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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(11): 105-120
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www.thepharmajournal.com
Received: 05-09-2021
Accepted: 30-10-2021

BS Thorat
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

SG Bhave
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

SG Shinde
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

RL Kunkerkar
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

BD Waghmode
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

SS Desai
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

VV Dalvi
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

UB Pethe
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

SV Sawardekar
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

Corresponding Author:
BS Thorat
Department of Agriculture and Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Maharashtra, India

CRISPR/Cas9: A versatile tool for genome editing in crop improvement

BS Thorat, SG Bhave, SG Shinde, RL Kunkerkar, BD Waghmode, SS Desai, VV Dalvi, UB Pethe and SV Sawardekar

Abstract
In a recent era of modernity, a lot of trends have been set in Science and Technology. Despite of vast discoveries always some gaps exists. To bridge up them new trends are set which are commonly known as emerging trends. Although a number of emerging trends have been established but they are specific in their nature to fix the emerging or previous issues. Now a days, in the biological sciences, CRISPR/Cas9 system an emerging approach has been agreed to accomplish the task associated with genome editing (DNA). CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat)-Cas9 is a multipurpose technology for genetic engineering that relies on the complementarity of the guide RNA (gRNA) to a specific sequence and the Cas9 endonuclease activity. It has broadened the agricultural research area, bringing in new opportunities to develop novel plant varieties with deletion of detrimental traits or addition of significant characters. This RNA guided genome editing technology is turning out to be a ground-breaking innovation in distinct branches of plant biology. CRISPR technology is constantly advancing including options for various genetic manipulations like generating knockouts; making precise modifications, multiplex genome engineering and activation and repression of target genes. This system used for improvement of plant nutrition's, enhancement of plant disease resistance and production of drought tolerant plants. This revolutionary system is easy to handle, eco-friendly and easily executable in every biological field including agriculture, health, virology and genetic disorders.

Keywords: CRISPR/Cas9, sgRNA, knockouts, mutation and DNA

Introduction
Over the next 30 years, the world is expected to look for ways to feed additional two billion people. This implies we are responsible to produce a lot more food. Among the different options, one way we might be able to accomplish this is by engineering plants to make them more nutritious, grow faster and more resilient to various stresses. Advancements in Genome editing technologies have revolutionized the fields of functional genomics and crop improvement (Arora and Narula, 2017) [7]. Functional genomics aims on identification of gene function that is achieved by reducing or completely disrupting the normal gene expression. Genome editing is a powerful technique for gene functional studies, genome modification in molecular research, gene therapy and crop breeding, and has the great advantage of imparting novel desired traits to genetic resources. However, the genome editing of plantlets remains to be established. (Sun *et al*., 2015) [91]. Once the desired alterations are achieved, transgenes can be crossed out from the improved variety. Traditionally, the crops were being improved by the conventional and mutation plant breeding techniques leading to point mutations, deletions, rearrangements, and gene duplications, which are now getting constrained by the declining of existing genetic variation of plants, hampering the production for future feeding (Chen and Gao, 2014) [21].
In a recent era of modernity, a lot of trends have been set in Science and Technology. Despite of vast discoveries always some gaps exists. To bridge up them new trends are set which are commonly known as emerging trends. Although a number of emerging trends have been established but they are specific in their nature to fix the emerging or previous issues (Hassan *et al*., 2017) [40]. Over a decade RNAi technology serves as a magic bullet in the genome editing (Das, 2018). RNAi or RNA interference is a sequence-specific method to silence genes by introducing small double-stranded RNA which mediates with nucleic acids and regulate gene expression. Targeted genome editing with site specific nucleases opens an efficient and precise pathway for reverse genetics, genome engineering and targeted transgene integration experiments (Das, 2018).

The advent of site-specific nucleases (SSNs) highlighted the importance of site directed mutagenesis over random mutagenesis (Osakabe *et al*., 2010; Sikora *et al*., 2011) [10, 89]. Random mutagenesis has also its own list of shortcomings too. It produces multiple undesirable rearrangements and mutations, which are expensive and very complex to screen. Gene editing uses engineered SSNs to delete, insert or replace a DNA sequence (Arora and Narula, 2017) [7]. There is an urgent need for efficient crop improvement strategies with novel genome editing techniques like engineered endonucleases/mega-nucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and type II clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) (Figure 1) paved the way for single nucleotide excision mechanism for crop improvement (Pabo *et al*., 2001; Boch *et al*., 2009; Moscou and Bogdanove, 2009) [81, 13, 76]. In mere 3 years, this technique has been trending vast with numerous examples of targeted mutagenesis and targeted regulation of transcriptional control in model as well as crop plants which clearly demonstrates the revolutionary aspects of this novel system (Khatodia *et al*., 2016) [104]. These genome editing technologies use programmable nucleases to increase the specificity of the target locus.

Genome editing modifies a specific genome in precise and predictable manner. There could be varieties of genes, which could be altered in different cell types and organisms with the aid of nucleases that offer targeted alterations. ZFNs is one of the oldest gene editing technologies, developed in the 1990s and owned by Sangamo BioSciences. ZFNs are premeditated restriction enzymes having sequence specific DNA binding zinc finger motifs and non-specific cleavage domain of Fok1 endonuclease. An array of 4-6 binding modules combines to form a single zinc finger unit. Each module recognizes a codon (Pabo *et al*., 2001) [81]. A pair of ZFNs together identifies a unique 18-24 bp DNA sequence and double stranded breaks (DSBs) are made by Fok1 dimer. FokI nucleases are naturally occurring type IIS restriction enzymes that introduce single stranded breaks in a double helical DNA. Hence FokI functions as a dimer, with each catalytic monomer (nickase) cleaving a single DNA strand to create a staggered DSB with overhangs (Pabo *et al*., 2001) [81]. ZFNs have been successfully employed in genome modification of various plants including tobacco, maize, soybean, etc. (Curtin *et al*., 2011; Ainley *et al*., 2013; Baltes *et al*., 2014) [27, 2, 8]. It was taken back due to some drawbacks such as time-consuming and expensive construction of target enzymes, low specificity and high off-target mutations that eventually made way for the new technology. TALENs turned out to be a substitute to ZFNs and were identified as restriction enzymes that could be manipulated for cutting specific DNA sequences. Traditionally, TALENs were considered as long segments of transcription activator-like effector (TALE) sequences that occurred naturally and joined the Fok l domain with carboxylic-terminal end of manipulated TALE repeat arrays (Christian *et al*., 2010) [25]. TALENs contain a customizable DNA-binding domain which is fused with non-specific Fok 1 nuclease domain (Christian *et al*., 2010) [25]. TALENs compared to ZFNs, involve the interaction of individual nucleotide repeats of the target site and amino acid sequences of TAL effector proteins. They can generate overhangs by employing Fok l nuclease domain to persuade site-specific DNA cleavage. It has been widely used to generate non-homologous mutations with higher efficiencies in diverse organisms (Joung and Sander, 2012) [56].

CRISPR/Cas9 targets specific genomic loci with the help of approximately 100 nucleotide (nt) guide RNA (gRNA) sequence. The sgRNA binds to the protospacer adjacent motif (PAM) on targeted DNA *via* Watson and Crick base pairing through 17-20 nt at the gRNA 50- end and guide Cas9 for specific cleavage (Tsai *et al*., 2015). Cas9 stimulates the DNA repair mechanism by introducing DSBs in the target DNA. Repair mechanism involves error prone non-homologous end joining (NHEJ) or homologous recombination (HR) to produce genomic alterations, gene knockouts and gene insertions (Figure 2). NHEJ by far is the most common DSB repair mechanism in somatic plant cells (Puchta, 2005) [84]. Random insertions or deletions by NHEJ in the coding region lead to frame shift mutations, hence creating gene knockouts. CRISPR technology holds potential for loss-of-function, gain-of-function and gene expression analysis.

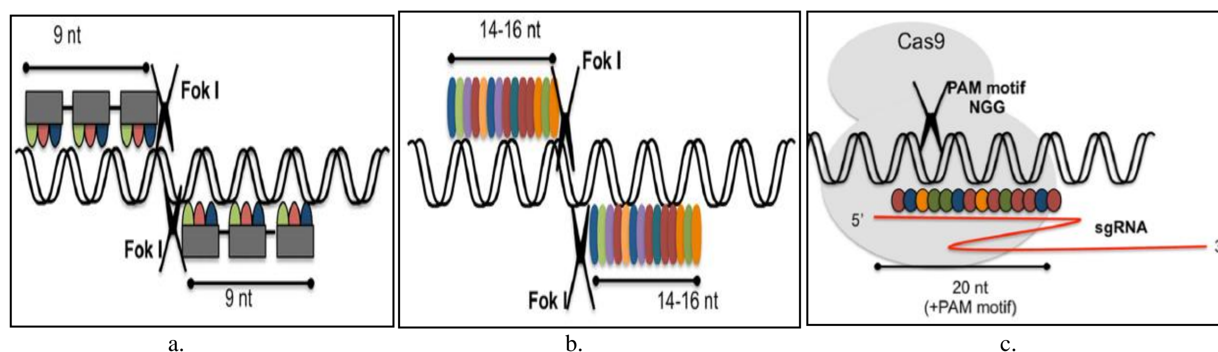

Fig 1: Various genome-editing tools. (A) Zinc-finger nucleases (ZFNs) act as dimer. Each monomer consists of a DNA binding domain and a nuclease domain. Each DNA binding domain consists of an array of 3-6 zinc finger repeats which recognizes 9-18 nucleotides. Nuclease domain consists of type II restriction endonuclease Fok1. (B) Transcription activator-like nucleases (TALENs): these are dimeric enzymes similar to ZFNs. Each subunit consists of DNA binding domain (highly conserved 33-34 amino acid sequence specific for each nucleotide) and Fok1 nuclease domain. (C) CRISPR/Cas9: Cas9 endonuclease is guided by sgRNA (single guide RNA: crRNA and tracrRNA) for target specific cleavage. 20 nucleotide recognition site is present upstream of protospacer adjacent motif (PAM).

The emergence of CRISPR technology supersedes ZFNs and TALENs and used widely as a novel approach from "methods of the year" in 2011 to "breakthrough of the year" in 2015 (Figure 2) for their captivated genome editing (Arora and Narula, 2017) [7].

