Review article

CRISPR-Cas9 system: A new-fangled dawn in gene editing

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ARTICLE INFO

ABSTRACT

Till date, only three techniques namely Zinc Finger Nuclease (ZFN), Transcription-Activator Like Effector Nucleases (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-Associated 9 (CRISPR-Cas9) are available for targeted genome editing. CRISPR-Cas system is very efficient, fast, easy and cheap technique for achieving knock-out gene in the cell. CRISPR-Cas9 system refurbishes the targeted genome editing approach into a more expedient and competent way, thus facilitating proficient genome editing through embattled double-strand breaks in approximately any organism and cell type. The off-target effects of CRISPR Cas system has been circumnavigated by using paired nickases. Moreover, CRISPR-Cas9 has been used effectively for numerous purposes, like knock-out of a gene, regulation of endogenous gene expression, live-cell labelling of chromosomal loci, edition of single-stranded RNA and high-throughput gene screening. The execution of the CRISPR-Cas9 system has amplified the number of accessible scientific substitutes for studying gene function, thus enabling generation of CRISPR-based disease models. Even though many mechanistic questions are left behind to be answered and the system is not yet fool-proof i.e., a number of challenges are yet to be addressed, the employment of CRISPR-Cas9–based genome engineering technologies will increase our understanding to disease processes and their treatment in the near future. In this review we have discussed the history of CRISPR-Cas9, its mechanism for genome editing and its application in animal, plant and protozoan parasites. Additionally, the pros and cons of CRISPR-Cas9 and its potential in therapeutic application have also been detailed here.

1. Introduction

With the evolution of whole genome sequencing methodologies, new challenges like discovery of specific gene functions and personalized medicine have intrigued scientists around the globe. To overcome these challenges, efficient and reliable tools are needed to derive information regarding the influence exerted by genotype on phenotype. In the era of modern scientific advancement, targeted genome editing technologies have outperformed other molecular tools in overcoming these challenges. Since then, targeted genome editing has become one of the most popular topics of investigation. As a result, components of the repair system were studied extensively and nucleases were modified in order to create artificial systems that would bind to a specific sequence of DNA and bring about desired modifications precisely. The speciality of these systems rests in the fact that it can work at the genome level, enabling in-situ alterations, which drives the development of a plethora of genome editing systems.

This manuscript compiles a thorough and detailed review of the existing genome editing techniques, with an appropriate emphasis on CRISPR-Cas9. Unlike other published reviews in this field, here we additionally deal with other systems like plant and protozoan parasites. We also shed light on the pros and cons of CRISPR-Cas9 and its potential in therapeutic application.

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1.1. Zinc Finger Nuclease (ZFN)

The classical *in-vivo* gene targeting method involves normal homologous recombination which is low in efficiency, time consuming and laborious. It was overcome with the advent of ZFN (Zinc Finger Nuclease) and TALEN (Transcription-Activator Like Effector Nucleases) as genome editing tools, carrying the catalytic domains of the restriction endonuclease *FokI*, which generates a Double Strand Break (DSB) with cohesive overhangs, which played a pivotal role in extracting gene function information by targeted genome editing. However, with the advancement of science, more updated and efficient gene manipulation technique has come in the limelight namely CRISPR (Cluster Regularly Interspaced Short Palindromic Repeats)/CRISPR associated system (Cas system), adapted from the bacterial adaptive immune system [1]. This advancement has made functional genomics more reliable by permitting precise alteration or inhibition of gene function at ease. Such data are of significant importance in the areas of medicine or therapeutics [2].

Zinc Finger nucleases are synthetic DNA binding proteins having two domains connected by a linker sequence - the first is an engineered zinc-finger DNA binding domain responsible for sequence specificity of a 24 bp stretch of DNA and the second is a restriction endonuclease *FokI mediated* DNA cleaving domain which cleave the sequence in 5–7 bp spacer sequence [3,4] (Fig. 1). The first domain consists of three sets of zinc fingers where each finger is constituted of approximately 30 amino acids linked to a single zinc atom and binds 3 bp of DNA. The cleavage domain is non-specific. It executes its function in a dimeric form, targeting the opposite strands.

1.2. Transcription activator like effector Nuclease (TALEN)

TALENs are based on highly repetitive sequences promoting homologous recombination *in vivo*. Similar to ZFNs, the TALENs also contain two domains – N-terminal transcription activator like effector (TALE) DNA-binding domain and the C-terminal catalytic domain of restriction endonuclease *FokI* (Fig. 2). The nuclease domain is associated with the DNA binding domain by a linker sequence and the two binding sites are separated by a 12–25 bp sequence known as spacer sequence. Additionally, TALENs also function in dimeric form to make a double stranded break and the binding sites are located on opposite strands. The DNA binding domains are composed of monomers containing tandem repeats, two of which are highly variable and each of which recognises and binds to a single nucleotide in the target sequence. This variability is responsible for the recognition of specific DNA sequences and thus can be comparatively easier to engineer than

ZFN. The target sequence of TALEN pairs is usually 30–40 bp in length.

Genome editing using ZFN and TALEN, has played an important role in targeted gene editing. However, their use has been limited by certain factors, which are their complexity, difficulty and expensiveness. Due to this, researchers have been compelled to produce a simple, reliable, efficient and an affordable approach for precise genome modification. It has therefore resulted in the development of the gold standard genome editing technology, the CRISPR-Cas9 system. The CRISPR-Cas9 system involves two components, Cas9, a signature endonuclease and guide RNA (gRNA) molecules. Table 4 shows a comparison between ZFNs, TALENs and CRISPR-Cas9 with respect to the major properties of the genome editing tools. In this review we bring forward the detailed overview of CRISPR-Cas9 system in the light of basic science and applications in genome engineering.

