 system adapted from *Streptococcus pyognesis*and repurposed for genome editing by modifying the DNA sequences by either deleting or introducing new gene sequences (Liang and Corn 2022).The CRISPR array is mainly composed of two components; first component includes two type of RNAs i.e CRISPR RNA or crRNA, Tracer RNA or tracrRNA and the other component is RNA dependent endonuclease i.e Cas (CRISPR associated protein) (Mohamadi et al., 2020).

The CRISPR immunity works in three phases, phase 1 is adaptation, phase 2 involves expression and processing and phase 3 proceeds with recognition and cleavage of the target (Mosterd et al., 2021).In phase 1 when the bacteria is infected by the phage the Cas, such as Cas1 and Cas2 recognize the Protospacer (a 20 -50 bp short sequence similar to the spacer sequence in CRISPR array) from the invading phage or virus and store it as a new spacer on

CRISPR locus as a result an memory for the invader is formed for future recognition (McGinn and Marraffini 2021) (Figure 3)..

Phase 2 transcription of CRISPR array occurs resulting into the formation of the long precursor RNA (pre-crRNA). The pre-crRNA is processed by RNase III and Cas proteins into crRNA, the crRNA further combines with the trans-activating CRISPR RNA (tracrRNA) to form a duplex form complex. Finally the Cas 9 protein binds to this duplex resulting into formation of the single guide RNA (sgRNA) (Munawar and Ahmad 2021).

Phase 3 involves the target recognition and cleavage with the help of Cas9. It contains a sequence known as PAM (Protospacer Adjacent Motif) which is adjacent to the target sequences in the foreign DNA in most of the CRISPR -Cas systems (Ding et al., 2021). It also contains a guide RNA, which is almost 20nt and leads Cas 9 to the target DNA adjacent to the PAM and has the sequence complementary to the part of the sequence of the invading DNA which is to be edited (Gupta et al., 2021). In many cases, a chimeric sgRNA, formed by the association of the crRNA and the tracr RNA into a single transcript is used, which is accurately functional in causing Cas mediated sequence-specific DNA cleavage. This simplified two-component system CRISPR Cas 9, can be used to target any DNA of interest merely by changing the 20nt guide RNA sequence (Liao and Beisel 2021).

The Cas9 enzyme consists of two well defined lobes: the alpha-helical recognition lobe (REC) and the nuclease lobe (NUC). The NUC has the HNH nuclease domain (motif with three conserved amino acids; Histidine (H) Asparagine (N) and Histidine (H), the RuvC nuclease domain and the CTD (C- terminal domain) (Palanivelu, 2021). The Cas 9 enzyme snips the target DNA 3bp upstream of the PAM with these two nuclease domains. The HNH like-nuclease domain cuts the target strand (the strand complementary to the guide RNA) of the DNA and the RuvC like-nuclease domain which cuts the non-target strand (strand opposite to the complementary strand of the guide RNA) (Chuang et al., 2021). The RuvC domain of the enzyme has three motifs: Motif I, Motif II, and Motif III. The HNH domain of the nuclease enzyme interrupts motif I and III, and the larger lobe made of alpha-helices (REC) interrupts motif I and II (Nasrallah et al., 2022). The two domains of the enzyme (REC and NUC) are connected to each other by two linkers, one formed by the arginine-rich bridge and the other formed by the disordered linker. The CTD domain of the enzyme contains a PAM- interacting site, which is essential for PAM recognition (Jiang and Doudna 2017).Studies show that mutating any of the two domains of the Cas 9 converts it to nickase while mutating any of the two domains stops its endonuclease activity but its RNA - guided DNA binding ability remains as it is (Deb et al., 2022). The Cas 9 enzyme is present in an

inactive state and undergoes extensive rearrangements on binding with the sgRNA, thus making the enzyme compatible for the recognition of the target DNA(Montecillo et al., 2020).

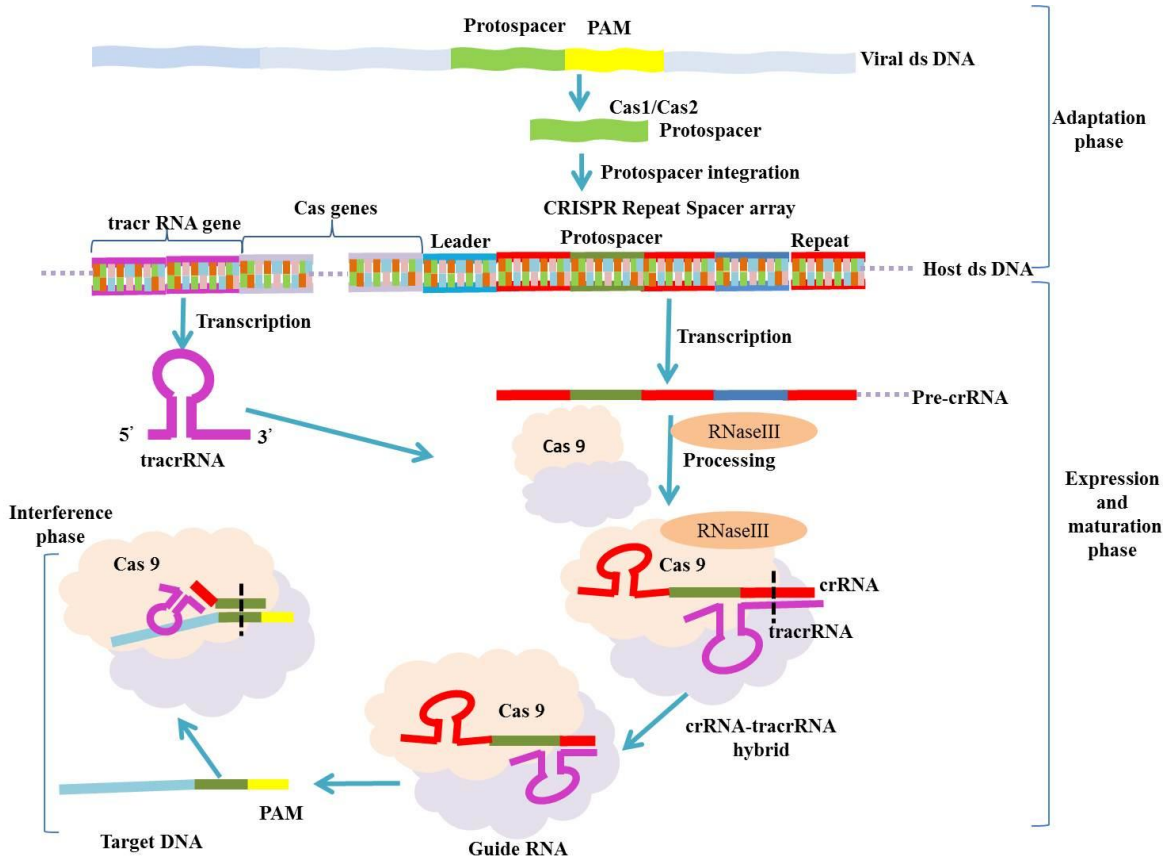

Figure 3: Mechanism of CRISPR-Cas9 activity highlighting three phases- Adaptation – Integration of protospacer in CRISPR array, Expression- Generation of Guide RNA (complex of Cas9-with crRNA and tracRNA, Interference phase- Degradation of target DNA

Binding of the Cas 9 with the sgRNA indicates preparedness of the enzyme to search for the complementary sites in the target DNA (David et al., 2022). The two prerequisite conditions for the recognition of the target sequence are: The complementary base pairing between the 20nt spacer and the complementary protospacer is the target DNA and the presence of PAM sequence adjacent to the target site (Karvelis et al., 2017). The most commonly studied and used CRISPR Cas 9 system for genome editing belongs to *Streptococcus pyogenes*which has a 5'-NGG-3' PAM sequence in the target DNA. The PAM sequence is of immense importance in the type II CRISPR Cas 9 system as it helps in distinguishing between the self and non-self-sequences. A single mutation in PAM can inhibit the cleavage of the target DNA (Gupta et al., 2021). DNA melting starts after the Cas 9 has found a suitable PAM in the target site,and is followed by the formation of RNA-DNA hybrid, and the formation of

the R-loop by the non-target DNA strand. The R-loop is formed when the guide RNA of the crRNA array, combines with the ds target DNA to form a RNA-DNA heteroduplex(Pacesa et al., 2022).The guide RNA pairs with the target DNA strand (complementary to the guide RNA), thus replacing the opposite non-target DNA strand (non-complementary to the guide RNA) (Gorski et al., 2017). The interaction of the R-loop with the target DNA and the complementary guide RNA, leads to the double stranded breaks, 3bp upstream of PAM by the HNH and the RuvC nuclease domains of the Cas 9 (Allemailem et al., 2024).

After the site specific double stranded break (DSB), the cell undergoes repair mechanism. Non homologous end joining (NHEJ) and homologous directed repair (HDR) in DNA repair mechanism are the two most commonly used methods to induce sequence specific changes during genome engineering (Yang et al., 2020) (Figure 4).