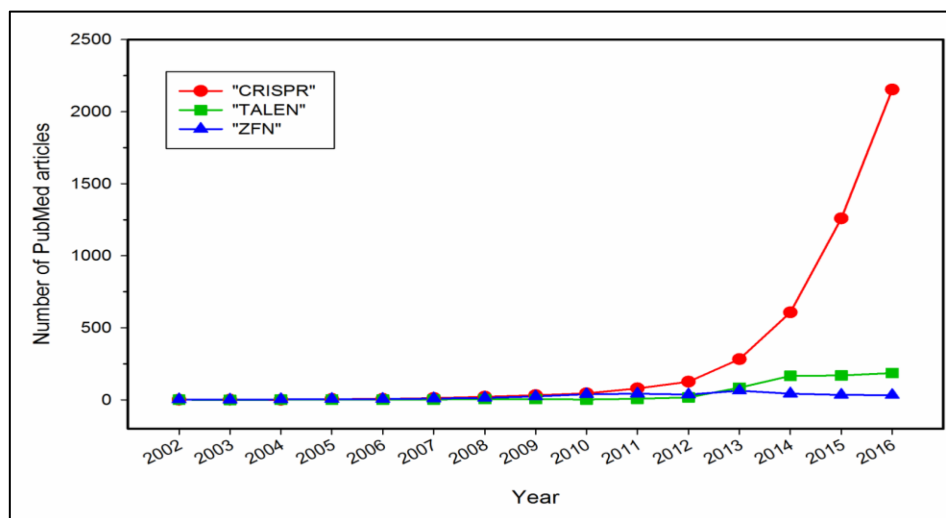

Fig 2: Graphical representation of research papers published in various publications.

Comparative Analysis of different genome editing techniques

CRISPR/Cas9 system has a lot of advantages over other old fashioned genome editing techniques. *i.e.*, Zinc finger nucleases (ZFNs) and Transcription activator like effectors nucleases (TALENs). In ZFNs and TALENs chimeric proteins are programmed against target DNA sequence in every experiment and this was the major issue associated for the wide spread of these genome editing techniques. But in CRISPR/Cas9 system there is no need to design Cas9 protein to target DNA sequence. It employs its own RNA guided nuclease proteins to cleave the specific target site (Mao *et al*., 2013; Hassan *et al*., 2017) [72, 40]. Additionally, success rate of CRISPR/Cas9 system is too high as compared to ZFNs and TALENs. CRISPR/Cas9 system has very low off target effects as compared to ZFNs and TALENs. Furthermore, CRISPR/Cas9 system can be used for multiple genome engineering. This concept is based on designing of many sgRNA which leads to the editing of multiple target sites simultaneously. Usually, multiplexing is used for more complex target genes. Any genomic region in DNA can be targeted by CRISPR Technology by designing sgRNA and specific desired results can be achieved. A brief comparison of genome editing tools is given below in TABLE 1 (Kim and Kim, 2014; Hassan *et al*., 2017) [58, 40].

In this review, we have summarized the broad applicability of Cas9 nuclease mediated targeted genome editing of plants for development of designer crops. The Cas9 system has been used extensively for gene knockouts, gene replacement, multiplex editing, interrogating gene function, and transcription modulation in animals and plants. The opportunities of the gene editing with Cas9/sgRNA for obtaining climate resilient and bio-energy agriculture and to develop non- GM plants along with regulating the uncertainty and the social acceptance of this new plant breeding technique have been prospected in this article.

Table 1: Comparison between different genome editing techniques

Sr. No.	Characteristics	CRISPR/Cas9 System	ZFN	TALEN
1.	Target region specifying unit	SgRNA	Zinc finger DNA binding domain	TALEN DNA binding domain
2.	Enzyme	Cas9 protein	Fok1	Fok1
3.	base pair length of target site	20-22	18-36	30-40
4.	Success rate	High (>99%)	Low (24%)	High (>99%)
5.	Modifications	More (20%)	Less (10%)	More (20%)
6.	Restriction site	PAM region (NGG or NAG)	G-rich	A and T are the first and last bases
7.	Types	Three	No	No

CRISPR/Cas9 System

The CRISPR/Cas9 system, an adaptive immunity system against foreign nucleic acid invaders in prokaryotes, exists in most archaea and numerous bacteria (Koonin and Makarova, 2009) **[59]**.

Background of CRISPR/Cas9 System

CRISPR progress in today's world as genome editing tool can be traced back to its origin in the late 1980s (Ishino *et al*., 1987) [46] and a decade of extensive experimentation since 2005 (Figure 3). CRISPR/Cas9 microbial adaptive immune system and its progress till date is the outcome of the work of numerous researchers around the globe. A series of comprehensive reviews (Bortesi and Fischer, 2015; Amitai and Sorek, 2016; Puchta, 2016) [15 4, 85] gives the detailed information of each aspect of CRISPR/Cas technology.

The word "CRISPR/Cas9" is as Clustered Repetitive Interspaced Short Palindromic Repeats. (Figure 4) Cas9 is a multi-domain nuclease protein (Mojica and Montoliu, 2016) [75]. CRISPR/Cas9 technology is a genome editing approach involving the insertion, deletion and substitution of a specific targeted genome sequence (Belhaj *et al*., 2015) [11].

Fig 3: Key discoveries and advances in CRISPR/Cas9 technology

It has a prokaryotic origin and initially it was reported in *Escherichia coli* (1987) and *Streptococcus pyogenes* (1991). Subsequently it is also recognized in archaea in 1993. In 2002, a group of Netherland "Francisco J.M. Mojica" coined the acronym "CRISPR". Finally, Jennifer Doudna introduced the CRISPR Cas9 system as a new efficient genome editing tool in 2013 in *Streptococcus pyogenes* and in the same year first report on genome editing in mice by employing CRISPR Cas9 system was published (Ishini *et al*., 1987; Hermans et.al., 1991; Cong *et al*., 2013; Mali *et al*., 2013) [46, 41, 26, 70]. For this purpose, mouse embryonic stem cells were harvested. CRISPR/Cas9 system is used as a type II adaptive immune system (Sorek *et al*., 2013) [90] in *Streptococcus pyogenes* to mask themselves from bacteriophages.

Fig 4: Structure of CRISPR loci with tracrRNA (Black) (Trans activating CRISPR RNA), spacers (boxes in different colour) and short palindromic repeats (boxes in black colour).

Assembly Units and Basic Strategy of CRISPR/Cas9 System

CRISPR Cas9 system cleaves the foreign DNA via mainly two components i.e., Cas9 endonuclease enzyme, sgRNA and CRISPR arrays where all immunological memories are engraved. (Figure 5A)

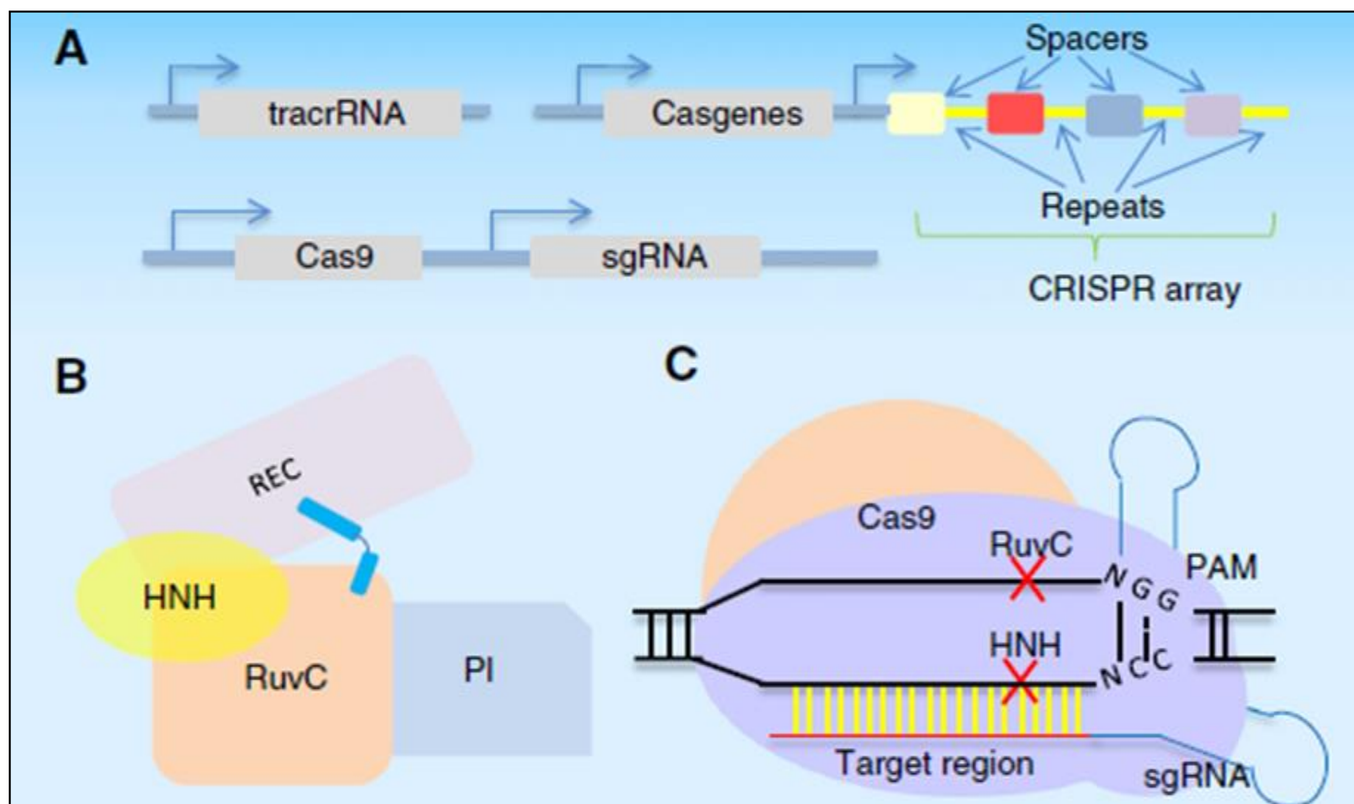

Fig 5: Components of the type II CRISPR/Cas9 system. A. Genomic structures of the native bacterial CRISPR/Cas system (top) and the engineered CRISPR/Cas9 system (bottom). tracrRNA, trans-activator RNA; sgRNA, single guide RNA; B. A schematic representation of the Cas9 protein structure. Domains include REC (large recognition lobe) and RuvC (a nuclease domain) which is linked with an arginine-rich region. HNH is a second nuclease domain. PI, PAM-interacting domain. C. The conformation of Cas9-sgRNA complex in the process of DNA cleavage.

