Journal Homepage: - www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI: 10.21474/IJAR01/22757
DOI URL: http://dx.doi.org/10.21474/IJAR01/22757

RESEARCH ARTICLE

CRISPR/CAS FOR BRINGING DESIRED TRAITS IN CROPS

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Manuscript Info

Manuscript History
Received: 10 December 2025
Final Accepted: 12 January 2026
Published: February 2026

Abstract

The projected surge in the global population, concomitant with escalati ng environmental perturbations, necessitates the sustainable production of high quality food to ensure long term food security. In order to overcome this major undertaking, adaptation of innovative, resilient and science driven agricultural strategies has been considered to be an essential step in plant biotechnology. Advanced biotechnological appro aches 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 pr otein 9 system has revolutionised the plant breeding system with except ional achievements. On that account, the present review re-evaluates and discusses the information on genome editing technologies specially CRISPR/Cas9 approach in detailtogether with complete mechanism of CRISPR/Cas, problem and future aspects of the approach aiming to develop desirable agronomic genetic traits.

Introduction:-

Plant breeders from decades are using several techniques namely, cross breeding, mutation breeding and transgenic breeding for enhancing quantitative and qualitative traits of the crop. Cross breeding involves the steps of crossing over between two genetic materials, selection of outstanding progeny and elimination of linked traits from subsequent generations by backcrossing (Mangrauthia et al., 2024). However, the incorporation of genetic variability subsequent to cross breeding is a time taking process and is sometimes hindered by the host genome itself (Swarup et al., 2021). Mutation breeding involves the use of chemical mutagens or physical irradiations for creating random mutations in the plant, to introduce desirable traits (Yali et al., 2022). Nevertheless, screening the large number of mutants is ardous (Sarsu et al., 2023). Transgenic breeding involves the transfer of exogenous genes into the crop through different transformation methods, 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 this technology (Marone et al., 2023). Thus, the adaptation of novel, expedited and efficient biotechnological techniques, to create advanced crops with better characteristics is a need of the hour, and is essential to enhance the agricultural yield and accelerates sustainable agricultural development (Patel et al., 2023).

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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 editing techniques leading to site directed mutagenesis has been developed during last few years. Zinc fingers are the naturally occuring sequence specific DNA binding transcription factors (TF) in eukaryotes (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 target DNA. ZFNs came into existence after the identification of FokI restriction enzymes, wherein it was figured out that the recognition and the cleavage domain of FokI are physically separable and therefore, recognition domain of interest can be incorporated. So, FokI cleavage domain cuts double stranded DNA by assembling different combination of zinc finger on them (Abdelrahman et al., 2021) (Figure 1). Using this strategy, zinc finger nucleases (ZFNs) were the first engineered endonucleases which are site directed and are created by the fusion of 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 (Limbalkar et al., 2025). Despitethe numerous uses, potential of ZFNs technology has not been completely harnessed, as its nucleases require protein engineering for recognizing the target DNA sequence (Liu et al., 2024). Therefore, due to construction complexity, high but variable off-target rate, high cost and skill, and difficulty in customization, its application in the field of genome editing gets restricted (Castro et al., 2021).