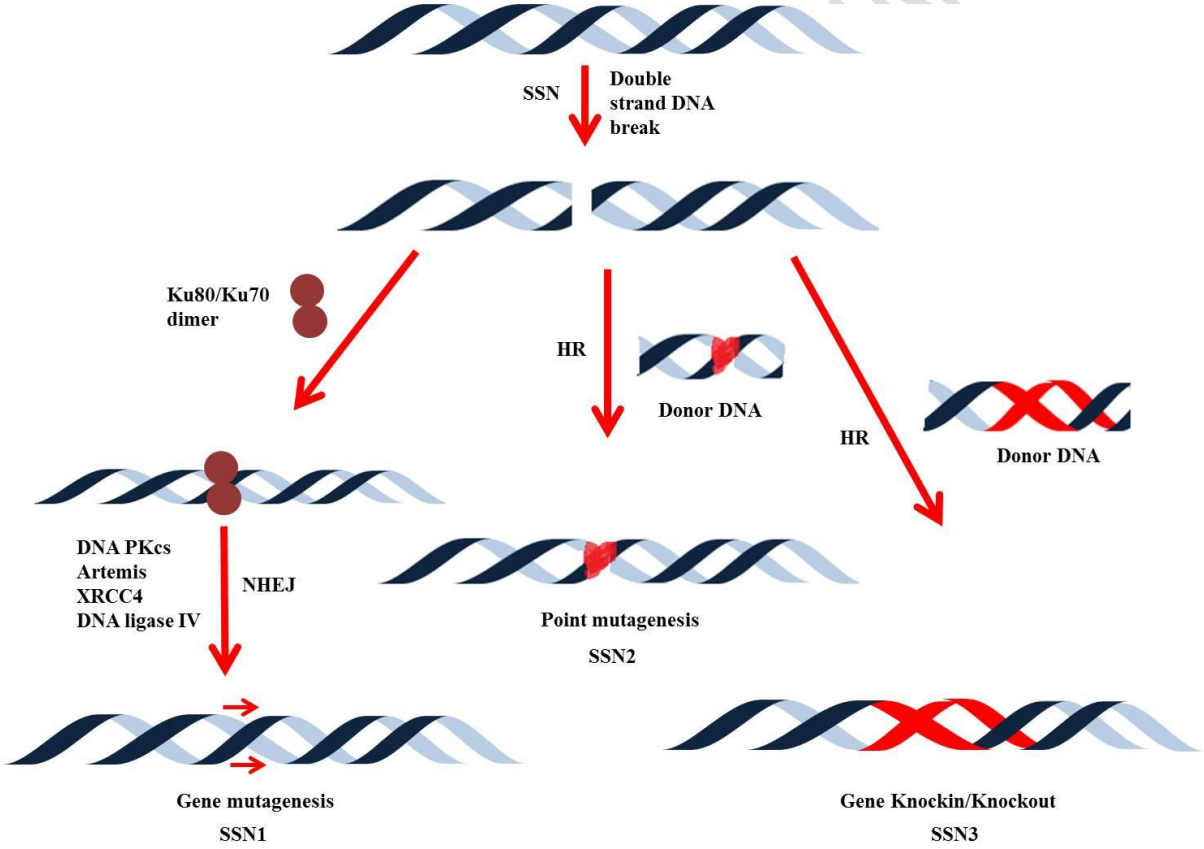

Figure 4: Categorization of site specific nuclease approach(SSN), depicting double stranded break repair (DSB) by non-homologous end joining (NHEJ) or homologous repair (HR) method in ZFNs / TALENs and CRISPR, SSN 1 result in small deletion/insertion via NHEJ, SSN 2 for point mutation via HR and SSN 3 for complete gene edit using HR.

All cells face double stranded breaks during cell cycle that are repaired preponderantly by the NHEJ. The DNA ends are identified, selected, polymerised, and ligated in a versatile manner by theproteins involved in the NHEJ mechanism. Ku70/Ku80 proteins bind to the blunt ends of cleaved double stranded DNA and ligation of the DNA occurs by ligase IV (Meyenberg et al., 2021). This adaptability licenses NHEJ to operate on a wide range of DNA end arrangements, ending with mutation in the repaired DNA (Chang et al., 2017). This repair mechanism occurs in the absence of any homologous DNA template, which makes the process little error prone also resulting into random mutations sometimes (Chatterjee and Walker, 2017).

HDR is another mechanism for the repair of the targeted DNA.As compared to NHEJ, HDR happens at lower and significantly more variable frequency. It uses externally added target DNA template or a homologous sequence for the repair mechanism. The externally added target DNA can either be single stranded or double stranded oligonucleotides. This mechanism creates more precise and specific genetic alteration (Jin et al., 2025).

Other systems of CRISPR

Although type II CRISPR/Cas system is most commonly used for genome editing, but there exist other systems also particularly in class 2 of CRISPR system such as Cas12(belonging from type V) and Cas13 (belongs from type VI). The system varies in structural organization, target specificity, cleavage mechanism, PAM requirement and repair pathway (Chaudhary et al., 2024).

Cas12- The system belongs from type V CRISPR system exhibiting cleavage system different to Cas9 system. Cas12 system (Figure 2).cleave both of the DNA strand by a single RuvC- like catalytic domain unlike Cas9 system where two nuclease domains are responsible for cleavage of the DNA strand (Lei et al., 2017). It recognizes T-rich PAM located at 5´ end of target sequence, unlike G-rich PAM of Cas9. The system generates staggered cuts in the DNA and follows HDR system for DNA repair (Teng et al., 2019).

Cas13

Cas13- Cas13 has been added to the CRISPR list very recently, it belongs toclass 2 type VI of CRISPR system. The system works only on RNA and is catalysed by its HEPN (Higher eukaryotes and prokaryotes nucleotide-binding domains. The Cas13 system involves the use of only crRNA for cleavage of RNA (Ashraf et al., 2022). Since the system works on the RNA only so no DNA strand cleavage occurs and hence no DSB mechanism takes place in Cas13 system and the system does not rely on host repair mechanism or pathway. But since the HEPN domain of the Cas13 system is present on the outer surface, there are high chances

of cleavage of RNA other than target RNA resulting in programmed cell deaths (Yang and Patel, 2024) (Figure 2)..

Cas14- very recently Doudna's group explored a new protein named as Cas14, a 400-700 amino acids small protein coding for small protein with molecular weight of 40-70 kd. The small size of this Cas14 protein make it efficient for targeting ssDNAusing only T-rich sequences without a requirement of PAM sequence, but cannot target dsRNA or ssRNA (Savage, 2019). For cleavage of ssDNA , similar to Cas9, Cas14 also require the tracrRNA and crRNA complex. In term of efficiency Cas14 system has proven to be more efficient and specific than Cas9, Cas12 and Cas13. Due to its small size it can be used to target any type of tissue and improves SNP (single nucleotide polymorphism) (Hillary and Ceasar, 2023) (Figure 2)..

Application of CRISPR/Cas 9 in plants

Concerning the surging need of food resources with increasing population, the food and agricultural researchers are showing significant attention to explore CRISPR/Cas technology, which was first used by Feng et al., 2013 for modifying genome of world's largest staple food crop, rice. Several studies have proven the efficiency of this technology in plant genome editing compared to other technologies existing previously. The CRISPR/Cas9 genome editing has been used to edit plant genome more precisely and efficiently (Chib et al., 2020). The main applications are as below:-

- **Crop trait improvement via knockout**: Eradicating the undesirable gene is the most promising approach for advancement in the field of genetic. The most widely used application of CRISPR/Cas9 comprises knocking out the unwanted genes. There have been several traits which are being enhanced and amended by the use of gene knockout. Better yield, quality, biotic- and abiotic-stress resistance, hybrid-breeding techniques and several other aspects of crop productivity have been enhanced using this approach (Rao and Wang, 2021). For eg. Knocking out of OsERF922 gene was done in rice to achieve bacterial blight disease resistance a major cause of loss of almost 50% rice yield (Wang et al., 2016)
- **Knock-In and Replacement by CRISPR/ Cas**:Several traits in the crop plants are altered by single-nucleotide substitutions, gene expression changes, or the addition of new genes with favorable traits. Introduction of new alleles without linkage drag or generating allelic variants that do not exist naturally has been made simple by knock-

ins and replacements via CRISPR/Cas system. Moreover, multiple traits can be modified via knock-inby introducing multiple genes in a single variety (Rozov et al., 2019). Herbicide tolerance in rice by knock-in of mutated acetolactate synthase (ALS) gene to confer resistance to herbicide bispyribac-sodium is one of the example for gene knock-in (Ouyang et al., 2025).

- **Use of Base editors for Genome editing**: As many agriculturally essential traits can be altered by single-nucleotide polymorphisms in either coding or noncoding regions, base editing is fairly convenient for plant breeding and improved crop development. For single-base substitution, base editing is developing as a substitute and is an effective, influential tool to HDR-mediated precise gene editing in plants (Molla et al., 2021). The most commonly used base editors include the cytidine base editor (CBE) and adenine base editor (ABE) (Bharat et al., 2020).
- **Fine-Tuning Gene Regulation via CRISPR/Cas in Plants**: Besides generating mutations in coding sequences, modifying gene expression is a beneficial method for investigating role of a gene and can significantly enable plant breeding (Sun et al., 2024). Gene expression can be modified at several levels, including transcription, mRNA processing, and mRNA translation. These processes are under the control of a series of cis-regulatory elements, which can be modified by genome editing. Till date, the alteration for modifying the gene expression mainly included promoters replacing and deleting cis-regulatory elements (Cui et al., 2023).
- **Antiviral Plant Breeding Strategies**: The viral infections are amongst the chief reasons that lead to damage of valuable crops in natural ecosystems. These infections significantly decrease harvest by displaying different symptoms in plants and therefore bring economic burden (Nazarov et al., 2020). The CRISPR/Cas system delivers a resistance mechanism that cleaves plasmids, DNA viruses , and RNA viruses. Opportunely, genetic engineering by the use of CRISPR/ Cas 9 has been supporting as a potent tool to increase plant resistance against a broad range of viral infections (Tyagi et al., 2021).
- **Highly efficient plant mutant libraries via CRISPR/Cas**: The most appreciated tool for functional genomics is whole-genome-scale mutant libraries. Traditional mutant libraries contain the data based on random mutations encouraged by agents such as irradiation, T-DNA insertions, ethyl methyl sulfonate (EMS) mutagenesis, and transposons (Santosh, 2020). The use of these methods to achieve stabilized mutations

requires many generations; moreover finding the genotypic and phenotypic associations amongst the mutants is a time-consuming and laborious process.The availability of high-quality, high-coverage, uniformly dispersed mutant libraries generated via CRISPR/Cas could simplify the expansion of advanced germplasm approaches as well as crop trait improvement (Tan et al., 2024).