Cas9 contains two domains namely HNH Domain and RuvC like Domain. (FIGURE 5B) The HNH domain cuts the complementary strand of crRNA, while the RuvC like domain cleaves the opposite strand of double strand of genomic DNA. The sgRNA is a synthetic chimeric RNA with a length of about 100nt. The crRNA and tracrRNA combinely form chimeric sgRNA (Doudna and Charpentier, 2014) [30]. CRISPR array is a genomic locus having series of 21-40 bp repeat sequences (direct repeats) interspaced by 25-40 bp variable sequences (spacers) (Jansen *et al*., 2002; Tang *et al*., 2002) [49, 93]. In 2005, three independent research groups (Bolotin *et al*., 2005; Mojica *et al*., 2005; Pourcel *et al*., 2005) [14, 74, 82] hypothesized the role of spacer elements as traces of past invasions of foreign DNA that provide immunity against phage infection. They also noted that spacers share a common end sequence, now known as PAM. Barrangou *et al*. (2007) [10] experimentally demonstrated the involvement of CRISPR arrays in resistance to bacteriophages in association with Cas genes. At every infection, new phage DNA gets incorporated into the CRISPR array building potential to fight the upcoming infection.

The CRISPR arrays, including the spacers, (Figure 5A) are transcribed during subsequent encounters with invasive DNA and are processed into small interfering CRISPR RNAs (crRNAs) approximately 40 nt in length, which combine with the transactivating CRISPR RNA (tracrRNA) to activate and guide the Cas9 nuclease (Barrangou *et al*., 2007) [10]. Role of CRISPR RNA (crRNA) in target recognition and trans-activating crRNA (tracrRNA in crRNA maturation & stabilization of Cas9 protein during complex formation. (Jinek *et al*., 2012) [54]. (FIGURE 5C) CRISPR and tracrRNA have non-coding ability and partially complement to each other. (Feng *et al*., 2013) [33]. The sgRNA having 5' end has a 20nt sequence that act as a guide sequence to identify the target sequence accompanied by a protospacer adjacent motif (PAM) sequence, which is often the consensus NGG (N-any nucleotide; G-Guinine). The loop structure at the 3' end of the sgRNA can anchor the target sequence by the guide sequence and form a complex with Cas9, which cleaves the double stranded DNA by forming double stranded break (DSB) at that site (Das, 2018). (Figure 6)

Once the DSB is generated, the host DNA breakage machinery activates and repaires the DNA double strand break with non-homologous end-joining (NHEJ) or homologous recombination (HR) mechanisms. (FIGURE 6) In NHEj, the host cellular DNA repair systems will tether the DNA double strand break by random insertion or deletion of short stretches of oligonucleotide bases. This mechanism results in the disruption of codon-reading frame followed by disrupting the gene expression. In HR, introduction of segment of DNA with regions having homology to the sequence of flanking both sides of the DNA double strand break will lead to the repair by host machinery through the incorporation of the extra segment of the DNA fragment (Chang *et al*., 2015) [19]. (Figure 6)

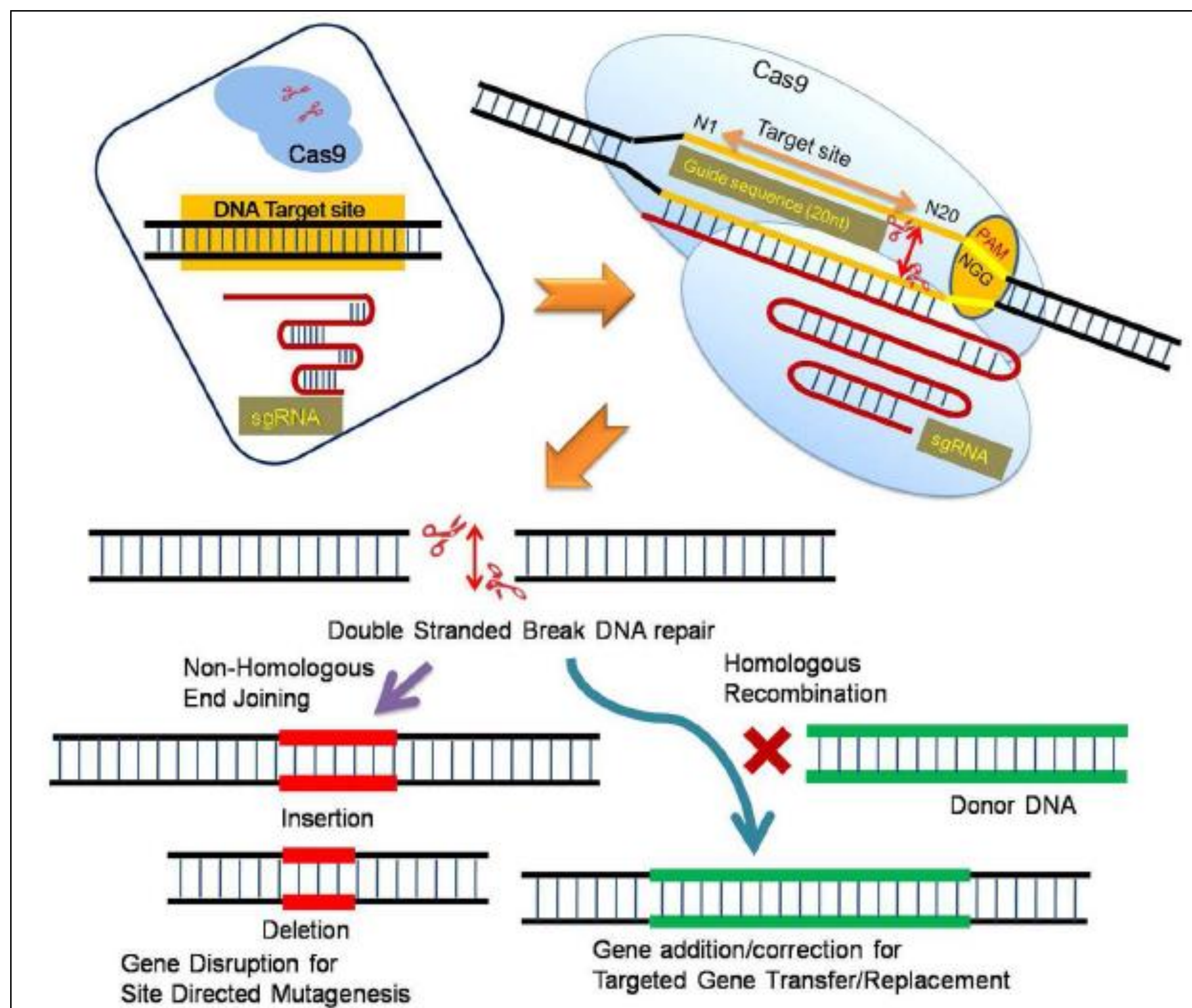

Fig 6: The basic strategy of Cas9/sgRNA system. The Cas9 is a RNA guided endonuclease consists of two nuclease domains namely HNH and RuvC. The target specificity of Cas9 depends upon the guide sequence (20nt) short guide RNA (sgRNA). The target sites must lay immediately 5' of a PAM (Protospacer Adjacent Motif) sequence of the form N20-NGG (or N20-NAG). The Cas9 nuclease induces double stranded breaks (DSB) at the target site which can be repaired either by Non-Homologous End Joining method or Homologous Recombination by cellular system which results in gene disruption by indels or gene addition/correction, respectively. (Khatodia *et al*., 2016) [104]

Classification of CRISPR/Cas System

The system has been categorized into three types (I, II, and III) (FIGURE 7) based on the *Cas* genes and CRISPR sequences present (Makarova *et al*., 2011; Bhya *et al*., 2011 and Barrangou, 2013) [68, 9]. Types I and III contain multiple CAS proteins that form a complex for degrading foreign DNA/RNA. Types I (FIGURE 7A) and III (FIGURE 7C) contain multiple CAS proteins that form a complex for degrading foreign DNA/RNA. Type II directs the cleavage of targeted foreign DNA using a single CAS9 protein, which makes it the system of choice for targeted genome engineering. In the Type II (FIGURE 7B) CRISPR-Cas9 system, CRISPR RNA (crRNA) hybridizes with a small trans-activating CRISPR RNA (trancrRNA) to form mature dual crRNA (Deltcheva *et al*., 2011 and Jinek *et al*., 2012) [29, 54]. The mature crRNA combines with Cas9 to form a functional complex. When the complex recognizes a short seed sequence in the vicinity of a typical 5'-NGG-3' protospacer-adjacent motif (PAM) by RNA/DNA base pairing, Cas9 cleaves the target DNA (Kuscu *et al*., 2014; Wu *et al*., 2014; Anders *et al*., 2014; Hsu *et al*., 2014) [60, 96, 5, 43]. Mature crRNA containing trancrRNA and crRNA can be replaced in the laboratory with a single synthetic guide RNA (sgRNA) (Jinek *et al*., 2012) [54]. Consequently, only sgRNA and Cas9 protein are needed to make genome editing simple and efficient. The CRISPR-Cas9 system has been widely applied in genetic studies of prokaryotes and eukaryotes over the past two years (Hsu *et al*., 2014) [44].

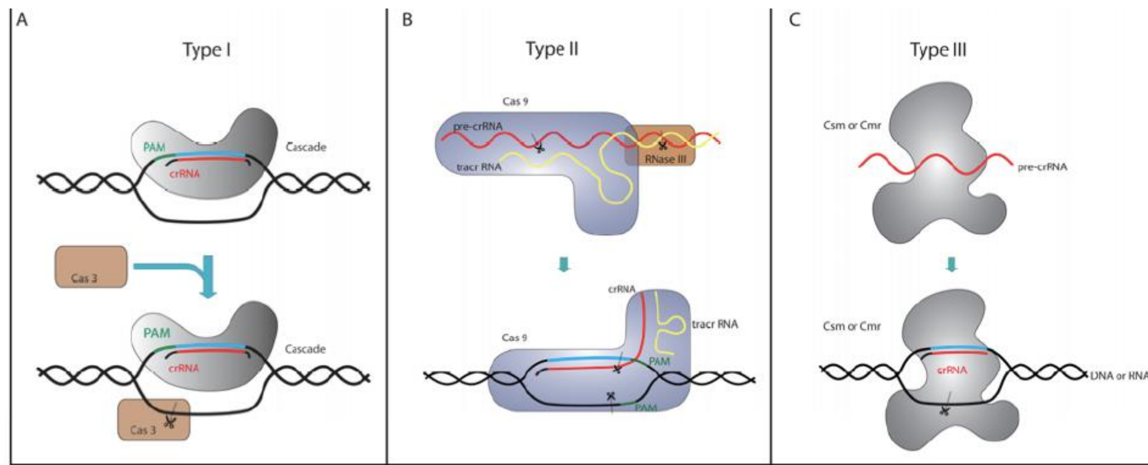

Fig 7: Three different types of CRISPR/Cas system representing the interference step (Richter *et al*., 2013)