1.3. CRISPR-Cas9 system

1.3.1. Evolution of the CRISPR-Cas 9 system

The chronicles for the discovery of CRISPR starts in 1987, when Ishino et al. [5] noted the presence of a 29 nucleotide repeat in *Escherichia coli*, which were interrupted by unrelated, non-repetitive short sequences (spacers). These were followed by several reports of similar sequences in other microbes. In 2000, the presence of similar repeats was reported in most prokaryotes and was then named 'CRISPR' by Ruud Jansen [6]. The significance of the spacers being derived from foreign sources was established in 2005 [7]. Thereafter 4500 CRISPR sequences from 67 strains representing both bacteria and archaea were sequenced [7]. In the same year, Pourcel et al. reported that CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, followed by the discovery of the Protospacer Adjacent Motif (PAM) sequences by Bolotin and others [8,9]. In 2013, it was reported that similar to eukaryotes, prokaryotes have an adaptive immune system and the CRISPR system is an integral part of it [10]. In 2008, the activity of CRISPR on DNA targets was established [11]. It was reported that spacers would be transcribed into mature CRISPR RNA (crRNAs) which can act as small guide RNAs (sgRNAs) [12]. The spacer-guided Cas9 can cleave target DNA generating Double Strand Breaks [13]. This was followed by the expression of Type II CRISPR in other organisms in 2011 [14]. In the same year, it was observed that trans-activating crRNA (tracrRNA) forms a duplex structure with crRNA and Cas9 and is essential for Cas9 activity and thereafter Caribou Biosciences, California started to use CRISPR technology for several therapeutic purposes [15]. In 2012, scientists concluded that CRISPR technology can be used in genome editing and *in-vitro* characterization of DNA targeting by Cas9 was performed [16]. The system was
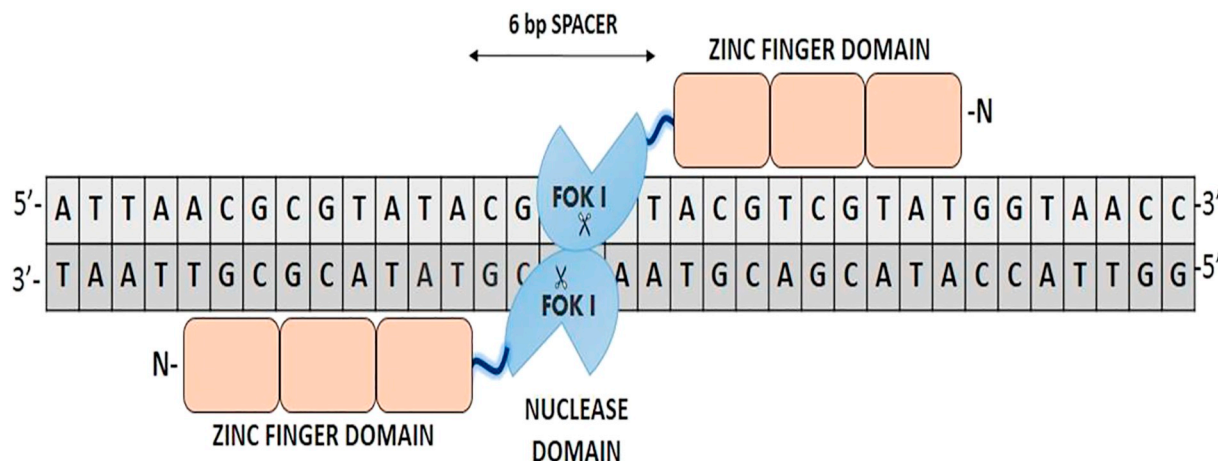
Fig 1. Schematic representation of Zinc Finger Nucleases: Each ZFN comprises a Zinc Finger protein domain (orange) at the N terminus and a *FokI* nuclease domain (blue) at the C-terminus. The *FokI* cleavage domain acts as dimer form to cleave the target sequence in 5–7 bp spacer sequence.
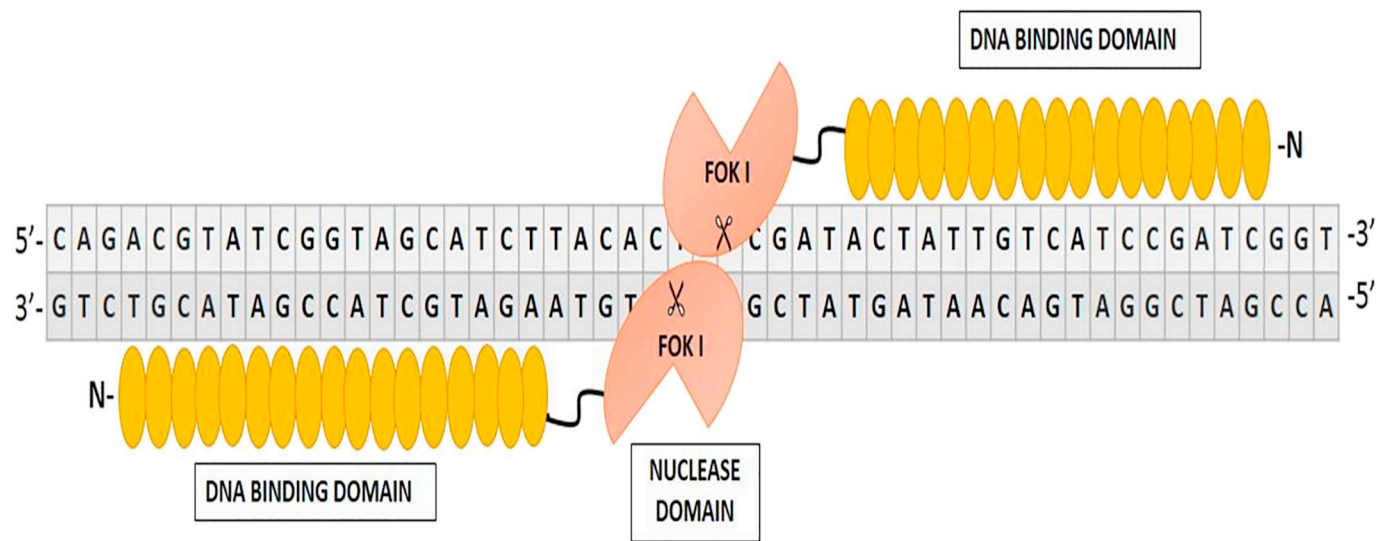
Fig 2. Schematic representation of Transcription Activator Like Effector Nucleases (TALENs): Each TALEN monomer consists of a nuclear location signal (NLS), 152 amino acids deletion N-terminal, 63 amino acids from C-terminal, the TALE repeat domains, and modified Fok I nuclease domain ELD/KKR. Each TALE repeat unit consists of 34 amino acids, in which the amino acids at positions 12 and 13 are called 'repeat-variable di-residues' (RVDs). The RVD determine binding specificity to DNA bases following the code that NG, NI, HD, and NN respectively recognized thymine, adenine, cytosine and guanine.

Table 1

Development of research on CRISPR-Cas system.