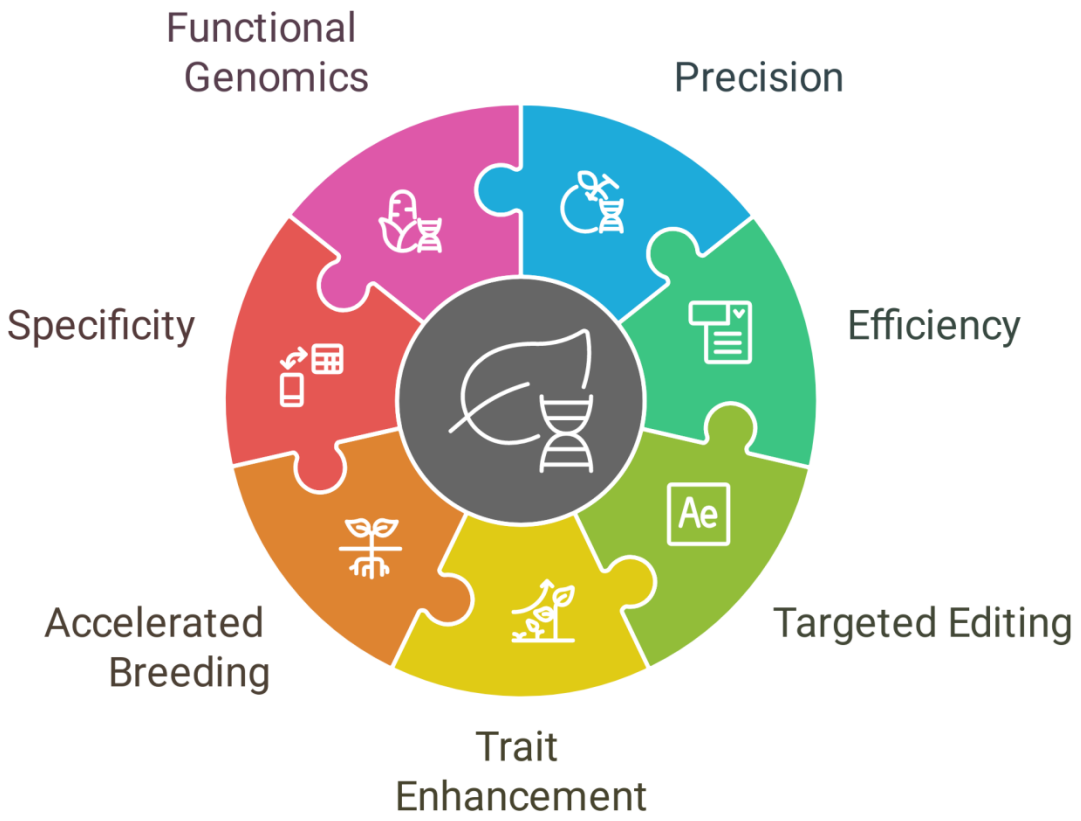

Figure5 : Impact of CRISPR/Cas 9 on genetic engineering in plants

Table1. Targeted mutagenesis using the CRISPR/Cas system in rice

Name	**Targeted Gene**	**Effect of editing on plant**	**Reference**
Rice (*Oryza sativa*)	*OsPHO1;2*	Improved phosphate uptake	Mayura et al., 2025
	OsALS	Herbicide resistance	Ouyang et al., 2025

	OsACA9	Disease resistance and regulates leaf senescence	Wang et al., 2024
	OsCPR5.1	Resistance against yellow mottle virus	Arra et al., 2024
	OsPUB9	Resistance against bacterial leaf blight	Kim et al., 2024
	OsCOP1	Improved UV protection	Hu et al., 2024
	OsCAT2	Improved scavenging of ROS	Shen et al., 2024
	OsMYB84	Controlled uptake and transport of copper	Ding et al., 2024
	OsNIP3	Reduced arsenic accumulation	Xu et al., 2024
	SD1, Wx	Increased semi-dwarf glutinous traits	Wang et al., 2024
	OsNAS2	Increased uptake and translocation of zinc	Ludwig et al., 2024
	OsRR22	Salinity tolerance	Sheng et al., 2023
	OsAUX5, OsWRKY78	Regulated amino acid accumulation	Shi et al., 2023
	Pi21, OsSULTR3;6	Resistance against rice blast	Yang et al., 2023
	OsCKX	Stress tolerance and enhanced growth	Zheng et al., 2023
	OsWRKY71, Bph15	Resistance from brown plant hopper	Li et al., 2023
	OsHPP04	Resistance against rice root-knot nematode	Huand et al., 2023
	OsTPP3	Improved salt resistance	Ye el al., 2023
	OsLCD	Low cadmium accumulation	Chen et al., 2023
	bHLH57	Increased yield even under cold condition	Zhang et al., 2023
	OsPUB7	Enhanced drought resistance	Kim et al., 2023
	OsGER4	Enhanced heat resistance	Nguyen et al., 2023
	NRAMP1, FRO2	Improved Fe uptake	Krishna et al., 2023
	OsPDR7, OsZIP9	Improved zinc accumulation	Lu et al., 2023

	OsGS2/GRF4	Improved size and yield	Wang et al., 2022
	PR10/Bet vl-like protein gene	Resistance from *Meloidogynegraminicola*	Li et al., 2022
	CRTL, PSY	Enhanced vitamin A content	Dong et al., 2020
	OsDEPI, OsROCs	Enhanced heat resistance	Malzahn et al., 2019
	OsSWEET 11, *OsSWEET 13*, *OsSWEET 14*, *Os8N3*	Resistance against Bacterial Blight	Olivaet al., 2019
	ISA-1	Reduced starch content and increased sugar content	Shufenet al., 2019
	eIF4G	Resistance against Rice Tungro Disease	Macoveiet al., 2018
	Waxy	Low Amylose content	Zanget al., 2018
	SBEI and *SBEIIb*	Increased amylose content and the resistant starch content	Sun et al., 2017
	OsAnn3	Increased relative electrical conductivity and reduced survival ratio after exposure to cold treatment	Shenet al., 2017
	OsNramp5	Reduced Cadmium content	Tang et al., 2017
	Gn1a, *DEP1*, *GS3*, and *IPA1*	Alteration in grain number, panicle architecture, grain size and plant architecture	Xuet al., 2016
	OsERF922	Resistance against Rice blast	Wang et al., 2016

309

310 Table 2.Targeted mutagenesis using the CRISPR/Cas system in wheat

Name	**Targeted Gene**	**Effect of editing on plant**	**Reference**
Wheat	*S*	Disease resistance	Waites et al., 2025

(*Triticum aestivum*)	*ω- and γ-gliadin*	Reduced immunotoxicity	Yu et al., 2024
	TaRR12	Drought resistance	Li et al., 2024
	TaRPK1	Enhanced yield	Rahim et al., 2024
	TaHKT1;5	Salt resistance	Wang et al., 2024
	TaHSFA1	Heat resistance	Wang et al., 2023
	ElF4E	Resistance against yellow mosaic virus	Kan et al., 2023
	TaPGK	Cold resistance	Zhang et al., 2023
	TaCIPK14	Resistance against stripe rust	He et al., 2023
	Tamyb10	Reduced pre-harvest sprouting	Zhu et al., 2023
	PSY	Enhanced vitamin and mineral level	Narayanan et al., 2023
	Ppd-1	Yield enhancement	Errum et al., 2023
	Sal1	Stress resistance	Mohr et al., 2022
	TaIPK1	Enhanced uptake of iron and zinc	Ibrahim et al., 2022
	TaPDI	Enhanced protein storage	Hu et al., 2022
	S	Stress resistance	Taj et al., 2022
	pinb, waxy, ppo and psy	Improved grain quality	Zhang et al., 2021
	TaASN	Reduced asparagine accumulation	Raffan et al., 2021
	TaSBElla	Regulated starch composition	Li et al., 2021
	TaNP1	Male sterility	Li et al., 2020
	TaQsd1	Enhanced seed dormancy	Abe et al., 2019
	TaMs1	Male sterility	Okada et al., 2019
	Gli-2	Low gluten wheat	Sanchez Leonset al., 2018
	TaERF3, TaDREB2	Drought Resistant	Kim et al., 2018
	Pinb, Waxy and *DA1*	Yield enhancement	Zhang et al., 2018