The first attempt to classify CRISPR/Cas system was done by Haft *et al*. (2005) (Arora and Narula, 2017) [7]. He defined 45 CRISPR-associated (Cas) protein families that are categorized into core proteins (Cas1, Cas2, Cas3, Cas4, Cas5, Cas6), 8 CRISPR/Cas subtypes and RAMP (repair associated mysterious protein) module in prokaryotic genomes. Makarova *et al*. (2011) [68] classified CRISPR/Cas systems into three types: type I, type II, and type III depending on the presence of signature Cas3, Cas9 and Cas10 proteins, respectively (Table 2). This system was divided into 10 subtypes depending on the presence of additional signature proteins. This three-type classification system is further modified into two class-five type classification systems depending on the type of signature proteins and CRISPR loci (Makarova *et al*., 2015) [69]. Major differences between CRISPR classes are based on the composition of crRNP complexes. Class 1 CRISPRs have multiple subunit effector complexes while class 2 CRISPRs concentrates most of their functions with single protein effectors. Class1 CRISPR systems, for example, have different nucleases for pre-crRNA processing, spacer sequence loading, and targeted cleavage processing. In class 2, a single protein performs all of these functions. Type IV and type V belongs to class I and class II systems respectively. Two subtypes of type V system and VI type is also recognized, elaborating the classification to two-class-six-type-19-subtype system (Shmakov *et al*., 2015; Table 2). Cas1 and Cas2 genes are ubiquitous in all CRISPR/Cas types (Makarova *et al*., 2011) [68]. CRISPR-Cpf1 (Class II, Type V CRISPR from *Prevoltella* and *Francisella1*) is an advanced tool that uses a single Cpf1 protein for crRNA processing, target site recognition, and DNA cleavage. *Cpf1* is functionally conserved to Cas9 protein but differs substantially in many aspects. The differences are as follows: it is a ribonuclease that processes precursor crRNA; it recognizes a thymine rich (like 50-TTTN-30) PAM sites (Zetsche *et al*., 2015a) [101]. PAM sequence is located upstream of the protospacer sequence and tracrRNA is not required for guiding Cas9 to the target site. The most important characteristic of *Cpf1* is the generation of 4 bp overhangs in contract to blunt ends produced by Cas9 (Zetsche *et al*., 2015a) [101]. These sticky ends would provide more efficient genomic insertions due to sequence complementarity into a genome. Among several proteins in the Cpf1 family, LbCpf1 from *Lachnospiraceae* bacterium ND 2006 and AsCpf1 from *Acidaminococcus* sp. BV3L6 act more effectively in human cells compared with other orthologs (Kim *et al*., 2016) [57]. Class 2 type VI is characterized by an effector protein C2c2 (Class 2, candidate 2). C2c2 contains two nucleotide binding (HEPN) conserved domains, which lacks homology to any known DNA nuclease (Abudayyeh *et al*., 2016) [1]. HEPN domains function as RNases, hence it is visualized as a new RNA targeting tool guided by a single crRNA which can be engineered to cleave ssRNA carrying complementary protospacers. Hence, C2c2 does not target DNA (Abudayyeh *et al*., 2016) [1]. C2c2 is similar to type III-A and III-B systems in having HEPN domains that are biochemically characterized as ssRNA specific endoribonucleases but there is a significant line of difference between these two types. Cas10- Csm in type IIIA and Csx in type III B have less target specificity and have to dimerize to form active sites. C2c2, in contrast, contains two HEPN domains and function as monomeric endoribonuclease (Abudayyeh *et al*., 2016) [1]. Cas9 analogs of C2c2, dC2c2 can be produced by alanine substitution of any of the four predicted HEPN domain. Further examination is required to clarify the mechanism of the C2c2 system and the class of pathogens against which it can protect bacteria. Currently, type VI system is found in *Carnobacterium gallinarum, Leptotrichia buccalis, L. shahii, L. wadei, Listeria newyorkensis, L. seeligeri, L. weihenstephanensis, Paludibacter propionicigenes,* and *Rhodobacter capsulatus* (Choi and Lee, 2016) [24].

Table 2: Classification of CRISPR/Cas9 System

Class	Type	Subtypes	Organism harboring respective types	Signature Cas proteins	Other core proteins
Class 1*	I	I-A	*Archaeoglobus fulgidus*	Cas3, Cas8	Cas1, Cas2, Cas5, Cas6, Cas7
		I-B	*Clostridium kluyveri*	Cas3, Cas8	Cas1, Cas2, Cas5, Cas6, Cas7
		I-C	*Bacillus halodurans*	Cas3, Cas8	Cas1, Cas2, Cas5, Cas7
		I-D	*Cyanothece spp.*	Cas3, Cas8	Cas1, Cas2, Cas5, Cas6, Cas7
		I-E	*Escherichia coli*	Cas3, Cas8	Cas1, Cas2, Cas5, Cas7
		I-F	*Yersinia pseudotuberculosis*	Cas3, Cas8	Cas1, Cas2, Cas5, Cas6, Cas7
		I-U	*Geobacter sulfurreducens*	Cas3, Cas8	Cas1, Cas2, Cas5, Cas6, Cas7
	III	III-A	*Staphylococcus epidermidis*	Cas3, Cas8	Cas1, Cas2, Cas5, Cas6, Cas7

		III-B	*Pyrococcus furiosus*	Cas3, Cas8	Cas1, Cas2, Cas5, Cas6, Cas7
		III-C	*Methanothermobacter thermaautotrophicus*	Cas3, Cas8	Cas5, Cas7
		III-D	*Roseiflexus spp.*	Cas3, Cas8	Cas5, Cas7
	IV	IV	*Acidithiobacillus ferrooxidans*	Cas3, Cas8	Cas5, Cas7
Class 2*	II	II-A	*Streptococcus thermophilus*	Cas3, Cas8	Cas1, Cas2
		II-B	*Legionella pneumophila*	Cas3, Cas8	Cas1, Cas2
		II-C	*Neisseria lactamica*	Cas3, Cas8	Cas1, Cas2
	V	V	*Francisella cf. Novicida*	Cas3, Cas8	Cas1, Cas2
	VI	VI	*Leptotrichia shahii*	Cas3, Cas8	Cas1, Cas2

*Makarova *et al*., 2011, 2015 [68, 69].

Mechanism of CRISPR/Cas9 System

The adaptive immunity of CRISPR/Cas9 system consists of three phases: adaptation, expression and interference (**Figure 8**).

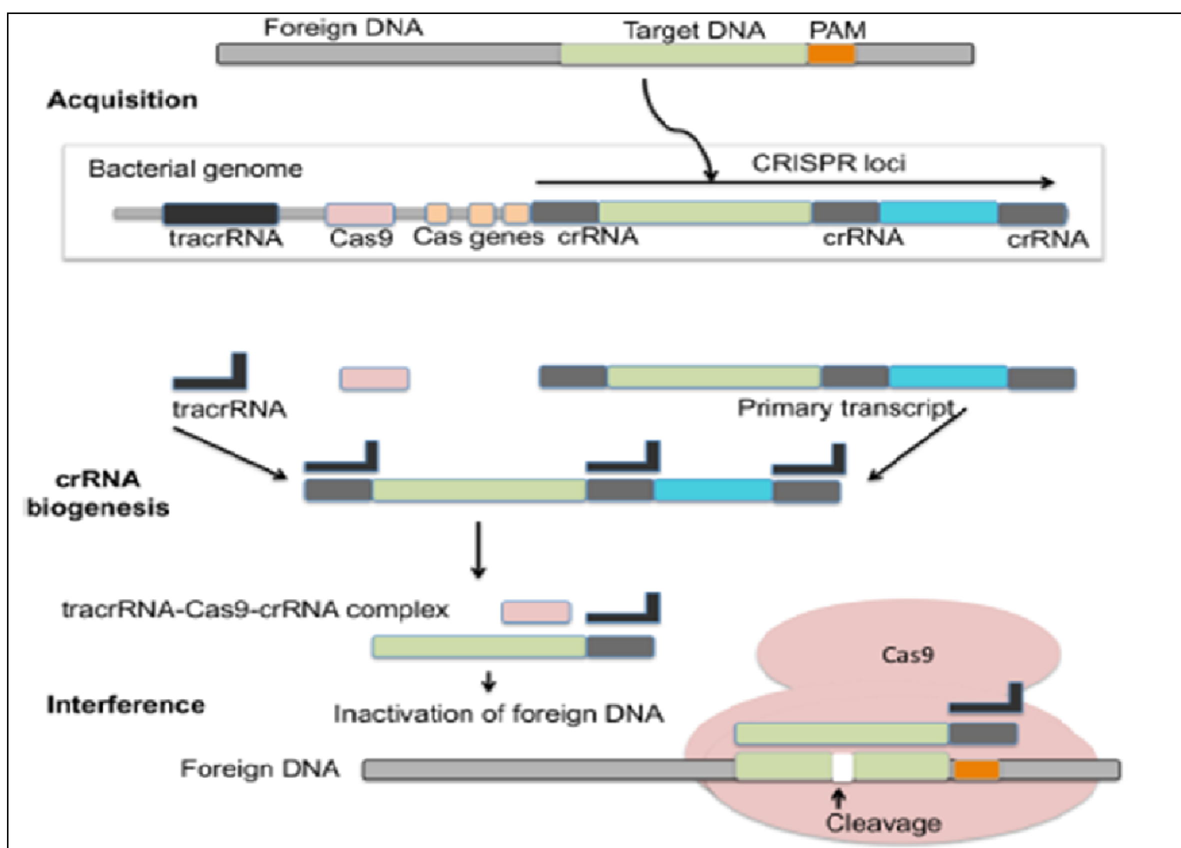

Fig 8: In the acquisition phase foreign DNA gets incorporated into the CRISPR loci of bacterial genome. CRISPR loci is then transcribed into primary transcript and processed into crRNA with the help of tracrRNA during crRNA biogenesis. During interference, Cas9 endonuclease complexed with a crRNA and cleaves foreign DNA near PAM region.