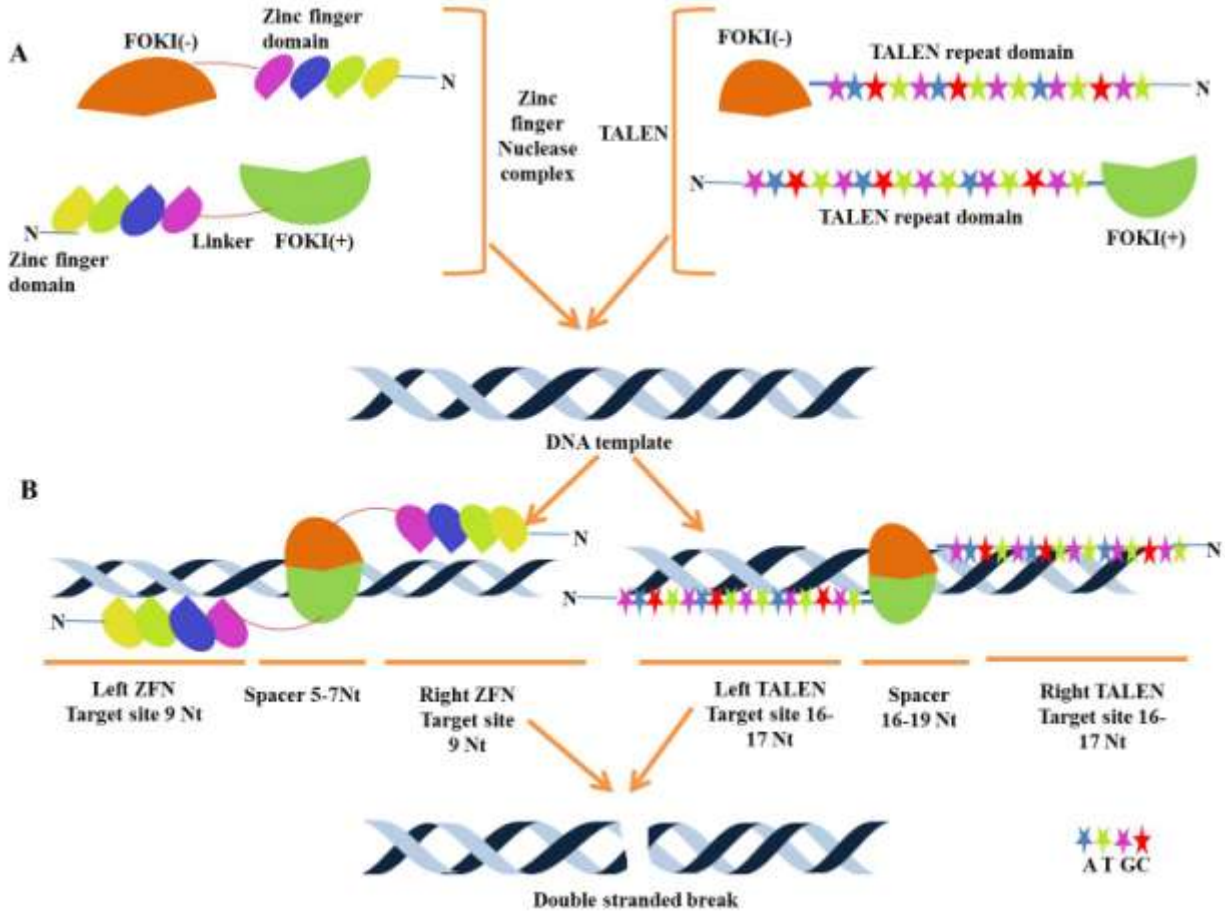

Figure 1: Comparative mechanism of Zinc finger nucleases (ZFN) and Transcription activator-like effector nucleases (TALENS). A) dimers of ZNF and TALENS (Left and Right with FOKI endonuclease domain) B) recognition and attachment of ZFN and TALENS on target DNA, causing double stranded break.

Transcription activator-like effector nucleases (TALENs):-

TALENs arose from studies of bacterial plant pathogen of the genus *Xanthomonas*, which produces and secretes proteins into plant host cell. In the host cells, these proteins bind upstream of the host gene and regulate its

transcription in a way that promotes the bacterial infection (Teperet al., 2023). TALENs are chimeric enzymes formed by the fusion of transcription activator like (TALE) protein 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. These repeats differ at two hyper variable amino acid positions that determine the specific nucleotide to which each nucleotide 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 involves no re-engineering of proteins (Mishra et al., 2023), the 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 to create DSB (Bhagtaney and Sundarrajan 2023). Thus, to overcome the shortcomings of these techniques, a new technique known as CRISPR came into existence.