	TaEDR1	Resistance against Powdery Mildew	Zhang et al., 2017

311

312 Table 3.Targeted mutagenesis using the CRISPR/Cas system in other important staple crops

Name	**Targeted Gene**	**Effect of editing on plant**	**Reference**
Sorghum (*Sorghum bicolor)*	SbLG1	Yield improvement	Brant et al., 2021
	sbFT	Yield improvement	Char et al., 2020
	SbGA20Ox5	Yield improvement	Char et al., 2020
	CAD	Yield and quality improvement	Liu et al., 2019
	K1C	Quality improvement	Li et al., 2018
Maize *(Zea mays)*	*ZmHDT103*	Drought resistance	Wang et al., 2024
	ZmHSPs	Enhanced heat resistance	Li et al., 2024
	Zmpdrp1	Virus resistance	Xie et al., 2024
	Cry1F	Pest resistance	Kumari et al., 2024
	RZ2MS9	Stress tolerance	Figueredo et al., 2023
	ZeSWEET1b	Enhanced nutrient uptake and accumulation	Wu et al., 2023
	ZmAGO18b	Southern leaf blight resistance	Dai et al., 2023
	MCMV	Reduction in viral infections	Lei et al., 2023
	ZmG6PDH1	Enhanced cold resistance	Li et al., 2023
	ZmMYB69	Quality improvement	Qiang et al., 2022
	Zmbadh2a, *Zmbadh2b*	Improved sugar and acid metabolism	Wang et al., 2021
	SbLG1	Yield improvement	Brant et al., 2021
	SbBADH2	Quality improvement	Suebpongsang et al., 2020

	CRTL, PSY	Enhanced vitamin A content	Dong et al., 2020
	Wx1	Low amylose content	Qi et al., 2020
	MS8	Male sterility	Chen et al., 2018
	ARGOS8	Enhanced grain yield in drought condition	Shi et al., 2017
	ALS1 and *ALS2*	Resistance against chlorosulfuron herbicides	Svitashevet al., 2016
Soybean (*Glycine max*)	*GmFAD2*	Increased fatty acid	Zhou et al., 2023
	GmHsp90A2	Heat resistance	Jianing et al., 2022
	GmSNAP11, α-SNAP	Soybean cyst nematode resistance	Shaibu et al., 2022; Usovsky et al., 2023
	Glyma05g29080	White mold resistance	Zhang et al., 2022
	GmUGT	Resistance against insects (leaf chewing)	Zhang et al., 2022
	GmF3H1, *GmF3H2* and *GmFNSII-1*	Increased isoflavone content and resistance to soya bean mosaic virus	Zhang et al., 2020
	GmFAD2	Increased oleic acid and decreased linoleic and α-linolenic acid	Do et al., 2019
	GmFT2a, GmFT5a	Late flowering	Caiet al., 2018
Barley *(Hordeum vulgare)*	*GW2.1*	Improved yield	Kis et al., 2024
	Hina	Increased grain hardness	Jiang et al., 2022
	HGGT, HPT	Increased vitamin A biosynthesis	Zeng et al., 2020
	HvITPK1	Increased phosphate levels	Vlčko and Ohnoutková, 2020
	HvMORC1	Resistance to *Blumeriagraminis* and *Fusarium graminearum*	Kumar et al., 2018
	HvCKX1	Improved plant productivity and decreased total grain biomass	Holubovaet al., 2018

Challenges

Genome editing technologies like CRISPR/Cas system offer several benefits for the agriculture due to their less-complicated, robust and multiplex targeting. Due to their high efficacy and accuracy, these systems are used to overcome the confines that existed while using the conventional breeding methods for the development of the disease resistant, high yielding and better agricultural crops. In spite of several benefits and enormous application, CRISPR/Cas 9 has quite few shortcomings. The development of the disease resistant crops by the use of CRISPR/Cas 9 system is obstructed by a few important hindrances.

These hindrances include:

(i) **High fitness cost**: Some fitness cost may be caused due to the direct targeting of the host susceptible genes as they are linked with the other growth and developing genes in plants. Moreover, the disruption of the any host susceptible genes may affect the formation of several products in the pathway and eventually other products in the plant due to the mutation in the desired target gene. This may cause the insufficiency of several important nutrients and may cause abnormal phenotypic changes. Nevertheless, the fitness cost may modify based on the targeting susceptible gene/s and additionally is governed by numerous elements, i.e. architects of disease susceptibility, defense suppressors, genes or pre-penetration factors involved in the replication machinery. Use of base editing methods, promoter targeting to create immediate alleles, introduction and designing of susceptible gene variants and knock-in of desired characters is the possible solution to control this phenomenon. Research has been intensively working on natural or synthetic pathogen inducible promoters or regulatory elements from the past two decades. By the use of regulatory elements, pathogen inducible CRISPR/ Cas 9 systems can be shaped, which can quickly knockout a host susceptible gene involved in synthesis of a specific micronutrient or a sugar promoter and eventually evading fitness consequences. Without regarding the barriers of the species, CRISPR/Cas 9 editing can be used for the production of the desired host susceptible gene mutants in most of the plants of interest (Wang et al., 2022).

(ii) **Off- target mutations**: Another challenge faced by the CRISPR/ Cas9 system, mainly in the construction of transgene-free crops is off-target mutations. Off-targeting can happen due

to misguide by the gRNA or may be gRNA independent in nature and refers to the modifications in the DNA at non-specific, unintended and unwanted sites (Hajiahmadi et al., 2019). It has become a major barrier in the production of targeted mutations at the desired sites. Several methods are being developed in largely two directions to find a solution to this problem of off-targeting: evolving a technique for distinguishing off-target mutations and developing the CRISPR/Cas system with high precision. Today numerous bioinformatics tools, such as CasOFFinder (http://www.rgenome.net/cas-offinder/) and CCTop (https://crispr.cos.uniheidelberg.de) and several other tools which include SELEX, IDLV capture, Guide-seq, HTGTS, BLESS, Digenome-seq (Wang et al., 2023) and DISCOVER (Zou et al., 2023) have been established as a measure against this matter. As each tool has its individual positive and negative aspects, the researchers have to select their analytical tool depending on their nature of work and their need. On the other hand, many enhancements are being made in the CRISPR/Cas 9 system to reduce the off-target mutations. Firstly, Cas 9 proteins including eSpCas9 eSpCas9, HiFiCas9 and HypaCas9 and Sniper Cas9 were created to expand the target specificity of the enzyme. eSpCas9 (Kim et al 2020), HF-Cas9 and HypaCas9 were technologically advanced by structural alterations to enhance specificity, whereas Sniper Cas9 was screened from a library of SpCas9 mutants that displayed enhanced specificity (Moon et al., 2019). The improved Cas proteins exhibited extraordinarily reduced off-target levels whereas retaining on-target activity. Enhanced specificity has also been achieved by gRNA engineering. The synthesis of guide RNA has itself delivered convenient and adaptable opportunities to advance the CRISPR/Cas system. Chemical synthesis, *in vitro* transcription, or intracellular transcription systems can be used for the synthesis of the gRNAs. Guide RNAs can be engineered in numerous ways, including chemical alterations, modifications in the spacer length, sequence alterations in the spacer or scaffold, blending with additional DNA or RNA components, and partial replacement with DNA. The engineered guide RNAs are responsible for enhanced genome editing efficiency and target specificity , regulation of biological toxicity, sensitive and specific molecular imaging, multiplexing, and genome editing flexibility (Zhou et al., 2023). Lately, off-target mutations have also been discovered in rice due to cytosine base editors, but no off-target mutations were detected in adenine base editors. This shows that the new tools devised also need developments (Jin et al., 2019). Off- targeting has been removed in many of the important crops.

(iii) **Commercialization of the crops**: The safety and commercialization of the crops generated by the mutations caused by the CRISPR/Cas system are associated with the humans and other living organisms. The crops generated by the editing using the CRISPR/ Cas9 system are transgene-free and thus do not contain any foreign element in their genome. Therefore these crops would not be considered as transgenic and thus their adoption for commercial cultivation becomes easier. But, the adoption of the genome-edited crops is an issue. Concerning about the problem, several countries are debating on this issue due to rules pertaining to GMO crops, while many countries have adopted these genome-edited crops (Turnbull et al., 2021).

(iv) **Resistance against viruses**: DNA/RNA viruses editing: earlier, CRISPR/Cas 9 has been very useful in creating the virus-resistant crops by accurately and efficiently mutating the genetic material of the DNA- and RNA-based viruses. But due to compromise with the virus immunity in several plants, the ability and efficiency of this system has been questioned. Therefore, the expansion of a well-organized, effective and openly and satisfactorily acceptable form of CRISPR such as CRISPR/Cas13a is immediately needed.

(v) **Plants with unknown genome**: The use of CRISPR/Cas 9 system cannot be used on the plants whose genomes are still not known. Moreover, its applicability is also limited for the plants in which the functions of certain proteins are not known.

(vi) **Delivery systems**: Present delivery systems are restricted to explicit plant species, genotypes, and tissues. In addition, more or less all the existing methods need tissue culture, an extensive and laborious process. Refining the current delivery systems and emergence of new systems will be crucial in reducing obstacles to low-cost application of gene editing in plants (Zhang, 2019). To increase the range of delivery systems, both Agrobacterium and plant genes could be manipulated to advance the Agrobacterium-mediated transformation. Plant germline or meristematic cells can be used for establishing genotype-independent, tissue culture–free delivery systems for the delivery of the CRISPR/ Cas 9 in plants (Gordon-Kamm et al., 2021). The emergence of the sperm cells, the egg cells and the zygote as the realistic target for delivery is a boon for the CRISPR/Cas technology. The limitation of the species specificity can be avoided by the use of pollen mediated transformation and the regeneration using pollination and artificial hybridization. Moreover, the use of shoot apical meristem for the delivery of the CRISPR/ Cas is evident as the stem cells are destined to

differentiate into gametes. New delivery systems grounded on nanotechnology and virus particle–like structures too embrace a potential for crop improvement (Abdallah et al., 2025)

(viii) **Limited PAM sequences**: The action of CRISPR/Cas system requires the PAM sequence for the identification and cleavage of the target DNA, and thus the missing of the PAM sequence causes a problem in the action the system. Moreover, there are very limited PAM sites present.