Adaptation involves the invading DNA from virus or plasmids that are cleaved into small fragments and incorporated into CRISPR locus. CRISPR loci are transcribed and processed to generate small RNA (crRNA), which guide the effector endonucleases to target the viral material by base complementarity (Barrangou *et al*., 2007; Yosef *et al*., 2012) [100, 10]. DNA interference in Type II CRISPR/Cas system requires a single Cas9 protein (Hale *et al*., 2009; Zetsche *et al*., 2015b) [39, 102]. Cas9 is a huge protein possessing multiple domains (RuvC domain at the amino terminus and the HNH nuclease domain positioned in middle) and two small RNAs namely crRNA and tracrRNA. Cas9 assists adaptation, participates in pre-crRNA processing to crRNA and introduce targeted DSBs guided by tracrRNA and double stranded RNA specific RNase III (Jackson *et al*., 2014; Mulepati *et al*., 2014) [47, 77]. As compared to type II CRISPR, the unique features of type III CRISPR are the cleavage of both DNA and RNA, and its association with the cleavage protein Cas10. The cleavage is a transcription-dependent DNA sequence modification that also contains a transcriptionally active promoter (Samai *et al*., 2015) [86]. Cas10 system enables bacteria to acquire viral spacer elements enabling a type of resistance against foreign DNA under special conditions. This resistance to foreign/viral DNA prevents activation of the lytic pathway, which is detrimental to the host cell. These sequences could also alter the physical characteristics of the cell, potentially providing a survival advantage for the host cell (Samai *et al*., 2015) [86].

Applications of CRISPR/Cas9 System

CRISPR/Cas9 system has been widely used in various organisms for gene mutation, gene expression repression or activation and epigenome editing. (FIGURE 9) CRISPR technology holds potential for loss-of-function, gain-of-function and gene expression analysis. CRISPR has versatile applications in plant biology and is readily developed and successfully applied to produce high quality agriculturally sustainable products (TABLE 3).

The major applications of CRISPR/Cas9 system include gene knockouts in organisms for identifying the function of single or multiple gene targets (e.g., enzyme genes or micro RNAs)

via gene mutation. Research was done for investigating the capability of CRISPR/Cas9 system as a genome editing tool in Arabidopsis, tobacco, rice, wheat, maize, sorghum, tomato, *Brassica spp*, potato, soybean, sweet orange, grape, apple, strawberry, banana, sugarcane and cassava.

Advantages of CRISPR/Cas9 system

CRISPR/Cas9 system is more advantageous than other techniques *viz*., ZFN and TALEN. Like other genome editing tools, CRISPR/Cas9 system does not require any protein engineering or cloning step. Any number of sgRNA can be produced by *in vitro* transcription using two complementary annealed oligonuclitides (Cho *et al*., 2013) [23]. CRISPR/Cas9 system brings the genome editing within the budget of any molecular biology laboratory. Unlike ZFNs and TALENs, the CRISPR/Cas9 system can cleave methylated DNA. Approximately 70% of CpG/CpNpG sites are methylated in plants, particularly the CpG islands found in promoters and proximal exons (Vanyushin *et al*., 2011) [94]. Thus CRISPR/Cas9 system can be used as a versatile tool for genome editing in plant. Multiplex editing by this method requires monomeric Cas9 protein & any number of different sequence-specific gRNAs. In contrast, multiplex editing with ZFNs or TALENs requires separate dimeric proteins specific for each target site. Gene knockout over RNAi CRISPR targets endogeneous genes that are impossible to specifically target using RNAi technology. These advantages of CRISPR/Cas9 system make it most popular tool for genome editing.

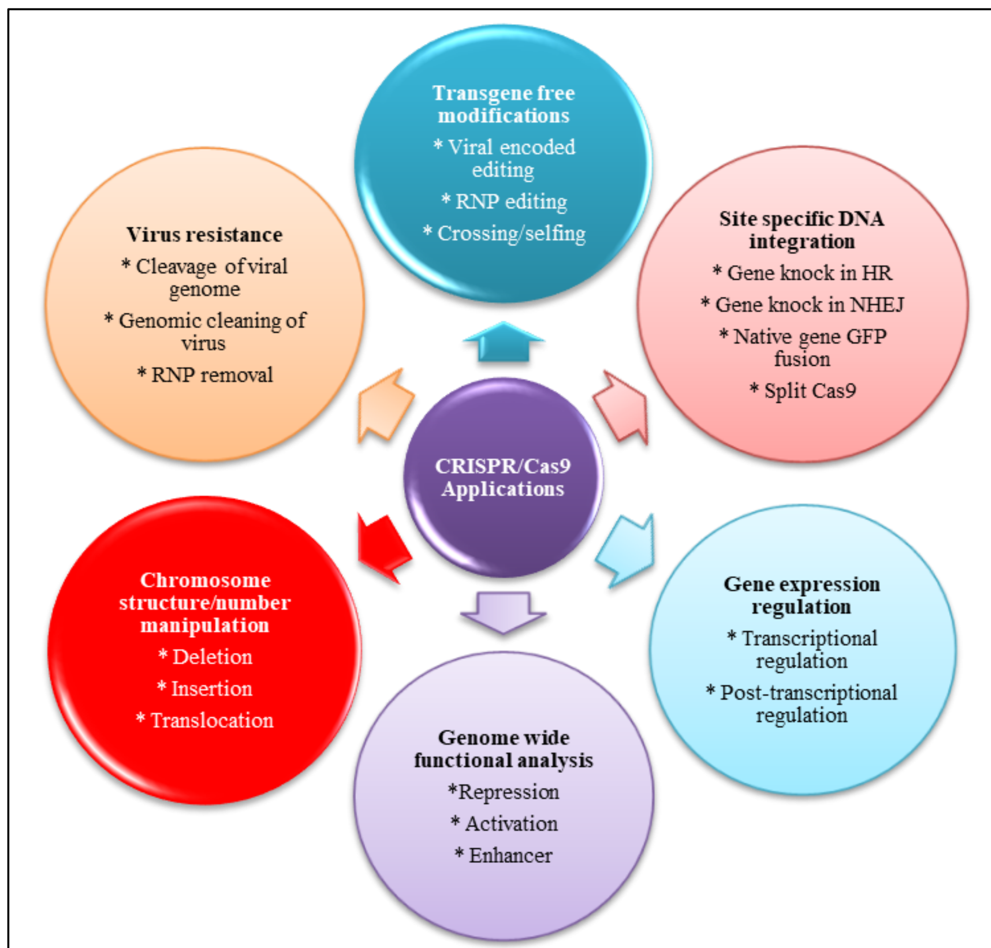

Fig 9: Applications of CRISPR/Cas9 system

Challenges of CRISPR/Cas9 System

Although CRISPR system has proved to be best in the current scenario but still it is in its infancy. Most vital drawback is off-target effects in which specific designed sgRNA does not bind to target region in genome and creates non specificity. (Fu *et al*., 2013) [34] But this can be overcome by employing online in silico analysis tools (Yang, 2014) [98]. Several reports have shown some modifications to increase CRISPR/Cas9 target selection including changes in PAM recognizing domain to add other PAM motifs and incorporation of purines or pyrimidine's as a first nucleotide of sgRNA (Dang, *et al*., 2015) [28]. Immuno-suppression is also another issue which limits its fidelity (Chew, *et al*., 2016) [22] but it can be resolved by genome optimization. Also it is difficult to know the influence of chromatin structure as well as side effects on nearby gene. Another challenge is an identifying the mechanisms involved in the different effects of different sgRNAs on mutation frequency.

Table 3: List of targeted gene *via* CRISPR/Cas9 system in different crops.

Plant species	Targeted gene; gene function	Cas9 promotor; codon optimization of Cas9	sgRNA promotor	Transformation method	Multiplex strategy; transformant type	Mutant efficiency; type of mutants	Reference
Arabidopsis thaliana	*AtPDS3;* Phytoene desaturase, *AtFLS2;* Flagelin sensitive 2, *AtRACK1c;* Receptor for activated C kinase, *CHLI1, CHLI2;* magnesium chelatase subunit 1 *TRY, CPC and ETC2;* trichome density regulators *AtCRU3* *Atlg16210, Atlg56650, Atlg55580* *AP1, TT4* *FT, SPL4* *BRI1, JAZ1, GAI*	CaMV35SPDK; plant OsUBQ1; human P35S; maize CaMV35S; plant Ubi, CaMV35S; plant AtUBQ1, SPL; plant AtICU2; human 2x35S; human	AtU6 OsU3 AtU6-26, AtU6-29 U6-26 AtU3b, AtU3d, AtU6-1, AtU6-29 AtU6-26 AtU6 AtU6-26	PEG Protoplast co-transfection and *Agrobacterium* infiltration *Agrobacterium* mediated transformation Agro-transformation by floral dip *Agrobacterium* mediated transformation *Agrobacterium* infiltration *Agrobacterium* mediated transformation *Agrobacterium* mediated transformation Agro-transformation by floral dip	Transient method Sequential cloning Golden gate/ Gibson assembly - - - -	1.1-5.6% 81.2; Chimeric 82.6%; Chimeric - - - -	Feng *et al*., 2013 [33]; Li *et al*., 2013 [62]; Mao *et al*., 2013 [72]; Feng *et al*., 2014 [32]; Jiang *et al*., 2014 [53]; Xing *et al*., 2014 [97]; Hyun *et al*., 2015; Johnson *et al*., 2015 [55]; Ma *et al*., 2015 [67]; Mao *et al*., 2016 [73]
Oryza sativa	*OsSWEET;* disease susceptibility gene, *OsSWEET14* *OsWaxy;* amylase synthase *Gn1a, DEP1, GS3 and IPA1;* regulators of grain number, panicle architecture, grain size, plant architecture. *ROC5, SPP, YSA* *OsMYB1* *OsPDS, OsMPK2, OsBADH2*	OsUbi; rice ZmUbi; rice Maize ubiquitin promoter CaMV35S; human OsUBQ1; human 2x35S; rice	OsU6.1, OsU6.2 OsU3, OsU6a, OsU6b, OsU6c U6a OsU6-2 OsU3 OsU6	*Agrobacterium* and PEG mediated transformation - *Agrobacterium* transformation in embryonic cell *Agrobacterium* mediated *Agrobacterium* mediated Particle bombardment	Sequential cloning Golden gate/ Gibson assembly - - -	12.5% 85.4%; biallelic, homozygous, heterozygous 42.5% (Gn1a), 67.5% (DEP1), 57.5% (GS3)	Feng *et al*., 2013 [33]; Jiang *et al*., 2013 [52]; Mao *et al*., 2013 [72]; Shan *et al*., 2013[87]; Zhou *et al*., 2014 [103]; Ma *et al*., 2015 [67]; Li *et al*., 2016 [63].