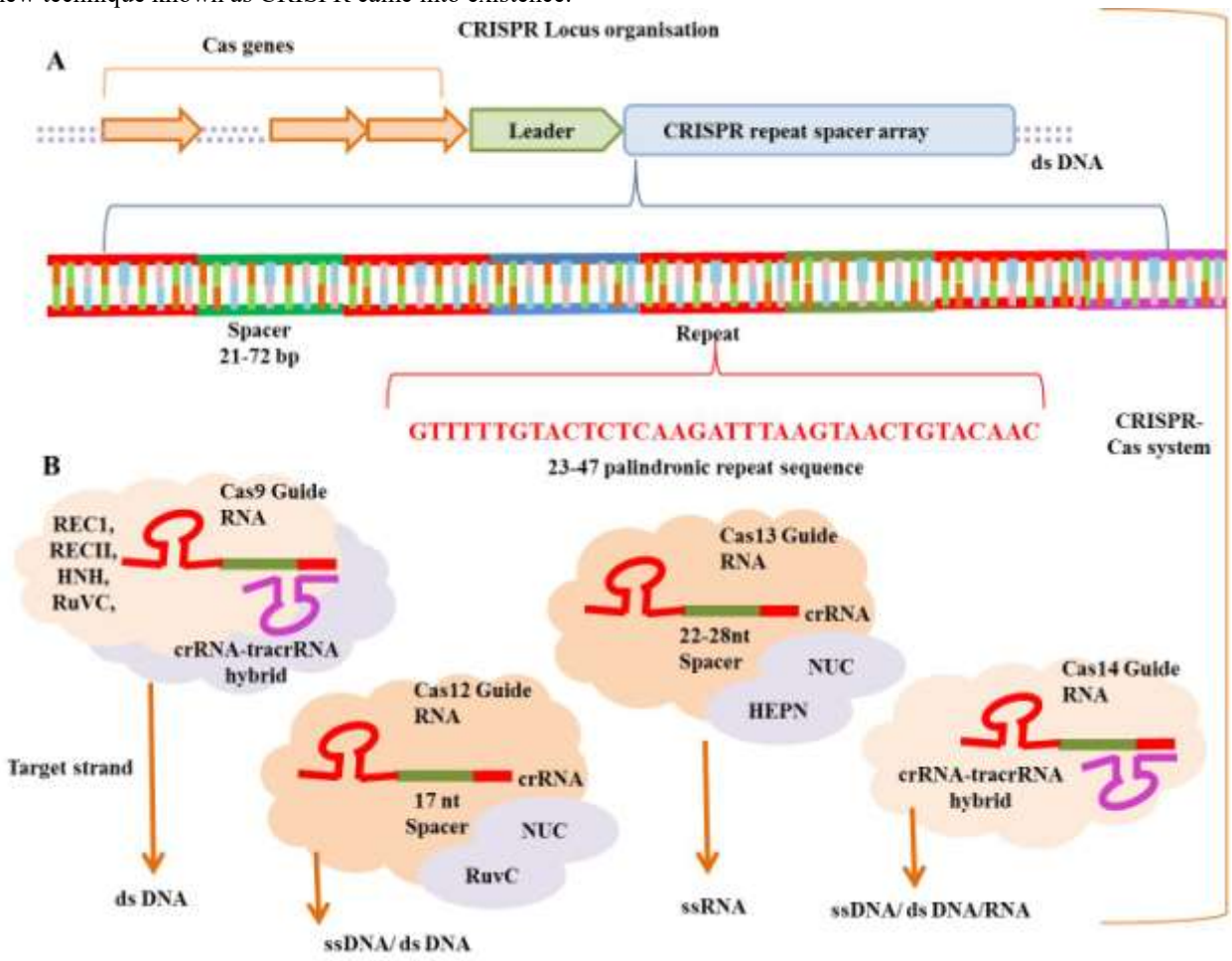

Figure 2: CRISPR/Cas system A) CRISPR locus organization incorporating Cas gene, leader sequence (upstresm to CRISPR repeat (Red) spacer array B) complex of different Cas proteins like 9, 12, 13 and 14 forming the guide RNA . NUC- Nuclease lobe, HNH- Ribonuclease domain and RuVC-Ribonuclease, REC1 and 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 system (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 practical benefit 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 providing protection against invading viruses (Garcia-Robledoet al., 2020). CRISPR system has been classified into two; Class 1 and Class 2, further categorized into three types each. Class 1 (Types I, III, and IV) utilizes multiple Cas proteins for RNA guided cleavage, while Class 2 (Types II, V, and VI) relies on only one endonuclease protein for the DNA cleavage (Hillary and Ceasar 2023). The most commonly used system is type II CRISPR/Cas 9 system adapted from *Streptococcus pyogenes* and repurposed for genome editing by modifying the DNA sequences by either deletion or insertion of new gene sequences (Liang and Corn, 2022). The CRISPR array is mainly composed of two components; first component comprises of two types of RNAs i.e CRISPR RNA or crRNA, Tracer RNA or tracrRNA and the other component consists of 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 site (Mosterd et al., 2021). In phase 1, when the bacteria is infected by the phage, the Cas, such as Cas1 and Cas2 recognizes the protospacer (a 20 -50 bp short sequence similar to the spacer sequence in CRISPR array) from the invading phage or virus and stores it as a new spacer on CRISPR locus generating memory for the invader for future recognition (McGinn and Marraffini, 2021) (Figure 3).

Phase 2 involves the transcription of CRISPR array, resulting into the formation of the long precursor RNA (pre-crRNA), which 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. Finally, the Cas 9 protein binds to this duplex resulting into the formation of the single guide RNA (sgRNA) (Munawar and Ahmad 2021). Phase 3 involves the target recognition and cleavage with the help of Cas9. There is a sequence known as PAM (Protospacer Adjacent Motif), adjacent to the target sequences in the foreign DNA in most of the CRISPR/Cas systems (Ding et al., 2021). The CRISPR system also contains a guide RNA (almost 20 nt) that 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 to create 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 20 nt 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 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 reveal that mutation in any of these two domains of the Cas 9 converts it to nickase (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).