(ix) **Low HDR efficacy**: A challenge of HDR-mediated gene editing is that it needs synchronized introduction of DSBs and delivery of a repair template to one site inside the genome. There are numerous possible ways to increase the frequency of HDR in plant cells, for example, management of DNA repair pathways (Ahmad et al., 2022)

Conclusion

The CRIPSR/Cas system has permitted cost-effective and efficient gene editing compared with prior technologies, comprising zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), making it available to many scientists. The ease, flexibility, and sturdiness of CRISPR/Cas systems make genome editing an influential tool for efficient crop improvement via gene knockout, knock-in, replacement, point mutations, fine-tuning of gene regulation, and other alterations at any gene locus in crops. This system can be further extended to the crops with complex genomes or to the crops with unknown genomes to further extend the technology to a broader prospect, by investigating and developing more efficient delivery systems.

Author contributions

MKD conceived the review and provided the possible outline. SC, TS, SK, collected the information and wrote the first draft of the manuscript. MKD edited and finished the final draft of the manuscript.

Declarations

Consent for publication

We hereby give our informed consent for the publication of this manuscript and any accompanying materials, including images or data that may directly or indirectly disclose our identity, as part of the publication process.

Conflict of interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript.

References

1. Abdelrahman, M., Wei, Z., Rohila, J. S., & Zhao, K. (2021). Multiplex genome-editing technologies for revolutionizing plant biology and crop improvement. *Frontiers in Plant Science*, *12*, 721203.
2. Ahmad, Y., Haider, S., Iqbal, J., Abbasi, B. A., Yaseen, T., &Mahmood, T. (2022). The mechanisms of genome editing technologies in crop plants. In *Principles and practices of OMICS and genome editing for crop improvement* (pp. 295-313). Cham: Springer International Publishing.
3. Aksoy, E., Yildirim, K., Kavas, M., Kayihan, C., Yerlikaya, B. A., Çalik, I., ...&Demirel, U. (2022). General guidelines for CRISPR/Cas-based genome editing in plants. *Molecular biology reports*, *49*(12), 12151-12164.
4. Ali, A., Zafar, M. M., Farooq, Z., Ahmed, S. R., Ijaz, A., Anwar, Z., ...&Maozhi, R. (2023). Breakthrough in CRISPR/Cas system: Current and future directions and challenges. *Biotechnology Journal*, *18*(8), 2200642.
5. Allemailem, K. S., Almatroudi, A., Rahmani, A. H., Alrumaihi, F., Alradhi, A. E., Alsubaiyel, A. M., ...& Khan, A. A. (2024). Recent updates of the CRISPR/Cas9 genome editing system: Novel approaches to regulate its spatiotemporal control by genetic and physicochemical strategies. *International Journal of Nanomedicine*, 5335-5363.
6. Arra, Y., Auguy, F., Stiebner, M., Chéron, S., Wudick, M. M., Miras, M., ...&Albar, L. (2024). Rice Yellow Mottle Virus resistance by genome editing of the Oryza sativa L. ssp. japonica nucleoporin gene OsCPR5. 1 but not OsCPR5. 2. *Plant Biotechnology Journal*, *22*(5), 1299-1311.
7. Ashraf, S., Ghouri, M. Z., Javed, M. A., Zafar, H., Ali, H., Qari, S. H., & Ahmad, A. (2022). RNA editing with CRISPR/Cas13. In *The CRISPR/Cas Tool Kit for Genome Editing* (pp. 219-254). Singapore: Springer Singapore.
8. Bhagtaney, L., &Sundarrajan, P. (2023). An overview of tools for genome editing: ZFNs, mega nucleases, and TALENs. *CRISPR/Cas-mediated genome editing in plants*, 37-64.
9. Bharat, S. S., Li, S., Li, J., Yan, L., & Xia, L. (2020). Base editing in plants: current status and challenges. *The Crop Journal*, *8*(3), 384-395.
10. Bhardwaj, A., & Nain, V. (2021). TALENs—an indispensable tool in the era of CRISPR: a mini review. *Journal of Genetic Engineering and Biotechnology*, *19*(1), 125.
11. Brant, E. J., Baloglu, M. C., Parikh, A., &Altpeter, F. (2021). CRISPR/Cas9 mediated targeted mutagenesis of LIGULELESS-1 in sorghum provides a rapidly scorable phenotype by altering leaf inclination angle. *Biotechnology journal*, *16*(11), 2100237.
12. Chang, H. H., Pannunzio, N. R., Adachi, N., &Lieber, M. R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nature reviews Molecular cell biology*, *18*(8), 495-506.
13. Char SiNian, C. S., Wei Jialu, W. J., Mu Qi, M. Q., Li Xianran, L. X., Zhang ZhanyuanJ, Z. Z., Yu Jianming, Y. J., & Yang Bing, Y. B. (2020). An Agrobacterium-delivered CRISPR/Cas9 system for targeted mutagenesis in sorghum.
14. Chatterjee, N., & Walker, G. C. (2017). Mechanisms of DNA damage, repair, and mutagenesis. *Environmental and molecular mutagenesis*, *58*(5), 235-263.

15. Chaudhary, E., Chaudhary, A., Sharma, S., Tiwari, V., &Garg, M. (2024). Different classes of CRISPR-Cas systems. In *Gene editing in plants: CRISPR-cas and its applications* (pp. 73-94). Singapore: Springer Nature Singapore.
16. Chen, H., Ye, R., Liang, Y., Zhang, S., Liu, X., Sun, C., ...& Yi, J. (2023). Generation of low-cadmium rice germplasms via knockout of OsLCD using CRISPR/Cas9. *Journal of Environmental Sciences*, *126*, 138-152.
17. Cheng, H., Jeong, E., & Cho, S. W. (2025). Applications of multiplexed CRISPR–Cas for genome engineering. *Experimental & Molecular Medicine*, *57*(7), 1373-1380.
18. Chib, S., Thangaraj, A., Kaul, S., Dhar, M. K., & Kaul, T. (2020). Development of a system for efficient callus production, somatic embryogenesis and gene editing using CRISPR/Cas9 in Saffron (Crocus sativus L.). *Plant methods*, *16*(1), 47.
19. Chuang, C. K., & Lin, W. M. (2021). Points of view on the tools for genome/gene editing. *International Journal of Molecular Sciences*, *22*(18), 9872.
20. Cui, Y., Cao, Q., Li, Y., He, M., & Liu, X. (2023). Advances in cis-element-and natural variation-mediated transcriptional regulation and applications in gene editing of major crops. *Journal of Experimental Botany*, *74*(18), 5441-5457.
21. David, S. R., Maheshwaram, S. K., Shet, D., Lakshminarayana, M. B., &Soni, G. V. (2022). Temperature dependent in vitro binding and release of target DNA by Cas9 enzyme. *Scientific reports*, *12*(1), 15243.
22. Deb, S., Choudhury, A., Kharbyngar, B., &Satyawada, R. R. (2022). Applications of CRISPR/Cas9 technology for modification of the plant genome. *Genetica*, *150*(1), 1-12.
23. Ding, J., Ji, C., Yu, L., Wang, C., Ding, G., Wang, S., ...&Cai, H. (2024). OsMYB84, a transcriptional regulator of OsCOPT2 and OsHMA5, modulates copper uptake and transport and yield production in rice. *The Crop Journal*, *12*(2), 456-469.
24. Ding, Y., Zhu, J., Zhao, D., Liu, Q., Yang, Q., & Zhang, T. (2021). Targeting cis-regulatory elements for rice grain quality improvement. *Frontiers in Plant Science*, *12*, 705834.
25. Dong, O. X., Yu, S., Jain, R., Zhang, N., Duong, P. Q., Butler, C., ...& Ronald, P. C. (2020). Marker-free carotenoid-enriched rice generated through targeted gene insertion using CRISPR-Cas9. *Nature communications*, *11*(1), 1178.
26. Errum, A., Rehman, N., Uzair, M., Inam, S., Ali, G. M., & Khan, M. R. (2023). CRISPR/Cas9 editing of wheat Ppd-1 gene homoeologs alters spike architecture and grain morphometric traits. *Functional & integrative genomics*, *23*(1), 66.
27. Feng, Z., Zhang, B., Ding, W., Liu, X., Yang, D. L., Wei, P., ...& Zhu, J. K. (2013). Efficient genome editing in plants using a CRISPR/Cas system. *Cell research*, *23*(10), 1229-1232.
28. Ganger, S., Harale, G., &Majumdar, P. (2023). Clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) systems: Discovery, structure, classification, and general mechanism. In *CRISPR/Cas-Mediated Genome Editing in Plants* (pp. 65-97). Apple Academic Press.
29. Garcia-Robledo, J. E., Barrera, M. C., &Tobón, G. J. (2020). CRISPR/Cas: from adaptive immune system in prokaryotes to therapeutic weapon against immune-related diseases: CRISPR/Cas9 offers a simple and inexpensive method for disease modeling, genetic screening, and potentially for disease therapy. *International Reviews of Immunology*, *39*(1), 11-20.
30. González Castro, N., Bjelic, J., Malhotra, G., Huang, C., &Alsaffar, S. H. (2021). Comparison of the feasibility, efficiency, and safety of genome editing technologies. *International Journal of Molecular Sciences*, *22*(19), 10355.
31. Gorski, S. A., Vogel, J., &Doudna, J. A. (2017). RNA-based recognition and targeting: sowing the seeds of specificity. *Nature Reviews Molecular Cell Biology*, *18*(4), 215-228.