Year	Major advances in understanding of CRISPR/Cas system	Reference
1987	Establishment of the existence of CRISPR sequences in *E. coli*	[5]
2000	CRISPR sequence were found in all over prokaryote family	[26]
2002	The CRISPR name was coined and signature Cas genes were established	[6]
2005	Adaptive immunity functions of CRISPR were established with confirmation of the spacer sequence being of foreign origin.	[7]
2005	CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies	[9]
2005	Specific RNA Processing by a CRISPR Endonuclease was shown	[27]
2007	CRISPR-Cas is bacterial immune system	[28]
2008	It was discovered that the mature crRNAs were obtained from mature spacers which can act as small guide RNAs	[12]
2008	CRISPR activity upon DNA targets were established	[11]
2010	Type II CRISPR-Cas cuts target DNA	[13]
2011	It was found that tracrRNA can form duplex structure with crRNA and Cas9	[15]
2011	Type II CRISPR can be expressed in other organisms.	[14]
2012	Scientists concluded that CRISPR technology can be used in genome editing. In vitro characterization of DNA targeting by Cas9 was done.	[16]
2013	Genome editing by CRISPR/Cas in mammalian genome.	[17]
2013	Human genome editing by CAS9	[18,19]
2014	First genome wide Cas9 screening	[22–24]
2014	Knock out via CRISPR/CAS9 in human cell	[20,21]
2014	CRISPR/Cas9 system was rapidly used as a principle tool for genome editing	[25]

simplified with the use of sgRNA to program CRISPR specificity. This marked the employment of CRISPR technology for the first time in human cells for genome editing. Cas9 based genome editing in eukaryotic cells was first established in 2013 [17–19]. In 2014, the first genome-wide Cas9 screening was executed by two independent groups [20–24]. Crystal structure of Cas9 complexed with guide RNA and target DNA was revealed [25]. Presently, CRISPR/Cas9 system is rapidly developing as a principle tool for targeted genome editing all around the globe (Table 1)

2. CRISPR-Cas locus

The CRISPR-Cas system is an adaptive immune system which provides resistance against foreign genetic elements in prokaryotes [10]. CRISPR locus has been found to be persistent in approximately 90% of archaea and 40% of bacterial genomes. The CRISPR array consists of several repeat sequences, interspaced by spacers. These spacers are unique segments obtained from foreign DNA which provide sequence-specific immunity against foreign DNA elements. A cluster of Cas genes are generally located next to such repeat-spacer units. New spacers can

also be introduced into the CRISPR locus during infection so that it can act as a memory during a subsequent encounter with the same invaders [28]. The number of the repeat-spacer units can vary from just a few to several hundred, the average number being 65. The thermophilic bacteria *Chloroflexus* sp., has been shown to contain the highest number of repeat-spacer units (374) in one of its four CRISPR loci. The length of repeat sequences can vary among different loci of the same genome. Recent findings reveal that the repeat sequences range from 18–50 nucleotides (nt) whereas spacer sequences range from 17–84 nt long [29]. There is a 20 nt long DNA target sequence or gRNA, adjacent to an upstream 3 nt sequence called as PAM which are the component of invading foreign element but not a part of the CRISPR locus.

3. Classification of CRISPR-Cas system

CRISPR-Cas system is classified based on phylogeny, sequence, locus organization and contents, resulting in three major types, **Type I**, **Type II** and **Type III**, among which the Type II system is mostly studied (Fig. 3). There are six subtypes identified for the type I system (Type I-A through Type I-F). They are defined by the presence of the signature
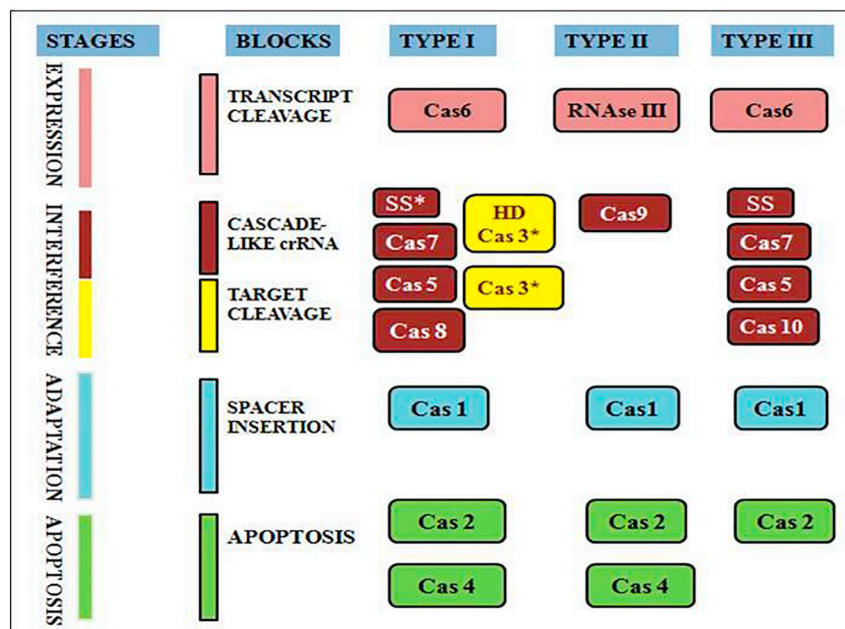
Fig 3. Schematic presentation of types of CRISPR-Cas system.

protein, Cas3, with both helicase and DNase domains responsible for degrading the target. Type II systems have been divided into two subtypes II-A and II-B. The Type II CRISPR-Cas systems encode Cas1 and Cas2, the Cas9 signature protein and sometimes a fourth protein (Csn2 or Cas4). Cas9 assists in adaptation, participates in crRNA processing and cleaves the target DNA assisted in assemblage by crRNA and an additional RNA called tracrRNA. While all Type I and II systems are known to target DNA, Type III systems target DNA and/or RNA. The Type III CRISPR-Cas systems contain the signature protein Cas10. Most Cas proteins are designed for the Type III-A or Type III-B complexes (otherwise also known as Csm and Cmr respectively) [30].

The Cas proteins are a highly diverse group of proteins consisting of approximately 45 Cas gene families found with a wide range of CRISPR subtypes. Cas 1 and Cas 2 are universal in all CRISPR loci, whereas Cas3, Cas9 and Cas10 are specific for type I, II and III CRISPR-Cas systems respectively. Among the CRISPR-Cas types, the type II system has received more attention than the rest because of its ability to induce double strand breaks in the target DNA (Fig. 4).