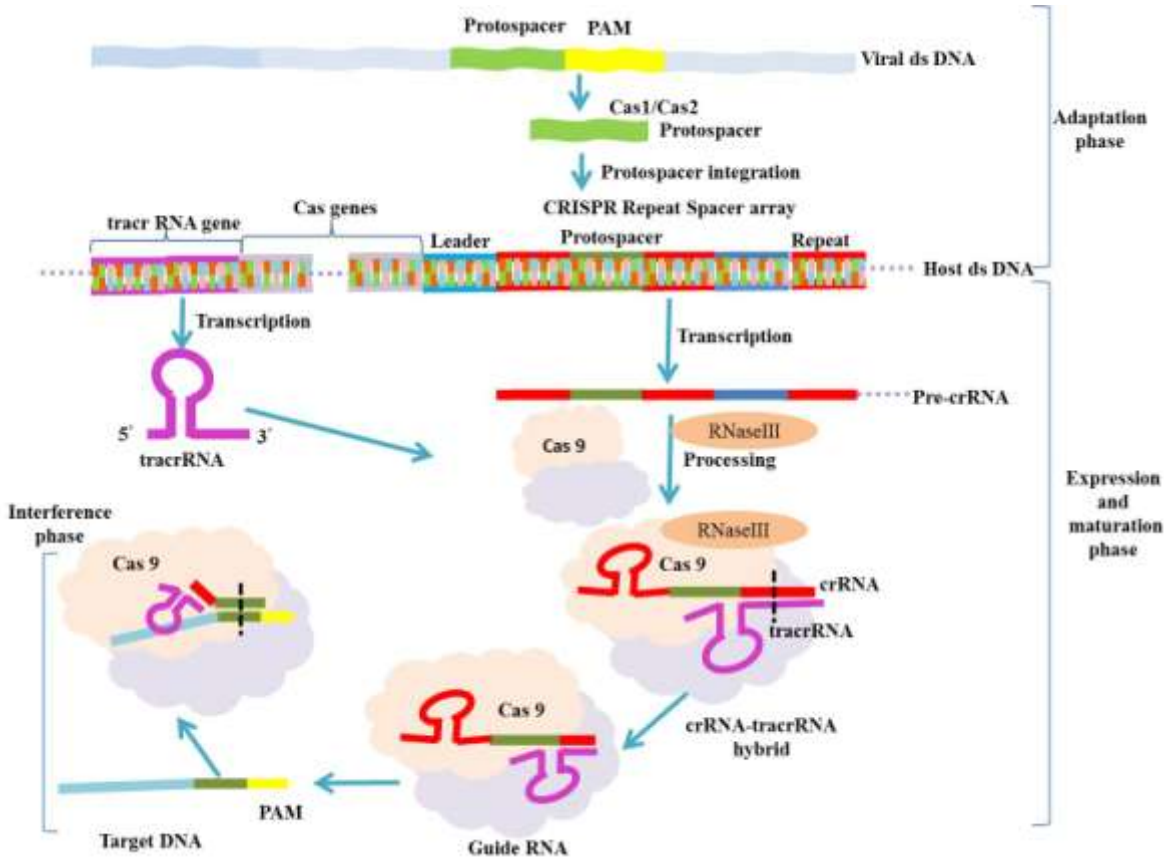

Figure 3: Mechanism of CRISPR-Cas9 activity highlighting three phases- Adaptation –Integration of protospacer in CRISPR array, Expression- Generation of Guide RNA (complex of Cas9-with crRNA and tracRNA, Interference phase- Degradation of target DNA

Binding of the Cas 9 with the sgRNA indicates preparedness of the enzyme for searchingr the complementary sites in the target DNA (David et al., 2022). The two pre-requisite conditions for the recognition of the target sequence are: the complementary base pairing between the 20 nt spacer and the protospacer in 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 belongs *to Streptococcus pyogenes* which has a 5'-NGG-3' PAM sequence in the target DNA. The PAM sequence sustains profound relevance in the type II CRISPR/Cas 9 system as it helps in distinguishing 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 with the recognition of suitable PAM in the target site by Cas 9, 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), 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 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).