Table 3: Continued

Plant species	Targeted gene; gene function	Cas9 promotor; codon optimization of Cas9	sgRNA promotor	Transformation method	Multiplex strategy; transformant type	Mutant efficiency; type of mutants	Reference
Camelina sativa	*FAD2* gene; key enzyme for synthesis of polyunsaturated fatty acids	CaMV35SP	U9P	*Agrobacterium* floral dip transformation	Golden gate	60.00%	Jiang et.al., 2016 [51]
Triticum aestivum	*TaMLO homologs;* repress resistance pathway to powdery mildew *TaERF3;* (wheat ethylene responsive factor 3), *TaDREB2* (wheat dehydration responsive factor 3)	ZmUbi, 2x35S; rice 2x35S; rice	TaU6 TaU6	Protoplast transformation Protoplast transformation	- Transient method	5.6% 0.013(TaERF3) and 0.97% (TaDREB2) actual 6.7%	Shan *et al*., 2013 [87]; Wang *et al*., 2014; Kim *et al*., 2017
Zea mays	*ZmIPK* *LIG1* (Liguleless gene), *Ms26 and Ms45* (male fertility gene), *ALS1 and ALS2* (acetolactate synthase gene) *Argonaute 18,* dihydroflavonol 4-reductase	2x35S; plant ZmUbi; maize PZmUbi; plant	ZmU3 ZmU6 PU6.1, PU6.2	*Agrobacterium* mediated Biolistic transformation Protoplast transformation	- Co-delivery Sequential cloning	- 77-100%; biallelic, heterozygous 70.00%	Liang *et al*., 2014 [66]; Char *et al*., 2016 [20]; Svitashev *et al*., 2016 [92]
Sorghum bicolor	*DsRed;* red fluorescent protein	CaMV35S; plant	AtU6	Agro-transformation of immature embryos	Golden gate	50.90%	Fan *et al*., 2015 [31]

Nicotiana benthamiana	*PDS gene;* phytoene desaturase, *PDS, PDR6;* phytoene desaturase, *NtPDR6; ABC* transporter *NbPDS* *NbPDS, NbPCNA* *NbPDS, NbIspH*	P35S; human 2xCaMV35S; tobacco 2xCaMV3; tobacco 35SPPDK; plant CaMV35S; human 35S; plant	AtU6 AtU6 AtU6-26 AtU6 PEBV AtU6-26	Leaf Agro-infiltration PEG Protoplast transfection *Agrobacterium* mediated transformation *Agrobacterium* infiltration *Agrobacterium* mediated transformation *Agrobacterium* mediated Transformation	Sequential cloning Transient method Restriction cloning Transient method Transient method Transient method	6.7; Chimeric 16.27-20.3 81.8-87.5%; chimeric - - -	Li *et al.*, 2013 [62]; Nekrasov *et al.*, 2013 [78]; Ali *et al.*, 2015 [3]; Gao *et al.*, 2015 [35]; Yin *et al.*, 2015 [99];
Solanum tuberosum	*StGBSS;* starch synthase gene *StMYB44*; transcription factor gene *StALS1*; (Herbicide resistance)	CaMV35S; plant CaMV35S; plant CaMV35S; plant	AtU6, StU6 AtU6 AtU6	PEG mediated protoplast transfection *Agrobacterium* mediated *Agrobacterium* mediated	Restriction cloning - -	67.00%	Butler *et al.*, 2015; Andersson *et al.*, 2016 [6]; Zhou *et al.*, 2017

Table 3: Continued

Plant species	Targeted gene; gene function	Cas9 promotor; codon optimization of Cas9	sgRNA promotor	Transformation method	Multiplex strategy; transformant type	Mutant efficiency; type of mutants	Reference
Camelina sativa	*FAD2* gene; key enzyme for synthesis of polyunsaturated fatty acids	CaMV35SP	U9P	*Agrobacterium* floral dip transformation	Golden gate	60.00%	Jiang et.al., 2016 [51]
Triticum aestivum	*TaMLO homologs;* repress resistance pathway to powdery mildew *TaERF3;* (wheat ethylene responsive factor 3), *TaDREB2* (wheat dehydration responsive factor 3)	ZmUbi, 2x35S; rice 2x35S; rice	TaU6 TaU6	Protoplast transformation Protoplast transformation	- Transient method	5.6% 0.013(TaERF3) and 0.97% (TaDREB2) actual 6.7%	Shan *et al.*, 2013 [87]; Wang *et al.*, 2014 [95]; Kim *et al.*, 2017
Zea mays	*ZmIPK LIG1* (Liguleless gene), *Ms26 and Ms45* (male fertility gene), *ALS1 and ALS2* (acetolactate synthase gene) *Argonaute 18,* dihydroflavonol 4-reductase	2x35S; plant ZmUbi; maize PZmUbi; plant	ZmU3 ZmU6 PU6.1, PU6.2	*Agrobacterium* mediated Biolistic transformation Protoplast transformation	- Co-delivery Sequential cloning	- 77-100%; biallelic, heterozygous 70.00%	Liang *et al.*, 2014 [66]; Char *et al.*, 2016 [20]; Svitashev *et al.*, 2016 [92]
Sorghum bicolor	*DsRed;* red fluorescent protein	CaMV35S; plant	AtU6	Agro-transformation of immature embryos	Golden gate	50.90%	Fan *et al.*, 2015 [31]
Nicotiana benthamiana	*PDS gene;* phytoene desaturase, *PDS, PDR6;* phytoene desaturase, *NtPDR6; ABC* transporter *NbPDS* *NbPDS, NbPCNA* *NbPDS, NbIspH*	P35S; human 2xCaMV35S; tobacco 2xCaMV3; tobacco 35SPPDK; plant CaMV35S; human 35S; plant	AtU6 AtU6 AtU6-26 AtU6 PEBV AtU6-26	Leaf Agro-infiltration PEG Protoplast transfection *Agrobacterium* mediated transformation *Agrobacterium* infiltration *Agrobacterium* mediated transformation *Agrobacterium* mediated Transformation	Sequential cloning Transient method Restriction cloning Transient method Transient method Transient method	6.7; Chimeric 16.27-20.3 81.8-87.5%; chimeric - - -	Li *et al.*, 2013 [62]; Nekrasov *et al.*, 2013 [78]; Ali *et al.*, 2015 [3]; Gao *et al.*, 2015 [35]; Yin *et al.*, 2015 [99];
Solanum tuberosum	*StGBSS;* starch synthase gene *StMYB44*; transcription factor gene *StALS1*; (Herbicide resistance)	CaMV35S; plant CaMV35S; plant CaMV35S; plant	AtU6, StU6 AtU6 AtU6	PEG mediated protoplast transfection *Agrobacterium* mediated *Agrobacterium* mediated	Restriction cloning - -	67.00%	Butler *et al.*, 2015; Andersson *et al.*, 2016; Zhou *et al.*, 2017

Table 3: Continued

Plant species	Targeted gene; gene function	Cas9 promotor; codon optimization of Cas9	sgRNA promotor	Transformation method	Multiplex strategy; transformant type	Mutant efficiency; type of mutants	Reference
Solanum esculentum	*SIAG07*; biogenesis of trans-acting short interfering RNAs, *Soliyc08g041770, Soliyc07g021170, Soliyc08g044760* *ANT1;* Anthocyanin biosynthesis	P35S; human 35S	AtU6 ANT1, AtU6	Agro-transformation of cotyledons *Agrobacterium* mediated Transformation	Golden gate Golden gate	48.00%; homozygous, biallelic, chimeric 57.10% heterozygous; 13.10% homozygous	Brooks *et al*., 2014 [16]; Cermak *et al*., 2015 [18]
Gossypium hirustum	*GhCLA1*gene (Cloroplastos alterados 1) and GhVP gene (Vacuolar H+ pyrophosphatase) *Gh EF1* gene (Elngation factor 1); *GhCLA1*gene (*Cloroplastos alterados* 1), *GhPDS* gene (*Phytoene desaturase*) and *GhARG* gene	2x35S 2x35S	AtU6 AtU6-29, AtU3b	*Agrobacterium* mediated transformation *Agrobacterium* mediated Transformation	Golden gate Golden gate/ Transient method	47.60-81.80% 0.82-63.93%; biallelic, deletions, insertions	Chen *et al*., 2015 [19]; Gao *et al*., 2017
Glycine max	*ALS1*; encode acetolactate synthase involved in amino acid biosynthesis *Glyma06g14180, Glyma08go2290, Glyma12g37050* *Glyma07g1450, GmDDM1s, GmMIRs*	EF1A2; soybean GmU6; plant 2x35S; human	AtU6-9-1 AtU6, AtU6-10 MtU6	Particle bombardment Protoplast mediated PEG transformation/ *Agrobacterium* mediated *Agrobacterium* mediated Transformation	- Golden gate -	59.00-76.00% 3.20-9.70% and 14.70-20.20%; biallelic, monoallelic -	Jacobs *et al*., 2015 [48]; Li *et al*., 2015; Sun *et al*., 2015 [91];
Brassica oleracea	*BoIC.GA4*; ortholog of Arabidopsis GA4a	CsVMW; human	AtU6-26	Agro-transformation of coteledonary petioles	Golden gate	10.00%	Lawrenson *et al*., 2015 [61]
Populus tomentosa	*PDS* gene (*Phytoene desaturase)*	2xP35S; plant	AtU3b, AtU3d	*Agrobacterium* mediated Transformation	Golden gate	50.90%	Fan *et al*., 2015 [31]
Citrus sinesis	*CsPDS* gene (*Phytoene desaturase)* *CsLOB1* (Citrus cancer resistance)	CaMV35S; human CaMV35S; human		*Agrobacterium* infiltration *Agrobacterium* infiltration	Golden gate Golden gate	-	Jia *et al*., 2014 [50]; Peng *et al*., 2017

Table 3: Continued

Plant species	Targeted gene; gene function	Cas9 promotor; codon optimization of Cas9	sgRNA promotor	Transformation method	Multiplex strategy; transformant type	Mutant efficiency; type of mutants	Reference
Fragaria vesca	*TAA1(Auxin biosynthesis gene) and ARF8 (Auxin response factor 8)*	UBQ	AtU6-26, FveU6-2	Protoplast mediated PEG transformation/ *Agrobacterium* mediated	Golden gate	49.00-75.00%	Zhou *et al*., 2018
Vitis vinifera	*VvMLO 7 ()Powdery mildew resistance gene* *ldnDH* Biosynthesis of Tartaric acid *(L-idonate dehydrogenase gene), VvPDS (Albino devoid green tissue)*	- -	- -	Protoplast transformation *Agrobacterium* mediated Transformation	- -	- -	Malnoy *et al*., 2016 [71]; Ren *et al*., 2016; Nakajima *et al*., 2017
Malus domestica	*MdPDS* Chlorophyll biosynthesis *(Phytoene desaturase)* *DIPM 1-4* (DspE-interacting protein) Fire blight resistant gene	2x35S CaMV 2x35S CaMV	AtU6-1 AtU6-1	*Agrobacterium* mediated transformation Protoplast transformation	Golden gate Golden gate	31.80%	Malnoy *et al*., 2016 [71]; Nishitani *et al*., 2016 [79]

Conclusion
CRISPR/Cas9 system is a promising tool for genome editing in plants due to its simplicity, efficiency, high specificity and fewer off-target effects. It holds potential for loss-of-function, gain-of-function and gene expression analysis. CRISPR has versatile applications in plant biology and is readily developed and successfully applied to produce high quality agriculturally sustainable products. This transformative tool has made possible to target any gene for required modifications in a more convenient way. It also reshaped the traditional techniques for discovering a particular gene function due to ease in execution, component designing flexibility and target specific mutations. RNA mediated DNA genome editing can accelerate plant breeding by introducing precise and predictable modifications directly in elite cultivars or accessions, saving the time-consuming backcrossing procedure in conventional breeding schemes. Mostly, CRISPR/Cas9 System is optimized in model organisms and now it is applicable in every field of Biological Sciences. This genome editing System has a huge capability for disturbing normal gene function, restoring errors and RNA based targeting against DNA sequence.