3.1. CRISPR Cas 9 as an adaptive immune system

The CRISPR activity requires a CRISPR locus including an array of repeat-spacer sequences and a set of CRISPR associated genes (Cas genes) which code for proteins essential for processing, functioning and cleavage activity (Fig. 5). The entire defence process can be classified into three phases:

- Acquisition or Adaptation of new spacers into CRISPR arrays
- Expression and the processing of CRISPR RNAs or crRNAs
- CRISPR interference

1. Acquisition or adaptation

The acquisition phase of CRISPR constructs the genetic memory of the cell. In this phase, new spacers obtained from the invading plasmids or foreign DNAs during the first encounter are incorporated into the array of CRISPR which allows the cell to adapt against the invaders present in the environment. Therefore, this phase is also termed as 'Adaptation'. The information stored in the spacers can be used to act against similar invaders on facing the second encounter. Spacer
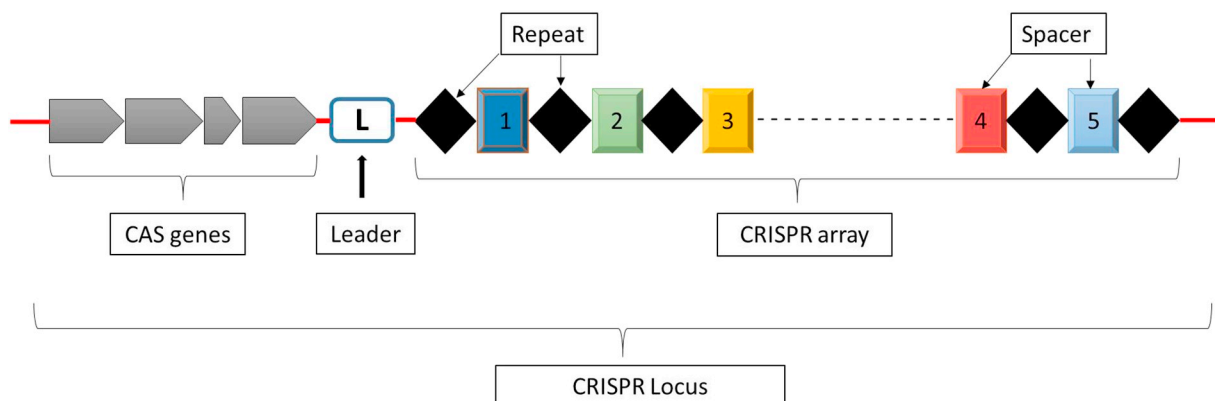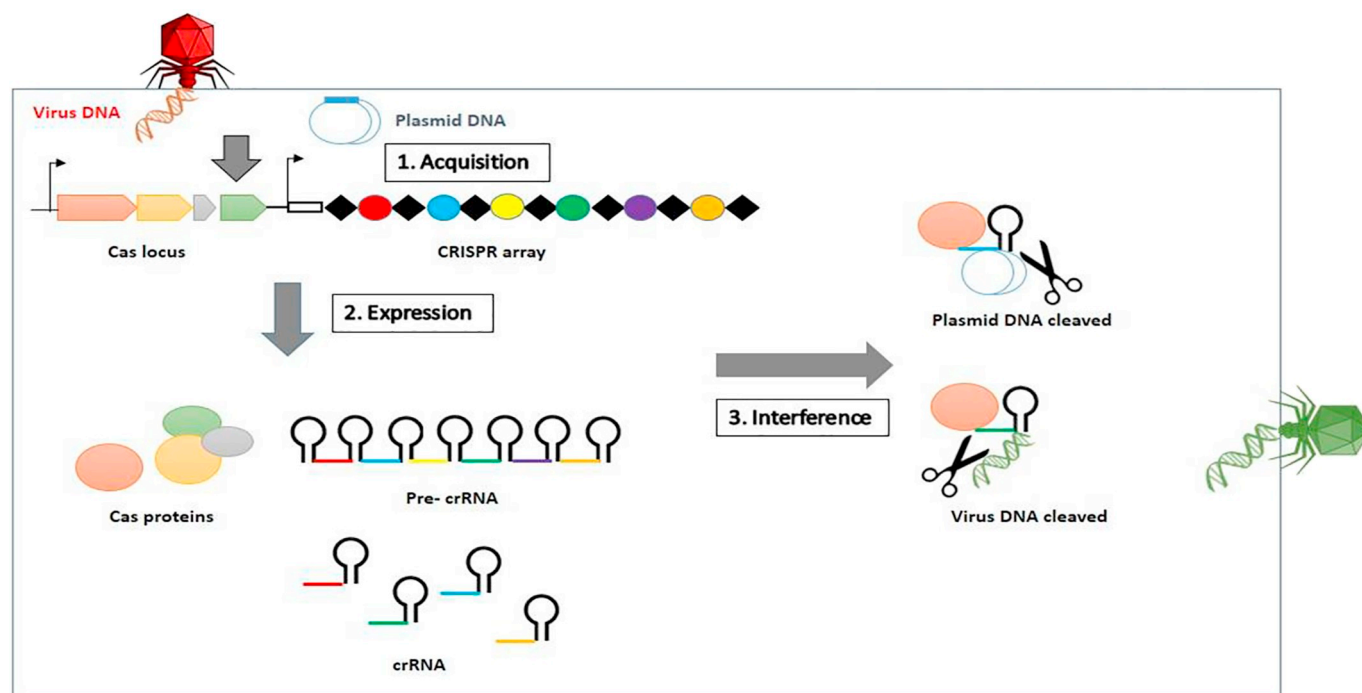

Fig 4. Schematic representation of CRISPR locus. Spacers are shown in colored boxes and unique spacers are shown in unique colors. Repeats are shown in black diamonds. Cluster of Cas genes (grey arrows) are located next to the repeat-spacer units or CRISPR array. A leader sequence (white box, L) is present between the cluster of Cas genes and CRISPR array.

Fig 5. Schematic representation of CRISPR-Cas9 immunity: **Acquisition**: Insertion of new spacers into the CRISPR locus. **Expression**: Transcription of CRISPR locus and processing of CRISPR-RNA (crRNA). **Interference**: Recognition and degradation of foreign elements by crRNA-Cas9 complex.

acquisition occurs in two types. Firstly, when the invader has not been previously encountered and secondly, when there is a pre-existing record of the invader in the CRISPR. This is divided into two steps, the protospacer selection and the generation of spacer. It is followed by incorporation into the array and synthesis of a new repeat sequence. Eventually, some spacer deletion may also occur to control the size of CRISPR array, although very little is known about that. Cas1 and Cas2 are the two most important proteins in spacer acquisition phase of CRISPR. They work as a complex, where a single dimer of Cas2 binds to two dimers of Cas1 to perform the activity. The presence of PAM, a prerequisite for the discrimination between the target and CRISPR array, is utilised in spacer acquisition [28]. It was shown that new spacers are inserted at the leader end of the CRISPR array. The palindromic sequence of many repeats provides direction and position during spacer acquisition into the array. The mechanism of spacer acquisition and sequence of the PAM motif varies among the different types of CRISPR systems.