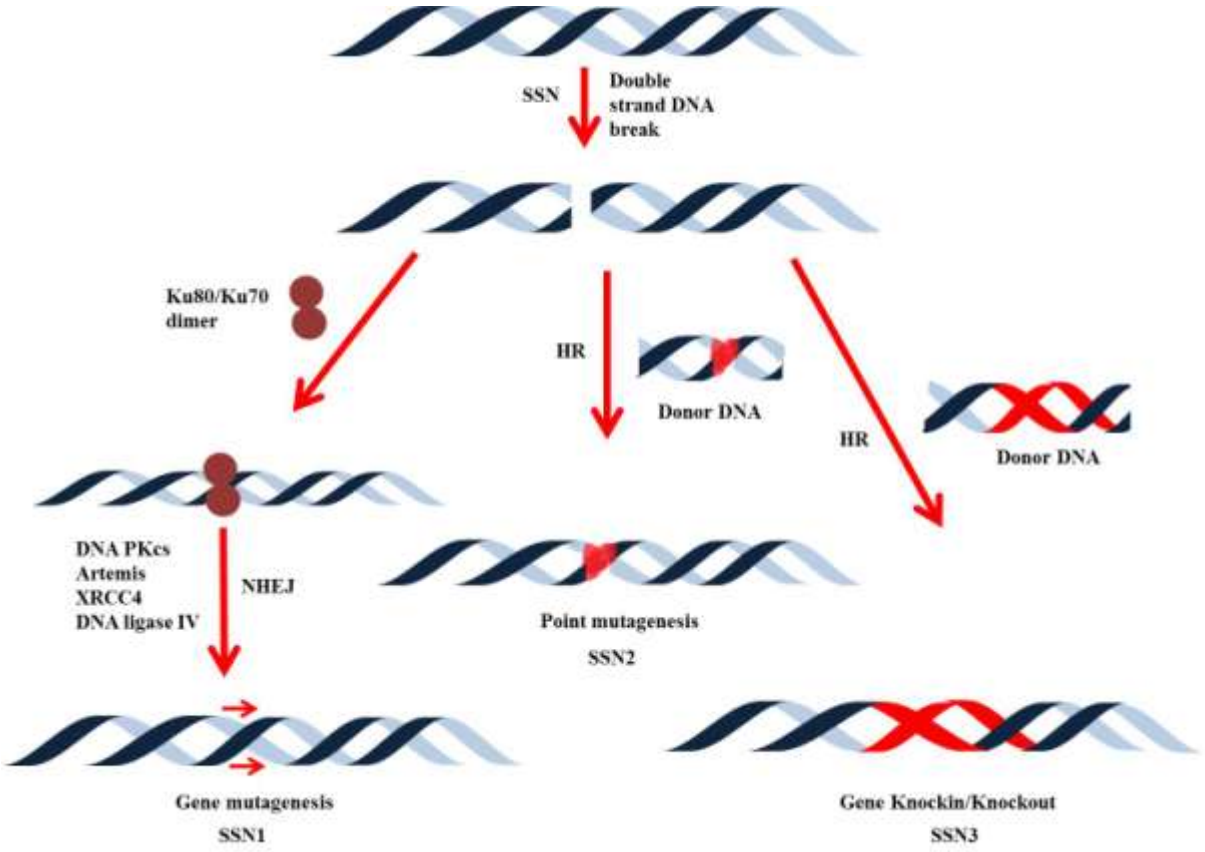

Figure 4: Categorization of site specific nuclease approach (SSN), depicting double stranded break repair (DSB) by non-homologous end joining (NHEJ) or homologous repair (HR) method in ZFNs / TALENs and CRISPR, SSN 1 result in small deletion/insertion via NHEJ, SSN 2 for point mutation via HR and SSN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

All cells face double stranded breaks during cell cycle that are repaired preponderantly by NHEJ. The DNA ends are identified, selected, polymerised and ligated in a versatile manner by the proteins 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 and sometimes resulting into random mutations (Chatterjee and Walker, 2017). HDR is another mechanism for the repair of the targeted DNA. As compared to NHEJ, HDR occurs 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 alterations (Jin et al., 2025).

Other systems of CRISPR:-

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

Cas12- The system belonging to type V CRISPR, exhibits different cleavage mechanism than Cas9. Cas12 proteins cleave both 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) (Figure 2).

Cas13- Recently, another Cas proteins- Cas13 has been introduced into the CRISPR associated gene editing mechanism. It belongs to class 2 type VI of CRISPR and works only on RNA. The CRISPR/Cas13 system involves only crRNA as guide RNA for the cleavage of target site, which is catalyzed by the activity of higher eukaryotes and prokaryotes nucleotide-binding domains (HEPN). Since there is no DNA break, the host repair mechanism is absent in this sytem. However, there are high chances of cleavage of non-target RNA as the HEPN domain of the Cas13 is present on the outer surface, resulting in programmed cell death (Ashraf et al., 2022; Yang and Patel, 2024) (Figure 2).

Cas14- Latterly, Doudna's group explored Cas14, a 400-700 amino acids small protein with molecular weight of 40-70 kd. The small size of Cas14 protein makes it efficient for targeting ssDNA using only T-rich sequences without the requirement of PAM sequence. However, it cannot target dsRNA or ssRNA (Savage, 2019). For cleavage of ssDNA, similar to Cas9, Cas14 also requires the tracrRNA and crRNA complex (Figure 2). In term of efficiency, Cas14 system has proven to be more efficient and specific than Cas9, Cas12 and Cas13. Moreover, 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).