32. Gupta, R., Gupta, D., Ahmed, K. T., Dey, D., Singh, R., Swarnakar, S., ...&Ghosh, D. (2021). Modification of Cas9, gRNA and PAM: key to further regulate genome editing and its applications. *Progress in Molecular Biology and Translational Science*, *178*, 85-98.
33. He, F., Wang, C., Sun, H., Tian, S., Zhao, G., Liu, C., ...&Guo, J. (2023). Simultaneous editing of three homoeologues of TaCIPK14 confers broad-spectrum resistance to stripe rust in wheat. *Plant Biotechnology Journal*, *21*(2), 354-368.
34. Hillary, V. E., &Ceasar, S. A. (2023). A review on the mechanism and applications of CRISPR/Cas9/Cas12/Cas13/Cas14 proteins utilized for genome engineering. *Molecular biotechnology*, *65*(3), 311-325.
35. Hu, J., Yu, M., Chang, Y., Tang, H., Wang, W., Du, L., ...& Ye, X. (2022). Functional analysis of TaPDI genes on storage protein accumulation by CRISPR/Cas9 edited wheat mutants. *International journal of biological macromolecules*, *196*, 131-143.
36. Hu, S., Chen, Y., Qian, C., Ren, H., Liang, X., Tao, W., ...& Huang, X. (2024). Nuclear accumulation of rice UV-B photoreceptors is UV-B-and OsCOP1-independent for UV-B responses. *Nature Communications*, *15*(1), 6396.
37. Huang, Q., Lin, B., Cao, Y., Zhang, Y., Song, H., Huang, C., ...&Zhuo, K. (2023). CRISPR/Cas9-mediated mutagenesis of the susceptibility gene OsHPP04 in rice confers enhanced resistance to rice root-knot nematode. *Frontiers in Plant Science*, *14*, 1134653.
38. Ibrahim, S., Saleem, B., Rehman, N., Zafar, S. A., Naeem, M. K., & Khan, M. R. (2022). CRISPR/Cas9 mediated disruption of Inositol Pentakisphosphate 2-Kinase 1 (TaIPK1) reduces phytic acid and improves iron and zinc accumulation in wheat grains. *Journal of Advanced Research*, *37*, 33-41.
39. Jiang, F., &Doudna, J. A. (2017). CRISPR–Cas9 structures and mechanisms. *Annual review of biophysics*, *46*, 505-529.
40. Jin, Y. Y., Zhang, P., & Liu, D. P. (2025). Optimizing homology-directed repair for gene editing: the potential of single-stranded DNA donors. *Trends in Genetics*..
41. Kan, J., Cai, Y., Cheng, C., Chen, S., Jiang, C., He, Z., & Yang, P. (2023). CRISPR/Cas9-guided knockout of eIF4E improves wheat yellow mosaic virus resistance without yield penalty. *Plant Biotechnology Journal*, *21*(5), 893.
42. Karvelis, T., Gasiunas, G., &Siksnys, V. (2017). Methods for decoding Cas9 protospacer adjacent motif (PAM) sequences: a brief overview. *Methods*, *121*, 3-8.
43. Kim, D., Alptekin, B., &Budak, H. (2018). CRISPR/Cas9 genome editing in wheat. *Functional & integrative genomics*, *18*(1), 31-41.
44. Kim, M. S., Ko, S. R., Jung, Y. J., Kang, K. K., Lee, Y. J., & Cho, Y. G. (2023). Knockout mutants of OsPUB7 generated using CRISPR/Cas9 revealed abiotic stress tolerance in rice. *International Journal of Molecular Sciences*, *24*(6), 5338.
45. Kim, M. S., Le, V. T., Jung, Y. J., Kang, K. K., & Cho, Y. G. (2024). OsPUB9 gene edited by CRISPR/Cas9 enhanced resistance to bacterial leaf blight in rice (Oryza sativa L.). *International Journal of Molecular Sciences*, *25*(13), 7145.
46. Krishna, T. A., Maharajan, T., &Ceasar, S. A. (2023). The role of membrane transporters in the biofortification of zinc and iron in plants. *Biological trace element research*, *201*(1), 464-478.
47. Kumar, K., Gambhir, G., Dass, A., Tripathi, A. K., Singh, A., Jha, A. K., ...&Rakshit, S. (2020). Genetically modified crops: current status and future prospects. *Planta*, *251*(4), 91.
48. Lei, C., Li, S. Y., Liu, J. K., Zheng, X., Zhao, G. P., & Wang, J. (2017). The CCTL (C pf1-assisted C utting and T aq DNA ligase-assisted L igation) method for efficient editing of large DNA constructs in vitro. *Nucleic acids research*, *45*(9), e74-e74.
49. Li, J., Jiao, G., Sun, Y., Chen, J., Zhong, Y., Yan, L., ...& Xia, L. (2021). Modification of starch composition, structure and properties through editing of TaSBEIIa in both winter and spring wheat varieties by CRISPR/Cas9. *Plant biotechnology journal*, *19*(5), 937-951.

580 550. Li, J., Wang, Z., He, G., Ma, L., & Deng, X. W. (2020). CRISPR/Cas9-mediated disruption
581 of TaNP1 genes results in complete male sterility in bread wheat. *Journal of genetics and*
582 *genomics*, *47*(5), 263-272.
583 551. Li, S., Zhang, Y., Liu, Y., Zhang, P., Wang, X., Chen, B., ...& Mao, H. (2024). The E3 ligase
584 TaGW2 mediates transcription factor TaARR12 degradation to promote drought resistance in
585 wheat. *The Plant Cell*, *36*(3), 605-625.
586 552. Li, X., Guo, T., Mu, Q., Li, X., & Yu, J. (2018). Genomic and environmental determinants
587 and their interplay underlying phenotypic plasticity. *Proceedings of the National Academy of*
588 *Sciences*, *115*(26), 6679-6684.
589 553. Li, X., Zhang, J., Shangguan, X., Yin, J., Zhu, L., Hu, J., ...&Lv, W. (2023). Knockout of
590 OsWRKY71 impairs Bph15-mediated resistance against brown planthopper in rice. *Frontiers*
591 *in Plant Science*, *14*, 1260526.
592 554. Li, Z., Huang, Q., Lin, B., Guo, B., Wang, J., Huang, C., ...&Zhuo, K. (2022). CRISPR/Cas9-
593 targeted mutagenesis of a representative member of a novel PR10/Bet v1-like protein
594 subfamily significantly reduces rice plant height and defense against
595 Meloidogynegraminicola. *Phytopathology Research*, *4*(1), 38.
596 555. Liang, J. R., & Corn, J. E. (2022). A CRISPR view on autophagy. *Trends in Cell*
597 *Biology*, *32*(12), 1008-1022.
598 556. Liao, C., &Beisel, C. L. (2021). The tracrRNA in CRISPR biology and technologies. *Annual*
599 *review of genetics*, *55*(1), 161-181.
600 557. Limbalkar, O. M., Srivastava, P., Reddy, K. R., Lali, S., Lal, S. K., Bishi, S. K.,
601 ...&Tribhuvan, K. U. (2025). Genome editing and its impact on crop improvement: current
602 approaches and future prospects. *Discover Plants*, *2*(1), 358.
603 558. Liu, G., Li, J., & Godwin, I. D. (2019). Genome editing by CRISPR/Cas9 in sorghum
604 through biolistic bombardment. In *Sorghum: Methods and protocols* (pp. 169-183). New
605 York, NY: Springer New York.
606 559. Liu, Q., Sun, Q., & Yu, J. (2024). Gene editing's sharp edge: Understanding zinc finger
607 nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered
608 regularly interspaced short palindromic repeats (CRISPR). *Transactions on Materials,*
609 *Biotechnology and Life Sciences*, *3*, 170-179.
610 560. Lu, M., Mingfeng, T., Yuxing, Z., &Longtao, T. (2023). Knocking-out OsPDR7 triggers up-
611 regulation of OsZIP9 expression and enhances zinc accumulation in rice. *Rice Science*, *30*(1),
612 36-49.
613 561. Ludwig, Y., DueñasJr, C., Arcillas, E., Macalalad-Cabral, R. J., Kohli, A., Reinke, R.,
614 &Slamet-Loedin, I. H. (2024). CRISPR-mediated promoter editing of a cis-regulatory
615 element of OsNAS2 increases Zn uptake/translocation and plant yield in rice. *Frontiers in*
616 *Genome Editing*, *5*, 1308228.
617 562. Macovei, A., Sevilla, N. R., Cantos, C., Jonson, G. B., Slamet-Loedin, I., Čermák, T.,
618 ...&Chadha-Mohanty, P. (2018). Novel alleles of rice eIF4G generated by
619 CRISPR/Cas9-targeted mutagenesis confer resistance to Rice tungro spherical virus. *Plant*
620 *biotechnology journal*, *16*(11), 1918-1927.
621 563. Malzahn, A. A., Tang, X., Lee, K., Ren, Q., Sretenovic, S., Zhang, Y., ...& Qi, Y. (2019).
622 Application of CRISPR-Cas12a temperature sensitivity for improved genome editing in rice,
623 maize, and Arabidopsis. *BMC biology*, *17*(1), 9.
624 564. Mangrauthia, S. K., Molla, K. A., Sundaram, R. M., Chinnusamy, V., &Bansal, K. C. (2024).
625 Genomics and genome editing for crop improvement. In *Transformation of agri-food*
626 *systems* (pp. 297-322). Singapore: Springer Nature Singapore.
627 565. Marone, D., Mastrangelo, A. M., &Borrelli, G. M. (2023). From transgenesis to genome
628 editing in crop improvement: applications, marketing, and legal issues. *International Journal*
629 *of Molecular Sciences*, *24*(8), 7122.