2. Expression of CRISPR RNA and Cas genes:

Acquisition of new spacers is followed by expression of CRISPR RNA (crRNAs) and Cas proteins. As mentioned earlier, there are three types of CRISPR systems, I, II and III classified based on the set of proteins used. The CRISPR transcription initiates in the leader region which contains the promoter elements, binding sites for regulatory proteins and elements important for spacer integration. A primary transcript, pre-crRNA is generated from the CRISPR array which is further processed into smaller units corresponding to a single spacer. Unlike other CRISPR types, type II employs Cas9 protein for processing of the pre-crRNA. The newly generated mature crRNA interacts with the short tracrRNA and guide the cas9 mediated cleavage of the target DNA.

3. CRISPR interference:

Once the crRNAs are generated, they recognize the invading target sequences through base complementarity. Following this, the crRNAs along with Cas proteins perform the target degradation process. As previously mentioned, the Cas 9 protein executes the same for a Type II CRISPR system. However, there are issues regarding the discrimination between specific and non- specific sequences followed by the occurrences of off-target mutations. In each type of CRISPR, the nucleases have two different domains acting together to perform the degradation. There is a recognition lobe for binding the tracrRNA which in turn interacts with the guide RNA-target DNA hybrid, and a nuclease lobe containing nuclease domains (HNH and RuvC) for degradation of the two strands of the target.

3.2. CRISPR Cas9 system as a genome editing tool

In the era of genome engineering, newer and updated technique to edit the genome is emerging day by day. CRISPR Cas9, the humble adaptive immune system of the prokaryotes, has drawn the researcher's interest. Consequently it has been modified to be implemented as a versatile, adaptable and target specific genome editing tool. This modified version of CRISPR Cas9 system employs Cas proteins guided by gRNA to cleave the target DNA sequence. There are two different components in the CRISPR/Cas9 system: gRNA and an endonuclease (Cas9).

3.3. Cas 9 and guide RNA

Cas 9 protein has six domains i.e. REC I, REC II, Bridge Helix, PAM Interacting, HNH and RuvC domains. Rec I domain is responsible for binding guide RNA. The role of the REC II domain is not yet well understood. The arginine-rich bridge helix is crucial for initiating cleavage activity upon binding of target DNA. The PAM-Interacting domain confers PAM specificity and is therefore responsible for initiating binding to target DNA. The HNH and RuvC domains are nuclease domains that cut single-stranded DNA. They are highly homologous to HNH and RuvC domains found in other proteins. The Guide RNA is comprised of a single stranded RNA (20 bases long) that forms a T-shape comprised of one tetraloop and two or three stem loops. The guide RNA is engineered to have a 5′ end that is complimentary to the target DNA sequence and provides for sequence specificity to its genome target.
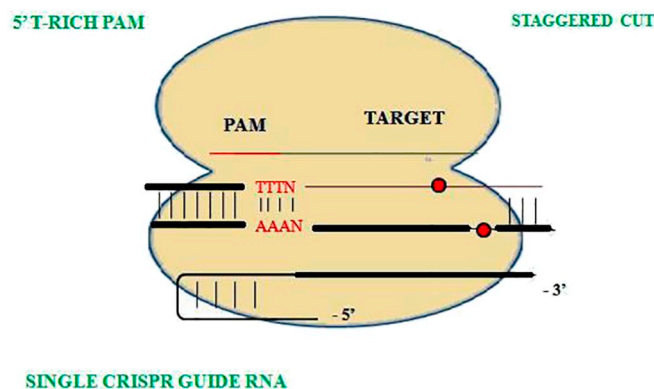
Fig 6. Cpf1, a new RNA guided nuclease: Cpf1 endonuclease recognizes a T-rich PAM bringing about cleavage at a site distal from the recognition site followed by generating sticky ends.

3.4. Cpf1 – an alternative to Cas9 endonuclease

A new RNA-guided nuclease known as **Cpf1** belonging to the CRISPR system of *Prevotella* and *Francisella* has been discovered. It is simpler than the Cas 9 endonuclease and appends the genome editing system with newer properties (Fig. 6). Cpf1 endonuclease recognizes a T-rich PAM bringing about cleavage at a site distal from the recognition site, this leads to the generation of sticky ends instead of blunt ones generated by Cas 9 making them easier to handle. In addition, Cpf1 does not require a tracrRNA, which further simplifies the designing process.

3.5. Mechanism of CRISPR-Cas

The gRNA binds with cas9 and induces conformational change and activates the protein. Once Cas9 is activated, it searches for target DNA by binding with a sequence that matches its PAM sequence. When Cas9 protein finds a target sequence, the protein will melt the bases immediately upstream of PAM and pair with the complementary region of gRNA. If the complementary region and the target region pair properly then Ruv C and HNH nuclease domain will cut the target DNA. Cas9 causes DSB, which are present 3-4 nucleotides upstream of the PAM sequence. DSBs may be repaired by two different pathways, NHEJ (Non-homologous end joining) DNA repair pathway, which is error-prone and produces inserts/deletions (Indels) at the DSB site that leads to frame-shifts or premature stop codon and HDR (Homology directed repair) pathway, that looks for the presence of a homologous DNA sequence and on finding one brings about homologous recombination and it is less error prone (Fig. 7). Since Cas9 functions as a general endonuclease, the only thing needed is gRNA, which can be synthesized chemically, transcribed *in vitro*, or cellularly expressed to provide specificity.

3.6. Limitations of the CRISPR Cas system

While experiments were being carried out to discover the complete potential of the CRISPR-Cas system for application in genome editing, it was reported that while the CRISPR system exhibited high specificity in bacterial cells, when coming to mammalian cells, the system showed a significantly high frequency of non-specific nuclease activity thus leading to mutagenesis in regions other than their particular targets. This arose as a major concern in the technology development process [31]. This was a severe limitation of CRISPR-Cas9 genome editing system. Strategies were devised to overcome this particular limitation. One such successful strategy was the use of paired nickases.