Application of CRISPR/Cas 9 in plants:-

Concerning the surging need of food resources with increasing population, the food and agricultural researchers are displaying significant attention towards 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 previously existing technologies. The CRISPR/Cas9 genome editing has been used to edit plant genome more precisely and efficiently (Chib et al., 2020). The main applications (Figure 5) are as below:-

- **Crop trait improvement via knockout**: Eradication of undesirable gene (Knock-out) using CRISPR/Cas is considered to be the most promising approach in the field of genetics. Several traits like better yield, quality, biotic- and abiotic-stress resistance, hybrid-breeding techniques and several other aspects of crop productivity have been amended (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 exists naturally has been made simple by knock-ins and replacements via CRISPR/Cas system. Moreover, multiple traits can be modified via knock-in by 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 examples 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, an effective and influential tool act as a substitute 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, modification of gene expression is a beneficial method for investigating the 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 of gene expression is mainly contributed by promoter replacement and deletion of cis-regulatory elements (Cui et al., 2023).
- **Antiviral plant breeding strategies**: The viral infections are amongst the chief reasons that lead to the damage of valuable crops in natural ecosystem. 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 emerging as a significant 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).

Figure 5: Impact of CRISPR/Cas9 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	Ding et al., 2024

		copper	
	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	Oliva et 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,	Alteration in grain number, panicle architecture,	Xuet al., 2016

	GS3, and IPA1	grain size and plant architecture	
	OsERF922	Resistance against Rice blast	Wang et al., 2016

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

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

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	Holubova et al., 2018

Challenges:-

Genome editing technologies like CRISPR/Cas system offer several benefits for the crop improvement due to their less-complicated, robust and multiplex targeting. Owing to their high efficacy and accuracy, these systems are used to overcome the constraints of conventional breeding methods for the development of disease resistant, high yielding and better agricultural crops. Despite bestowing substantial utility, CRISPR/Cas9 harbours few shortcomings. The development of the disease resistant crops by the use of CRISPR/Cas9 system is obstructed by a few critical hindrances.

These hindrances include:

(i) **High fitness cost**: Some fitness cost might 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 might 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 (Wang et al., 2022) Use of base editing methods, promoter targeting to create immediate alleles, introduction and designing of susceptible gene variants and knock-in of desired characters can be the possible solution to control this phenomenon. Extensive research has been carried out on natural or synthetic pathogen inducible promoters or regulatory elements from the past two decades. By the use of regulatory elements, pathogen inducible CRISPR/Cas9 systems can be shaped, which in turn quickly knockout a host susceptible gene involved in the synthesis of a specific micronutrient or a sugar promoter, eventually evading fitness consequences. Without concerning any barriers of the species, CRISPR/Cas9 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 the gRNA is 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 order to mitigate the issue of off-targeting: these include the evolvement of a technique for distinguishing off-target mutations and the development of 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 the need and nature of work. On the other hand, many enhancements are being made in the CRISPR/Cas9 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 has been screened from a library of SpCas9 mutants that displayed enhanced specificity (Moon et al., 2019). The improved Cas proteins have 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, 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 developed using the CRISPR/Cas9 editing 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, 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/Cas9 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 also 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 absence of the PAM sequence creating problem in the action of the system. Moreover, very limited numbers of PAM sites are 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 to 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 the crops. The CRISPR system is highly relevant for sustainable agriculture and ethical regulations, as this gene editing system works well with diverse plant species and generates transgene-free edited plants. This system can be further extended to the crops with complex genomes/ unknown genomes to further extend the technology to a broader prospect.Despite progress in CRISPR, certain challenges like off-targeting, efficient delivery and genotype dependent response remains areas of critical research. Therefore, further improvement to address these drawbacks will be critical for responsible implementation of CRISPR technology. Overall it is a powerful and indispensable tool for bringing global food security under changing climate, as it is capable of bringing desirable agronomic traits.

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.

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