66. Maurya, K., Mani, B., Singh, B., Sirohi, U., Jaskolowski, A., Sharma, S., ...&Giri, J. (2025). Editing cis-elements of OsPHO1; 2 improved phosphate transport and yield in rice. *Plant biotechnology journal*, *23*(9), 3864-3878.
67. McGinn, J., &Marraffini, L. A. (2019). Molecular mechanisms of CRISPR–Cas spacer acquisition. *Nature Reviews Microbiology*, *17*(1), 7-12.
68. Meyenberg, M., Ferreira da Silva, J., &Loizou, J. I. (2021). Tissue specific DNA repair outcomes shape the landscape of genome editing. *Frontiers in Genetics*, *12*, 728520.
69. Mishra, R., Agarwal, P., &Mohanty, A. (2023). Applications of genome editing techniques for the improvement of medicinal plants. In *Phytochemical genomics: Plant metabolomics and medicinal plant genomics* (pp. 545-569). Singapore: Springer Nature Singapore.
70. Mohamadi, S., Bostanabad, S. Z., &Mirnejad, R. (2020). CRISPR arrays: A review on its mechanism.
71. Mohr, T., Horstman, J., Gu, Y. Q., Elarabi, N. I., Abdallah, N. A., &Thilmony, R. (2022). CRISPR-Cas9 gene editing of the Sal1 gene family in wheat. *Plants*, *11*(17), 2259.
72. Molla, K. A., Sretenovic, S., Bansal, K. C., & Qi, Y. (2021). Precise plant genome editing using base editors and prime editors. *Nature Plants*, *7*(9), 1166-1187.
73. Montecillo, J. A. V., Chu, L. L., &Bae, H. (2020). CRISPR-Cas9 system for plant genome editing: current approaches and emerging developments. *Agronomy*, *10*(7), 1033.
74. Nambiar, T. S., Baudrier, L., Billon, P., &Ciccia, A. (2022). CRISPR-based genome editing through the lens of DNA repair. *Molecular cell*, *82*(2), 348-388.
75. Narayanan, Z., & Glick, B. R. (2023). Biotechnologically engineered plants. *Biology*, *12*(4), 601.
76. Nasrallah, A., Sulpice, E., Kobaisi, F., Gidrol, X., &Rachidi, W. (2022). CRISPR-Cas9 technology for the creation of biological avatars capable of modeling and treating pathologies: from discovery to the latest improvements. *Cells*, *11*(22), 3615.
77. Nazarov, P. A., Baleev, D. N., Ivanova, M. I., Sokolova, L. M., &Karakozova, M. V. (2020). Infectious plant diseases: etiology, current status, problems and prospects in plant protection. *Actanaturae*, *12*(3), 46.
78. Nguyen, T. T., Pham, D. T., Nguyen, N. H., Do, P. T., & To, H. T. M. (2023). The Germin-like protein gene OsGER4 is involved in heat stress response in rice root development. *Functional & Integrative Genomics*, *23*(3), 271.
79. Okada, A., Arndell, T., Borisjuk, N., Sharma, N., Watson-Haigh, N. S., Tucker, E. J., ...&Whitford, R. (2019). CRISPR/Cas9-mediated knockout of Ms1 enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. *Plant biotechnology journal*, *17*(10), 1905-1913.
80. Oliva, R., Ji, C., Atienza-Grande, G., Huguet-Tapia, J. C., Perez-Quintero, A., Li, T., ...& Yang, B. (2019). Broad-spectrum resistance to bacterial blight in rice using genome editing. *Nature biotechnology*, *37*(11), 1344-1350.
81. Ouyang, C., Jin, X., Zhao, H., Chen, S., Zhao, G., Li, D., ...& An, B. (2025). Generating Broad-Spectrum Resistance to ALS-Inhibiting Herbicides in Rice by CRISPR/Cas9-Mediated NHEJ. *Rice*, *18*(1), 86.
82. Pacesa, M., Loeff, L., Querques, I., Muckenfuss, L. M., Sawicka, M., &Jinek, M. (2022). R-loop formation and conformational activation mechanisms of Cas9. *Nature*, *609*(7925), 191-196.
83. Palanivelu, P. (2021). Assessment and Analyses of Homing Endonucleases and Mechanism of Action of CRISPR-Cas9 HNH Endonucleases. *Curr. Adv. Chem. Biochem*, *1*, 20-48.
84. Patel, A., Miles, A., Strackhouse, T., Cook, L., Leng, S., Patel, S., ...&Potlakayala, S. D. (2023). Methods of crop improvement and applications towards fortifying food security. *Frontiers in Genome Editing*, *5*, 1171969.

679 85. Qiang, Z., Sun, H., Ge, F., Li, W., Li, C., Wang, S., ...& Fu, Y. (2022). The transcription
680 factor ZmMYB69 represses lignin biosynthesis by activating ZmMYB31/42 expression in
681 maize. *Plant Physiology*, *189*(4), 1916-1919.
682 86. Raffan, S., Sparks, C., Huttly, A., Hyde, L., Martignago, D., Mead, A., ...&Halford, N. G.
683 (2021). Wheat with greatly reduced accumulation of free asparagine in the grain, produced by
684 CRISPR/Cas9 editing of asparagine synthetase gene TaASN2. *Plant Biotechnology*
685 *Journal*, *19*(8), 1602-1613.
686 87. Rao, M. J., & Wang, L. (2021). CRISPR/Cas9 technology for improving agronomic traits and
687 future prospective in agriculture. *Planta*, *254*(4), 68.
688 88. Rozov, S. M., Permyakova, N. V., &Deineko, E. V. (2019). The problem of the low rates of
689 CRISPR/Cas9-mediated knock-ins in plants: approaches and solutions. *International journal*
690 *of molecular sciences*, *20*(13), 3371.
691 89. Sánchez-León, S., Gil-Humanes, J., Ozuna, C. V., Giménez, M. J., Sousa, C., Voytas, D. F.,
692 &Barro, F. (2018). Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. *Plant*
693 *biotechnology journal*, *16*(4), 902-910.
694 90. Santosh, G. M. (2020). *Genome Editing by CRISPR/Cas9 for Potyvirus Resistance in*
695 *Tomato* (Doctoral dissertation, University of Agricultural Sciences, GKVK).
696 91. Sarsu, F., Penna, S., &Nikalje, G. C. (2023). Strategies for screening induced mutants for
697 stress tolerance. In *Mutation breeding for sustainable food production and climate*
698 *resilience* (pp. 151-176). Singapore: Springer Nature Singapore.
699 92. Savage, D. F. (2019). Cas14: big advances from small CRISPR proteins. *Biochemistry*, *58*(8),
700 1024-1025.
701 93. Shen, C., Que, Z., Xia, Y., Tang, N., Li, D., He, R., & Cao, M. (2017). Knock out of the
702 annexin gene OsAnn3 via CRISPR/Cas9-mediated genome editing decreased cold tolerance
703 in rice. *Journal of Plant Biology*, *60*(6), 539-547.
704 94. Shen, Y., Ye, Q., Wu, Z., Jiang, W., Wang, L., Zhang, Q., ...& Hu, L. (2024). Functional
705 characterization of OsCAT2 gene in rice that regulates ROS scavenging and plant growth and
706 development. *Plant Growth Regulation*, *103*(1), 165-175.
707 95. Sheng, X., Ai, Z., Tan, Y., Hu, Y., Guo, X., Liu, X., ...& Yuan, D. (2023). Novel salinity-
708 tolerant third-generation hybrid rice developed via CRISPR/Cas9-mediated gene
709 editing. *International Journal of Molecular Sciences*, *24*(9), 8025.
710 96. Shi, Y., Zhang, Y., Sun, Y., Xie, Z., Luo, Y., Long, Q., ...&Luo, J. (2023). Natural variations
711 of OsAUX5, a target gene of OsWRKY78, control the neutral essential amino acid content in
712 rice grains. *Molecular Plant*, *16*(2), 322-336.
713 97. Shufen, C., Yicong, C., Baobing, F., Guiai, J., Zhonghua, S., Ju, L. U. O., ...&Xiangjin, W.
714 (2019). Editing of rice isoamylase gene ISA1 provides insights into its function in starch
715 formation. *Rice Science*, *26*(2), 77-87.
716 98. Suebpongsang, P., Ekasingh, B., &Cramb, R. (2020). Commercialisation of rice farming in
717 northeast Thailand. In *White gold: The commercialisation of rice farming in the Lower*
718 *Mekong Basin* (pp. 39-68). Singapore: Springer Nature Singapore.
719 99. Sun, Y., Jiao, G., Liu, Z., Zhang, X., Li, J., Guo, X., ...& Xia, L. (2017). Generation of high-
720 amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching
721 enzymes. *Frontiers in plant science*, *8*, 298.
722 100. Sun, L., Lai, M., Ghouri, F., Nawaz, M. A., Ali, F., Baloch, F. S., ...&Shahid, M. Q. (2024).
723 Modern plant breeding techniques in crop improvement and genetic diversity: From
724 molecular markers and gene editing to artificial intelligence—A critical
725 review. *Plants*, *13*(19), 2676.
726 101. Swarup, S., Cargill, E. J., Crosby, K., Flagel, L., Kniskern, J., & Glenn, K. C. (2021). Genetic
727 diversity is indispensable for plant breeding to improve crops. *Crop Science*, *61*(2), 839-852.