3.7. Role of paired nickase

The Cas9 enzyme could create only one DSB under the guidance of an RNA molecule, but detailed knowledge of the structure and function of the protein has led to the creation of many variants of the existing form. This was essential as the enzyme was found to exhibit off-target mutagenesis at high frequencies in mammalian cells [31]. This aspect required special attention as its application for gene therapy was under consideration. Therefore, a loss of function mutant in one domain of Cas9 was constructed, (loss of RuvC and HNH domain function were caused by D10A and H840A mutations respectively). Due to its ability to break only one DNA strand it was named as nickase [16]. Double-strand breaks can be introduced through the use of paired nickases for cooperative genome engineering [18,19]. A major difference for this approach is that, when two Cas9 nickases are used, long overhangs are produced on each of the cleaved ends instead of blunt ends. This provides even greater control over precise gene integration and insertion. The paired nickase system is being developed to overcome the non-specificity of the wild type CRISPR-Cas system (Fig. 8). Since the target length is increased, the chances of a similar sequence being present in other regions of the genome are decreased. Since a double stranded break would be formed only if both targets match in close proximity, the probability of off-target DSBs is reduced. The individual nicks generated would be repaired by the high-fidelity Base Excision Repair mechanism thereby reducing off-target mutagenesis [32,33]. For the paired nickase system, the target site is selected such that it contains one PAM on each strand at a distance from each other. Thus, the probable PAM closest to the originally cloned target site is searched for in the complementary strand. A minimal distance of about 40-50 nucleotides are kept between the two cleavage sites [32,33].
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3.8. Applications of CRISPR-Cas genome editing in mammalian cells

The simplicity of the CRISPR-Cas9 system led to its establishment as a genome-editing technology, but the driving force for its application was mainly its necessity. With the development of knowledge about the hereditary material, came the observation that many of the commonly occurring, intractable diseases had their origins in the DNA code itself. Thus, the projected goal for any DNA modifying technology had been to correct these errors at their roots. Thereby, plasmid vectors were developed that could allow expression of the *S. pyogenes* Cas9 enzyme in mammalian systems, along with generation of a single gRNA system where a targeting sequence of about 20 nucleotides could be cloned into a larger segment that harbours the tracrRNA [1]. Transfection of this system into mammalian cell cultures revealed precise editing in the genome at levels comparable to those achieved by existing ZFNs and TALENs [34,35].

4. Recent developments in the technology

4.1. Functional screening of genes

For elaborate understanding of the functional aspect of the genome of any model organism, collection of genome-wide loss of function mutants had been generated. This could not be replicated in mammalian systems due to the difficulty in generation of knockouts and hence, silencing libraries created by RNA interference (RNAi) was the only option. This however, suffered the disadvantage of incomplete removal of the protein and had off-target effects. Thus, the CRISPR-Cas9 technology henceforth found application in functional analysis of genes in mammalian cells by the creation of a single gRNA library in lentiviral vectors. Screening of such libraries was performed by positive and negative selection of loss of function mutants [20,21]. Comparison with the existing RNA interference library revealed reduced off target effects in addition to creation of knockouts instead of temporary knock down.
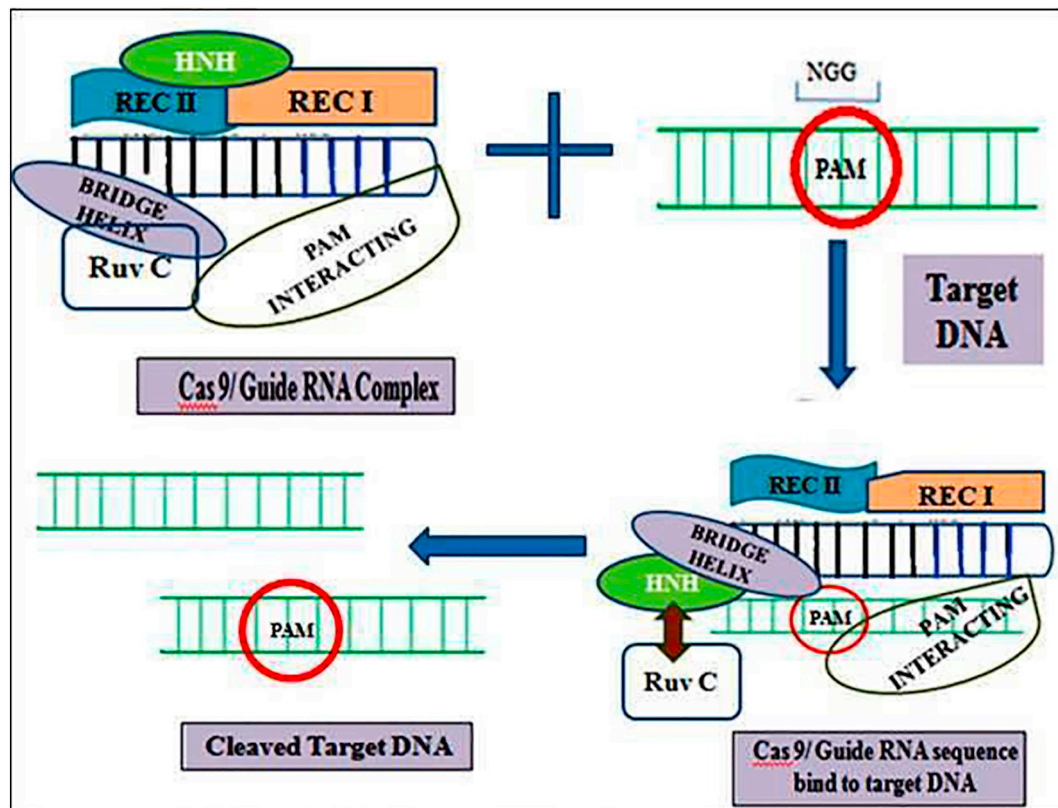
Fig 7. NHEJ and HR pathway: Double-strand breaks encouraged by a nuclease at a specific site can be repaired either by non-homologous end joining (NHEJ) or homologous recombination (HR) Repair by NHEJ regularly occurs in the insertion or deletion of arbitrary base pairs, causing gene knockout by disruption. If a contributor DNA is accessible, which is concurrently cut by the identical nuclease leaving attuned overhangs, gene insertion by NHEJ can also be accomplished. HR through a contributor DNA template can be subjugated to adapt a gene by introducing specific nucleotide replacement or to attain gene insertion.
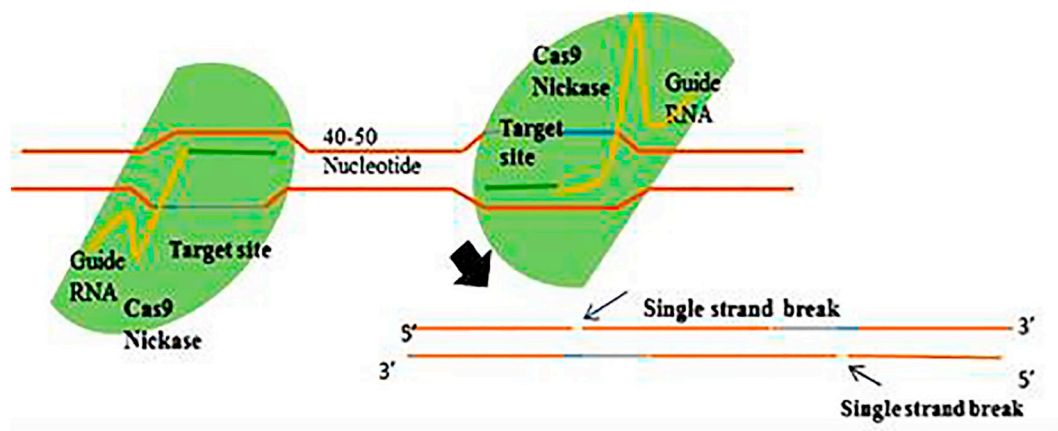
Fig 8. Paired nickases system: Schematic representation of DNA double-stranded breaks by a pair of sgRNAs guiding Cas9 nickases. Cas9 is able to cut merely the strand complementary to the sgRNA; a couple of sgRNA-Cas9n complexes can nick both strands at the same time. sgRNA compensate is defined as the remoteness between the PAM-distal (50) ends of the guide sequence of a known sgRNA pair; positive offset involves the sgRNA complementary to the top strand (sgRNA a) to be 50 of the sgRNA complementary to the base strand that produces a 50 overhang.