Acknowledgment
We would like to acknowledge all the Scientists of Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli and Chhatrapati Shahu Maharaj Research, Training and Human Development Institute (SARTHI), Pune for funding and proving necessary support and valuable suggestions while preparing this manuscript.

References
1. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DB *et al*. C2c2 is a single-component programmable RNAguided RNA-targeting CRISPR effector. Science 2016;353:557-566. doi: 10.1126/ science.aaf5573
2. Ainley WM, Sastry-Dent L, Welter ME, Murray MG, Zeitler B, Amora R *et al*. Trait stacking via targeted genome editing. *Plant Biotechnol. J*. 2013;11:1126-1134. doi: 10.1111/pbi.12107
3. Ali Z, Abulfaraj A, Idris A, Ali S, Tashkandi M, Mahfouz MM. CRISPR/Cas9-mediated viral interference in plants. Genome Biol. 2015;16:238-249. doi: 10.1186/s13059-015-0799-6
4. Amitai G, Sorek R. CRISPR-Cas adaptation: insights into the mechanism of action. Nat. Rev. Microbiol 2016;14:67-76. doi: 10.1038/nrmicro. 2015.14
5. Anders C, Niewoehner O, Duerst A, Jinek M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* 2014;513:569-573.
6. Andersson M, Turesson H, Nicolia A, Fält AS, Samuelsson M, Hof-vander P. Efficient targeted multiallelic mutagenesis in tetraploid potato (Solanum tuberosum) by transient CRISPR- Cas9 expression in protoplasts. Plant Cell Rep. 2016;36:117-128. doi: 10.1007/s00299-016- 2062-3
7. Arora L, Narula A. Gene editing and crop improvement using CRISPR-Cas9 system, Front. Plant Sci. 2017;8:1932, doi:10.3389/fpls.2017.01932.
8. Baltes NJ, Gil-Humanes J, Cermak T, Atkins PA, Voytas DF. DNA replicons for plant genome engineering. *Plant Cell* 2014;26:151-163. doi: 10.1105/tpc.113.119792
9. Barrangou R. CRISPR-Cas systems and RNA-guided interference. *WIREs RNA* 2013;4:267-278
10. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S *et al*. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 2007;315:1709-1712. doi: 10.1126/science.1138140
11. Belhaj K *et al*., Editing plant genomes with CRISPR/Cas9. Current opinion in biotechnology 2015;32:76-84.
12. Bhaya D, Davison M, Barrangou R. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* 2011;45:273-297.
13. Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S *et al*. Breaking the code of DNA binding specificity of TAL-type III effectors. Science 2009;326:1509-1512. doi: 10.1126/science.1178811
14. Bolotin A, Quinquis B, Sorokin A, Ehrlich SD. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extra-chromosomal origin. *Microbiology* 2005;151:2551-2561. doi: 10.1099/mic.0. 28048-0
15. Bortesi L, Fischer R. The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol. Adv*. 2015;33:41-52. doi: 10.1016/j.biotechadv.2014. 12.006
16. Brooks C, Nekrasov V, Lippman ZB, Van Eck J. Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. Plant Physiol 2014;166:1292-1297. doi: 10.1104/pp.114.247577
17. Butler NM, Atkins PA, Voytas DF, Douches DS. Generation and in heritance of targeted mutations in potato (Solanum tuberosum L.) Using the CRISPR/Cas System. PLoS ONE 2015, 10:e0144591. doi: 10.1371/journal.pone.0144591
18. Cermak T, Baltes NJ, Cegan R, Zhang Y, Voytas DF.. High frequency, precise modification of the tomato genome. *Genome Biol.* 2015;16:232-246. doi: 10.1186/s13059-015-0796-9
19. Chang ZY, Yan W, Liu DF, Chen ZF, Xie G, Lu JW. Research Progress on CRISPR/Cas, *Agric. Biotechnol*; 2015;23:1196-1206.
20. Char SN, Neelakandan AK, Nahampun H, Frame B, Main M, Spalding MH *et al*. An Agrobacterium-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. Plant Biotechnol. J. 2016;15:257-268. doi: 10.111/pbi.12611
21. Chen K, Gao C. Targeted genome modification technologies and their applications in crop improvements. Plant Cell Rep 2014;33:575-583.
22. Chew WL *et al*., A multifunctional AAV-CRISPR-Cas9 and its host response. Nat Meth 2016;13(10):868-874.
23. Cho SW, Kim S, Kim JM, Kim JS. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease, Nat. Biotechnol 2013;31:230-232.
24. Choi KR, Lee SY. CRISPR technologies for bacterial systems: current achievements and future directions. Biotechnol. Adv. 2016;34:1180-1209. doi: 10.1016/j.biotechadv.2016.08.002
25. Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A *et al*. Targeting DNA double-strand breaks with TAL effector nucleases. Genetics 2010;186:757-761. doi: 10.1534/genetics.110.120717
26. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N *et*

al. Multiplex genome engineering using CRISPR/Cas systems. Science 2013;339:819-823. doi: 10.1126/science.1231143

27. Curtin SJ, Zhang F, Sander JD, Haun WJ, Starker C, Baltes NJ *et al*. Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases. *Plant Physiol*. 2011;156:466-473. doi: 10.1104/pp.111.172981
28. Dang Y *et al*., Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency. *Genome biology*, 2015;16(1):280.
29. Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA *et al*. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 2011;471:602-607. doi: 10.1038/nature 09886
30. Doudna JA, Charpentier E. The new frontier of genome engineering with CRISPR-Cas9. Science 2014;346(6213):1258096.
31. Fan D, Liu TT, Li CF, Jiao B, Li S, Hou YS *et al*. Efficient CRISPR/ Cas9-mediated targeted mutagenesis in Populus in the first generation. Sci. Rep 2015;5:12217-12223. doi: 10.1038/srep12217
32. Feng Z, Mao Y, Xu N, Zhang B, Wei P, Yang DL *et al*. Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 2014;111:4632-4637. doi: 10.1073/pnas.1400822111
33. Feng Z, Zhang B, Ding W, Liu X, Yang DL, Wei P *et al*. Efficient genome editing in plants using a CRISPR/Cas system. Cell Res. 2013;23:1229-1232. doi: 10.1038/cr.2013.114
34. Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK *et al*. High frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol* 2013;31:822-826. doi: 10.1038/nbt.2623
35. Gao J, Wang G, Ma S, Xie X, Wu X, Zhang X *et al*. CRISPR/Cas9 mediated targeted mutaGenesis in Nicotiana tabacum. Plant Mol. Biol. 2015;87:99-110. doi: 10.1007/s11103-014-0263-0
36. Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P *et al*. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 2010;468:67-71. doi: 10.1038/nature09523
37. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE *et al*. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 2013;154:442-451. doi:10.1016/j.cell.2013.06.044
38. Haft DH, Selengut J, Mongodin EF, Nelson KE. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLOS Comput. Biol 2005;1:60. doi: 10.1371/journal. pcbi.0010060
39. Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, Wells L *et al*. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell 2009;139:945-956. doi: 10.1016/j.cell.2009.07.040
40. Hassan H, Sajid M, Ali Q, Nadeem T, Sehrai GH, Salman S. CRISPR CAS9 system: A novel genome editing tool, *Sci. Int. (Lahore)* 2017;29(3):639-644.
41. Hermans P *et al*., Insertion element IS987 from Mycobacterium bovis BCG is located in a hot-spot integration region for insertion elements in Mycobacterium tuberculosis complex strains. Infection and immunity 1991;59(8):2695-2705.
42. Hilton IB, D'Ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE. Epigenome editing by a CRISPR-Cas9- based acetyltransferase activates genes from promoters and enhancers. *Nat. Biotechnol.* 2015;33:510-517. doi: 10.1038/nbt.3199
43. Hsu PD *et al*. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol* 2013;31:827-832.
44. Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* 2014;157:1262-1278.
45. Huyn YB, Kim J, Cho SW, Choi Y, Kim JS, Coupland G. Site-directed mutagenesis in Arabidopsis thaliana using dividing tissue-targeted REGN of the CRISPR/Cas9 system to generate null alleles, Planta 2015;241:271-284.
46. Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatise isozyme conversion in Escherichia coli, and identification of the gene product. *J. Bacteriol*. 1987;169:5429-5433.
47. Jackson RN, Golden SM, Van-Erp PB, Carter J, Westra ER, Brouns SJ *et al*. Crystal structure of the CRISPR RNA- guided surveillance complex from Escherichia coli. Science 2014;345:1473-1479. doi: 10.1126/science.1256328
48. Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA. Targeted genome modifications in soybean with CRISPR/Cas9. BMC Biotechnol 2015;15:16. doi: 10.1186/s12896-015-0131-2
49. Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. Mol. Microbiol. 2002;43:1565-1575. doi: 10.1046/j.1365-2958.2002.02839.x
50. Jia HG, Wang N. Taregeted genome editing of sweet orange using Cas9/sgRNA., *PLoS One*, 2014;9:93806.
51. Jiang WZ, Henry IM, Lynagh PG, Comai L, Cahoon EB, Weeks DP. Significant enhancement of fatty acid composition in seeds of the allohexaploid, Camelina sativa, using CRISPR/ Cas9 gene editing. Plant Biotechnol. J. 2016;15:648-657. doi: 10.1111/pbi.12663
52. Jiang WZ, Zhou HB, Bi HH, Fromm M, Yang B, Weeks DP. Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, Tobacco, Sorghum and rice. Nucleic Acids Res 2013;41:e188. doi: 10.1093/nar/gkt780
53. Jiang W, Brueggeman AJ, Horken KM, Plucinak TM, Weeks DP. Successful transient expression of Cas9/sgRNA genes in Chlamydomonas reinhardtii. Eukaryot. Cell 2014;13:1465-1469. doi: 10.1128/EC. 00213-14
54. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012;337:816-821. doi: 10.1126/science.1225829
55. Johnson RA, Gurevich V, Filler S, Samach A, Levy A A. Comparative assessments of CRISPR-Cas nucleases cleavage efficiency in planta. Plant Mol. Biol. 2015;87:143-156. doi: 10.1007/s11103-014-0266-x
56. Joung JK, Sander JD.. TALENs: a widely applicable technology for targeted genome editing. Nat. Rev. Mol. Cell Biol 2012;14:49-55. doi: 10.1038/ nrm3486
57. Kim D, Kim J, Junho KH, Been KW, Yoon S, Kim J. Genome-wide analysis reveals specificities of Cpf1 nucleases in human cells. *Nat. Biotechnol* 2016;34:863-868. doi: 10.1038/nbt.3609