102. Taj, M., Sajjad, M., Li, M., Yasmeen, A., Mubarik, M. S., Kaniganti, S., & He, C. (2022). Potential targets for CRISPR/Cas knockdowns to enhance genetic resistance against some diseases in wheat (Triticumaestivum L.). *Frontiers in Genetics*, *13*, 926955.
103. Tan, W., Wang, Z., & Liu, L. (2024). The continuous improvement of the clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein system has led to its highly efficient application in plants. *Agriculture*, *15*(1), 29.
104. Tang, L., Mao, B., Li, Y., Lv, Q., Zhang, L., Chen, C., ...& Zhao, B. (2017). Knockout of OsNramp5 using the CRISPR/Cas9 system produces low Cd-accumulating indica rice without compromising yield. *Scientific reports*, *7*(1), 14438.
105. Teng, F., Li, J., Cui, T., Xu, K., Guo, L., Gao, Q., ...& Li, W. (2019). Enhanced mammalian genome editing by new Cas12a orthologs with optimized crRNA scaffolds. *Genome biology*, *20*(1), 15.
106. Teper, D., White, F. F., & Wang, N. (2023). The dynamic transcription activator-like effector family of Xanthomonas. *Phytopathology®*, *113*(4), 651-666.
107. Tyagi, S., Kumar, R., Kumar, V., Won, S. Y., &Shukla, P. (2021). Engineering disease resistant plants through CRISPR-Cas9 technology. *GM crops & food*, *12*(1), 125-144.
108. Varble, A., &Marraffini, L. (2022). The CRISPR-Cas system of Streptococcus pyogenes: function and applications. *Streptococcus pyogenes: Basic Biology to Clinical Manifestations [Internet]*. *2nd edition*.
109. Waites, J., Achary, V. M. M., Syombua, E. D., Hearne, S. J., &Bandyopadhyay, A. (2025). CRISPR-mediated genome editing of wheat for enhancing disease resistance. *Frontiers in Genome Editing*, *7*, 1542487.
110. Wang, F., Wang, C., Liu, P., Lei, C., Hao, W., Gao, Y., ...& Zhao, K. (2016). Enhanced rice blast resistance by CRISPR/Cas9-targeted mutagenesis of the ERF transcription factor gene OsERF922. *PloS one*, *11*(4), e0154027.
111. Wang, H., Feng, M., Jiang, Y., Du, D., Dong, C., Zhang, Z., ...& Liu, J. (2023). ThermosensitiveSUMOylation of TaHsfA1 defines a dynamic ON/OFF molecular switch for the heat stress response in wheat. *The Plant Cell*, *35*(10), 3889-3910.
112. Wang, M., Cheng, J., Wu, J., Chen, J., Liu, D., Wang, C., ...& Shi, W. (2024). Variation in TaSPL6-D confers salinity tolerance in bread wheat by activating TaHKT1; 5-D while preserving yield-related traits. *Nature Genetics*, *56*(6), 1257-1269.
113. Wang, Q., Gao, H., Liu, K., Wang, H., Zhang, F., Wei, L., ...& Yuan, H. (2024). CRISPR/Cas9-mediated enhancement of semi-dwarf glutinous traits in elite Xiangdaowan rice (Oryza sativa L.): targeting SD1 and Wx genes for yield and quality improvement. *Frontiers in plant science*, *15*, 1333191.
114. Wang, W., Wang, W., Pan, Y., Tan, C., Li, H., Chen, Y., ...& Ma, C. (2022). A new gain-of-function OsGS2/GRF4 allele generated by CRISPR/Cas9 genome editing increases rice grain size and yield. *The Crop Journal*, *10*(4), 1207-1212.
115. Wang, X., Wang, Z., Lu, Y., Huang, J., Hu, Z., Lou, J., ...& Chen, X. (2024). OsACA9, an autoinhibited Ca2+-ATPase, synergically regulates disease resistance and leaf senescence in rice. *International Journal of Molecular Sciences*, *25*(3), 1874.
116. Xu, H., Zhao, M., Zhang, Q., Xu, Z., &Xu, Q. (2016). The DENSE AND ERECT PANICLE 1 (DEP1) gene offering the potential in the breeding of high-yielding rice. *Breeding Science*, *66*(5), 659-667.
117. Xu, X., Sun, S. K., Zhang, W., Tang, Z., & Zhao, F. J. (2024). Editing silicon transporter genes to reduce arsenic accumulation in rice. *Environmental science & technology*, *58*(4), 1976-1985.
118. Yali, W., &Mitiku, T. (2022). Mutation breeding and its importance in modern plant breeding. *Journal of Plant Sciences*, *10*(2), 64-70.

119. Yang, H., & Patel, D. J. (2024). Structures, mechanisms and applications of RNA-centric CRISPR–Cas13. *Nature chemical biology*, *20*(6), 673-688.
120. Yang, H., Ren, S., Yu, S., Pan, H., Li, T., Ge, S., ...& Xia, N. (2020). Methods favoring homology-directed repair choice in response to CRISPR/Cas9 induced-double strand breaks. *International journal of molecular sciences*, *21*(18), 6461.
121. Yang, J., Fang, Y., Wu, H., Zhao, N., Guo, X., Mackon, E., ...& Li, R. (2023). Improvement of resistance to rice blast and bacterial leaf streak by CRISPR/Cas9-mediated mutagenesis of Pi21 and OsSULTR3; 6 in rice (Oryza sativa L.). *Frontiers in Plant Science*, *14*, 1209384.
122. Ye, N., Wang, Y., Yu, H., Qin, Z., Zhang, J., Duan, M., & Liu, L. (2023). Abscisic acid enhances trehalose content via OsTPP3 to improve salt tolerance in rice seedlings. *Plants*, *12*(14), 2665.
123. Yu, Z., Yunusbaev, U., Fritz, A., Tilley, M., Akhunova, A., Trick, H., &Akhunov, E. (2024). CRISPR-based editing of the ω-and γ-gliadin gene clusters reduces wheat immunoreactivity without affecting grain protein quality. *Plant Biotechnology Journal*, *22*(4), 892-903.
124. Zess, E., &Begemann, M. (2021). CRISPR-Cas9 and beyond: what's next in plant genome engineering. *In Vitro Cellular & Developmental Biology-Plant*, *57*(4), 584-594.
125. Zhang, J., Zhang, H., Botella, J. R., & Zhu, J. K. (2018). Generation of new glutinous rice by CRISPR/Cas9-targeted mutagenesis of the Waxy gene in elite rice varieties. *Journal of integrative plant biology*, *60*(5), 369-375.
126. Zhang, L., Xiang, Z., Li, J., Wang, S., Chen, Y., Liu, Y., ...& Chen, L. (2023). bHLH57 confers chilling tolerance and grain yield improvement in rice. *Plant, Cell & Environment*, *46*(4), 1402-1418.
127. Zhang, N., Wang, S., Zhao, S., Chen, D., Tian, H., Li, J., ...& Chen, F. (2023). Global crotonylatome and GWAS revealed a TaSRT1-TaPGK model regulating wheat cold tolerance through mediating pyruvate. *Science Advances*, *9*(19), eadg1012.
128. Zhang, S., Zhang, R., Gao, J., Song, G., Li, J., Li, W., ...& Li, G. (2021). CRISPR/Cas9-mediated genome editing for wheat grain quality improvement. *Plant Biotechnology Journal*, *19*(9), 1684.
129. Zhang, S., Zhang, R., Song, G., Gao, J., Li, W., Han, X., ...& Li, G. (2018). Targeted mutagenesis using the Agrobacterium tumefaciens-mediated CRISPR-Cas9 system in common wheat. *BMC Plant Biology*, *18*(1), 302.
130. Zhang, Y., Bai, Y., Wu, G., Zou, S., Chen, Y., Gao, C., & Tang, D. (2017). Simultaneous modification of three homoeologs of Ta EDR 1 by genome editing enhances powdery mildew resistance in wheat. *The Plant Journal*, *91*(4), 714-724.
131. Zheng, X., Zhang, S., Liang, Y., Zhang, R., Liu, L., Qin, P., ...& Zhang, Y. (2023). Loss-function mutants of OsCKX gene family based on CRISPR-Cas systems revealed their diversified roles in rice. *The Plant Genome*, *16*(2), e20283.
132. Zhu, Y., Lin, Y., Fan, Y., Wang, Y., Li, P., Xiong, J., ...& Zhang, C. J. (2023). CRISPR/Cas9-mediated restoration of Tamyb10 to create pre-harvest sprouting-resistant red wheat. *Plant biotechnology journal*, *21*(4), 665.