4.2. Creation of cellular and animal models for diagnosis and gene therapy

The complicacies involved in the genome editing technologies before CRISPR-Cas9, was a major hurdle for them to be used for any therapeutic purposes. CRISPR-Cas technology came as a revolution in this field by permitting precise mimicking of various modifications of DNA in cell and animal models that could range from single point mutations to chromosomal translocations thereby leading to generation of both transgenic cell lines and animal systems [36–40]. This application has been made effective not only for diagnosis but also for

correction of erroneous insertions or deletions, thus leading to an important aspect of gene therapy [17]. Thus, diseases that had polygenic sources could now be recreated in models with ease.

4.3. Transcriptional studies using fusion proteins

The inactive dCas9 protein when targeted to a particular genomic location could inhibit the binding of the RNA polymerase, thereby stalling transcription. It could hence function in a manner similar to RNA interference and this application came to be known as CRISPR

Table 2

Application of CRISPR-Cas9 system in different plants.

Species	Transformation method	Promoters (Cas9, gRNA)	Target	Reference
Arabidopsis thaliana	PEG protoplast transfection, Leaf agroinfiltration	CaMV35SPDK, AtU6	PDS3, FLS2	[49]
Nicotiana benthamiana	PEG-protoplast transfection Leaf agroinfiltration	CaMV35SPDK, AtU6	PDS3	[49]
Arabidopsis thaliana	Leaf agroinfiltration	CaMV35S, AtU6	Co-transfected GFP	[61]
Nicotiana benthamiana	Leaf agroinfiltration	CaMV35S, AtU6	Co-transfected GFP	[61]
Oryza sativa	PEG-protoplast transfection	CaMV35S, OsU6	SWEET14	[61]
Nicotiana benthamiana	Leaf agroinfiltration	CaMV35S, AtU6	PDS	[58]
Nicotiana benthamiana	Leaf agroinfiltration	CaMV35S, CaMV35S	PDS	[62]
Triticum Aestivum	Agrotransfection of cells fromimmature embryos	CaMV35S, CaMV35S	PDS, INOX	[62]
Oryza sativa	PEG-protoplast transfection	2xCaMV35S, OsU3	PDS, BADH2, MPK2, Os02g23823	[59]
Triticum Aestivum	PEG-protoplast transfection	2xCaMV35S,TaU6	MLO	[59]
Oryza sativa	Particle bombardment of callus	2xCaMV35S, OsU3	OsPDS, OsBADH2	[59]
Oryza sativa	PEG-protoplast transfection	CaMV35S, OsU3 or OsU6	MPK5	[60]
Arabidopsis Thaliana	Agro-transformation by floral dip	AtUBQ1, AtU6	Co-transfected GUUS	[63]
Arabidopsis Thaliana	Agro-transformation by floral dip	AtUBQ1, AtU6	TT4, CHLI1, CHL12	[63]
Oryza sativa	Agro-transformation of Callus	OsUBQ, OsU6	OsMYB1	[63]
Arabidopsis Thaliana	Agro-transformation by floral dip	2xCaMV35S, AtU6	BRI1, GAI, JAZ1	[57]
Oryza sativa	Agro-transformation of Callus	CaMV35S, OsU6	ROC5, SPP, YSA	[57]
Oryza sativa	Agro-transformation of Callus	ZmUbi, OsU3	CAO1, LAZY1	[64]
Nicotiana benthamiana	PEG-protoplast transfection	2xCaMV35S, AtU6	PDS, PDR6	[65]
Nicotiana tabacum	Agro-transformation of leaf discs	2xCaMV35S, AtU6	PDS, PDR6	[65]
Oryza sativa	PEG-protoplast transfection	ZmUbi, OsU6	KO1, KOL5, CPS4,CYP99A2; CYP76M5, CYP76M6	[66]
Oryza sativa	Agro-transformation of Callus	ZmUbi, OsU6	SWEET1a-1b-11-13	[66]
Triticum Aestivum	PEG-protoplast transfection	ZmUbi,TaU6	MLO-A1	Wang et al. [22,24]
Zea mays	PEG-protoplast transfection	2xCaMV35S, ZmU3	IPK	[67]
Citrus sinensis	Leaf agroinfiltration	CaMv35S, CaMV35S	PDS	[68]
Arabidopsis Thaliana	Agro-transformation by floral dip	CaMV35S, AtU6	Co-transfected GFP	([68], [69])
Sorghum Bicolor	Agro-transformation of immature embryos	OsActin1, OsU6	Co-transfected DsRed	([61], [68])
Arabidopsis Thaliana	Agro-transformation by floral dip	PcUBI4-2	ADH1, TT4, RTEL1	[70]
Oryza sativa	Agro-transformation of callus	2xCaMV35S, ZmU3	PDS, PMS3, EPSPS, DERF1, MSH1, MYB5, MYB1, ROC5, SPP, YSA	[71]
Oryza sativa	Agro-transformation of callus	2xCaMV35S, AtU6	BAL	[72]
Solanum lycopersicum	Hairy root transformation by *A. rhizogenes*	CaMV35S, AtU6	GFP, SHR	[73]
Solanum lycopersicum	Agro-transformation of cotyledons	2xCaMV35S, AtU6	SlAGO7, Solyc08g041770, Solyc07g021170, Solyc12g044760	[74]
Marchantia polymorpha	Agro-transformation of sporelings	CaMV35S or MpEF, MpU6	ARF1	[75]
Hordeum vulgare	Agrobacterium mediated transformation	ZmUbi, TaU6	HvPM19	[76]
Brassica oleracea	Agrobacterium mediated transformation	CsVMV, AtU626	BolC.GA4.a	[76]
Populustomentosa	Agrobacterium mediated transformation	AtU3b, AtU3d, AtU6-1 AtU6-29	PtoPDS	[77]
Zea mays	Biolistic-mediated transformation	maize U6 polymerase III promoter, maize UBIQUITIN1 promoter	LIG, ALS2MS26, MS45	[78]
Petunia			Petunia nitrate reductase (NR)	[79]
Triticum aestivum	Particle bombardment of embryos		TaGASR7, TaGW2, TaDEP1,TaNAC2, TaPIN1, TaLOX2,	[80]