58. Kim H, Kim JS. A guide to genome engineering with programmable nucleases. *Nature Reviews Genetics*, 2014;15(5):321-334.
59. Koonin EV, Makarova KS. CRISPR-Cas: an adaptive immunity system in prokaryotes. F1000 Biol. Rep. 2009;1:95.
60. Kuscu C, Arslan S, Singh R, Thorpe J, Adli M. Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. *Nat. Biotechnol*. 2014;32:677-683.
61. Lawrenson T, Shorinola O, Stacey N, Li C, Ostergaard L, Patron N *et al*. Induction of targeted, heritable mutations in barley and Brassica oleracea using RNA-guided Cas9 nuclease. Genome Biol. 2015;16:258-271. doi: 10.1186/s13059-015-0826-7
62. Li J, Norville JE, Aach J, McCormack M, Zhang D, Bush J *et al*. Multiplex and homologous recombination-mediated genome editing in Arabidopsis and *Nicotiana benthamiana* using guide RNA and Cas9. Nat. Biotechnol 2013;31:688-691. doi: 10.1038/nbt.2654
63. Li L, Liu Y, Chen B, Xu K, Zhang F, Li H *et al*. A genome-wide association study reveals new loci for resistance to clubroot disease in Brassica napus. Front. Plant Sci 2016;7:1483. doi: 10.3389/fpls.2016.01483
64. Li M, Li X, Zhou Z, Wu P, Fang M, Pan X *et al*. Reassessment of the four yield related genes Gn1a, DEP1, GS3, and IPA1 in rice using a CRISPR/Cas9 system. Front. Plant Sci. 2016;7:377. doi: 10.3389/fpls.2016.00377
65. Li Z, Liu Z, Xing A, Moon BP, Koellhoffer JP, Huang, L. Cas9- guide RNA directed genome editing in soybean. Plant Physiol. 2015;169:960-970. doi: 10.1104/pp.15.00783
66. Liang Z, Zhang K, Chen K, Gao C. Targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system. J. Genet. Genomics 2014;41:63-68. doi: 10.1016/j.jgg.2013.12.001
67. Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R *et al*. A robust CRISPR-Cas9 system for convenient high-efficiency multiplex genome editing in monocot and dicot plants. Mol. Plant. 2015;8:1274-1284. doi: 10.1016/j.molp. 2015.04.007
68. Makarova KS, Haft DH, Barrangou R, Brouns SJJ, Charpentier E, Horvath P. Evolution and classification of the CRISPR-Cas systems. *Nat. Rev. Microbiol*. 2011;9:467-477. doi: 10.1038/nrmicro3569
69. Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ *et al*. An updated evolutionary classification of CRISPR-Cas systems. Nat. Rev. Microbiol. 2015;13:722-736. doi: 10.1038/nrmicro3569
70. Mali P *et al*. RNA-guided human genome engineering via Cas9. *Science* 2013;339(6121):823-826.
71. Malnoy M, Viola R, Jung MH, Koo OJ, Kim S, Kim JS *et al*. DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front. Plant Sci 2016;7:1904. doi: 10.3389/fpls.2016.01904
72. Mao YF, Zhang H, Xu NF, Zhang BT, Gou F, Zhu JK. Application of the CRISPR-Cas system for efficient genome engineering in plants. Mol. Plant 2013;6:2008-2011. doi: 10.1093/mp/sst121
73. Mao YF, Zang ZJ, Feng ZY, Wei PL, Zang H, Botella JR. Development of germ-line-specific CRISPR/Cas9 system to improve the production of heritable gene modifications in Arabidopsis, *Plant Biotechnol* 2016;14:519-532.
74. Mojica FJ, Díez-Villaseñor C, García-Martínez J, Soria E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J. Mol. Evol*. 2005;60:174-182. doi: 10.1007/s00239-004- 0046-3
75. Mojica FJ, Montoliu L. On the Origin of CRISPR-Cas Technology: From Prokaryotes to Mammals. *Trends in microbiology* 2016;24(10):811-820.
76. Moscou MJ, Bogdanove AJ. A simple cipher governs DNA recognition by TAL effectors. Science 2009;326:1501. doi: 10.1126/science.1178817
77. Mulepati S, Heroux A, Bailey S. Crystal structure of a CRISPR RNAguided surveillance complex bound to a ssDNA target. Science 2014;345:1479-1484. doi: 10.1126/science.1256996
78. Nekrasov V, Staskawicz B, Weigel D, Jones JD, Kamoun S. Targeted mutagenesis in the model plant Nicotiana benthamiana using Cas9- guided endonuclease. *Nat. Biotechnol*. 2013;31:691-693. doi: 10.1038/nbt.2655
79. Nishitani C, Hirai N, Komori S, Wada M, Okada K, Osakabe K. Efficient genome editing in apple using a CRISPR/Cas9 system. Sci. Rep. 2016;6:31481. doi: 10.1038/srep31481
80. Osakabe K, Osakabe Y, Toki S. Site-directed mutagenesis in Arabidopsis using custom-designed zinc finger nucleases. Proc. Natl. Acad. Sci. 2010;107:12034-12039. doi: 10.1073/pnas.1000234107
81. Pabo CO, Peisach E, Grant RA. Design and selection of novel Cys2His2 zinc finger proteins. *Annu. Rev. Biochem*. 2001;70:313-340. doi: 10.1146/ annurev.biochem.70.1.313
82. Pourcel C, Salvignol G, Vergnaud G. CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* 2005;151:653-663. doi: 10.1099/mic.0.27437-0
83. Puchta H, Fauser F. Synthetic nucleases for genome engineering in plants: prospects for a bright future. Plant J. 2013;78:727-741.
84. Puchta H. The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. *J. Exp. Bot*. 2005;56:1-14. doi: 10.1093/jxb/ eri025
85. Puchta H. Using CRISPR/Cas in three dimensions: towards synthetic plant genomes, transcriptomes and epigenomes. Plant J. 2016;87:5-15. doi: 10.1111/tpj. 13100
86. Samai P, Pyenson N, Jiang W, Goldberg GW, Hatoum-Aslan A, Marraffini LA. Co-transcriptional DNA and RNA cleavage during type III CRISPR–Cas immunity. Cell 2015;161:1164-1174. doi: 10.1016/j.cell.2015.04.027
87. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z. Targeted genome modification of crop plants using a CRISPR-Cas system. Nat. Biotechnol 2013;31:686-688. Doi: 10.1038/nbt.2650
88. Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E *et al*. Discovery and functional characterization of diverse class 2 CRISPR/Cas systems. Mol. Cell 2015;60:385-397. doi: 10.1016/j.molcel.2015. 10.008
89. Sikora P, Chawade A, Larsson M, Olsson J, Olsson O. Mutagenesis as a tool in plant genetics, functional genomics, and breeding. Int. J. Plant Genomics 2011, 142-153. doi: 10.1155/2011/314829

90. Sorek R, Lawrence CM, Wiedenheft B. CRISPR-mediated adaptive immune systems in bacteria and archaea. Annual review of biochemistry 2013;82:237-266.
91. Sun X, Zheng H, Chen R, Jiang Q, Song G, Zang H, Xi Y. Targeted mutagenesis in soybean using the CRISPR-Cas9 system, Sci. Rep 2015;5:10342. DOI:10.1038/srep10342.
92. Svitashev S, Young JK, Schwartz C, Gao H, Falco SC, Cigan AM. Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. Plant Physiol 2016;169:931-945. doi: 10.1104/pp.15.00793
93. Maurya S, Dhaker GL, Dhaker DL, Singh MK. CRISPR/Cas9 system a novel tool for the management of plant diseases: a review. Int J Pharm Pharm Sci. 2019;4(5):31-4.
94. Vanyushin BF, Ashapkin VV. DNA methylation in higher plants: past, present and future, Biochim Biophys Acta 2011;1809:360-368.
95. Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C. Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat. Biotechnol 2014;32:947-951. doi: 10.1038/nbt. 2969
96. Wu X. *et al*. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nat. Biotechnol*. 2014;32:670-676.
97. Xing HL, Dong L, Wang ZP, Zhang HY, Han CY, Liu B. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol. 2014;14:327. doi: 10.1186/s12870-014-0327-y
98. Yang Y. Genome-wide prediction of highly specific guide RNA spacers for the CRISPR-Cas9 mediated genome editing in model plants and major crops. in Plant and Animal Genome XXII Conference 2014.
99. Yin KQ, Han T, Liu G, Chen T, Wang Y, Alice YZ. A geminivirus-based guide RNA delivery system for CRISPR/Cas9 mediated plant genome editing, Sci. Rep 2015;5:14926.
100. Yosef I, Goren MG, Qimron U. Proteins and DNA elements essential for the CRISPR adaptation process in Escherichia coli. Nucleic Acids Res 2012;40:5569-5576. doi: 10.1093/nar/gks216
101. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P *et al*. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR/Cas system. *Cell* 2015a;163:759-771. Doi: 10.1016/j.cell.2015. 09.038
102. Zetsche B, Volz SE, Zhang F. A Split-Cas9 architecture for inducible genome editing and transcription modulation. Nat. Biotechnol. 2015b;33:139-142. Doi: 10.1038/nbt.3149.
103. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B. Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice. *Nucleic Acids Res* 2014;42:10903-10914. doi: 10.1093/nar/ gku806.
104. Khatodia S, Bhatotia K, Passricha N, Khurana SMP, Tuteja N. The CRISPR/Cas Genome-Editing Tool: Application in Improvement of Crops. Front. Plant Sci. 2016;7:506. doi: 10.3389/fpls.2016.00506.
105. Tang TH, Bachellerie JP, Rozhdestvensky T, Bortolin ML, Huber H, Drungowski M *et al*. Identification of 86 candidates for small nonmessenger RNAs from the archaeon *Archaeoglobus fulgidus*. *Proc. Natl. Acad. Sci*., 2002;99:7536-7541. doi: 10.1073/pnas.112047299