(continued on next page)

Table 2 (*continued*)

Species	Transformation method	Promoters (Cas9, gRNA)	Target	Reference
Apple	Protoplast transformation		DIPM-1, DIPM-2, DIPM-4	[81]
Grape	Protoplast transformation		MLO-7	[81]
Apple	Agrobacterium mediated transformation	35SCaMV, AtU6-1	phytoene desaturase (PDS) gene	[82]
Zea mays	Biolistic-mediated transformation	maize U6 polymerase III promoter, maize UBIQUITIN1 promoter	ARGOS8	[83]
Solanum lycopersicum	*Agrobacterium tumefaciens*-mediated transformation	CaMV 35S, AtUBQ, AtU626	SlPDS and SlPIF4	[84]
Oryza sativa	Rice callus transformation		OsBEIIb	[85]

Interference and demonstrated lower off-target effects. This property was efficient only in prokaryotes as the eukaryotic transcriptional machinery is more complicated and requires involvement of various transcriptional activators and repressors for regulation. Fusion of dCas9 with repressor domains like KRAB and SID, that modify the structure of the chromosome, permitted efficient epigenetic silencing in human and yeast systems [41–44]. Similarly, fusion of the transcriptional activators VP16 and p65 activation domain enabled their recruitment on complimentary promoters, leading to activation of transcription and could also help in mapping of enhancer elements.

4.4. Fluorescence imaging of genome

The understanding of epigenetic mechanisms caused a shift in importance, from the DNA code to its structural organization within the cell. Intensive research is yet being pursued in this aspect to elucidate all the possible structural modifications that occur in the genome and their subsequent effects in the cells. In order to identify the various changes in chromatin states, it was essential to visualize the genetic material in the cell. Techniques such as Fluorescence In-situ Hybridization (FISH) were thus developed that could label DNA for visualization. The sample preparation itself served as a major drawback for the technique as it compromised on real time imaging. Fusion of fluorescent molecules with the dCas9 protein was able to serve the purpose efficiently and also allowed for live cell imaging [45]. That included, combination with multiplexing abilities of the protein the technique can be improved further.

4.5. Antimicrobial and antiviral applications

Drug resistance is creeping within bacterial populations due to improper use of antimicrobials. It is another sector where the applications of this system are being focused. Bacterial populations undergo constant mutation as a defence mechanism required for their survival against antibiotics. Due to the medicines being generalized in action, and used inappropriately, the bacteria develop resistance against them and transfer them horizontally to other populations. Recent studies showed that delivering the CRISPR Cas system designed against the resistance genes of bacteria, delivered by means of bacteriophages, helped in targeting the drug resistant candidates thereby making them sensitive, while causing no harm to the virulent populations [46,47]. In a similar manner, sequence specific antivirals are also under consideration, modifying genes involved in host-virus interaction.

4.6. Applications of CRISPR/Cas genome editing in plants

Unlike animals, targeted genome editing was not achievable in plants for several years. Very recently natural mutations (during tissue culture or hybridization) and induced mutations using X-rays [48] and fast neutrons bombardment or chemical factors such as ethyl methane sulfonate [49–51], sodium azide [52], and diepoxybutane [53] were used to create non-targeted mutation in plant genome. Apart from the

physical factor-induced mutations, Agrobacterium T-DNA mediated forward and reverse genetics studies was the other method of choice to create non targeted mutation in plant genome. The only way to targeted mutation was RNA interference (RNAi) [54]. Knockdown using RNAi, cannot achieve permanent effect on the genome thus this method cannot be used very efficiently for crop improvement [55,56]. Recent discovery of targeted genome editing tools such as ZFN, TALEN and lately CRISPR has revolutionised plant research. Conventional and marker assistant breeding was the only way for introduction of a new trait in the cultivated variety. The CRISPR/Cas9 system represents a flexible approach for genome editing in plants. It provides a valuable tool for both basic research in plant and offers opportunities for crop improvement. In the year 2013 several reports were published discussing the first application of CRISPR/Cas9-based genome editing in plants [36,57–60]. Thus, as CRISPR/Cas9 system can easily modify common crop plants, it is predicted to be the way to move forward with plant breeding programs (Table 2).

There are several reports of important agronomic traits, being incorporated in plant genome, using CRISPR-Cas9 based genome editing. The acetolactate synthase gene of wheat was modified to obtain chlorosulfuron (herbicide) resistant wheat. This was achieved by changing a single amino acid residue through CRISPR-Cas [78]. Also, CRISPR-Cas9 guided targeted insertion of phosphinotricin acetyl transferase gene into maize was achieved which in turn conferred resistance to natural herbicide Bialaphos [86]. Due to the hexaploid nature, creating mutation in bread wheat is a big challenge. Simultaneous mutation of three homoalleles in hexaploid bread wheat using CRISPR-Cas9 system confers heritable resistance to powdery mildew, a trait not found in natural populations [24]. In a nutshell, several economically important traits have been incorporated into various crop plants. Such traits include yield level, nutritional value, stress tolerance, pest and herbicide resistance. Plant species such as rice, wheat, maize, tomato apple, model legumes Lotus, Medicago etc. have all been successfully transformed.

4.7. Obstacles in CRISPR-Cas9 technology application in plants

The biggest obstacle in the field of CRISPR-Cas9 modified plants is that they are considered genetically modified organisms (GMOs), which are strictly regulated in some countries. Two approaches have been described with potential to bypass this problem, that involves delivery of a mixture of Cas9-encoding mRNA and gRNAs or pre-assembled ribonucleoprotein complexes [87]. However, the transient mutation by this approach is less efficient. Further developments of the existing procedures are needed to modify plants with CRISPR-Cas9, in order to have them not being classified as GMO.

Another problem is the off-target effect of CRISPR-Cas9 system itself. Till date, only limited large scale data are available to predict that an off-target effect of CRISPR-Cas9 in plant is low. Nonetheless, CRISPR-Cas9 is the most promising tool which can be used to incorporate heritable traits in plants. It is probably the easiest method that leads towards high yield production of various crops, to meet the

demands of a fast growing world population.

5. Application of CRISPR-Cas system in genome engineering of parasites

In many parts of the world, parasitic diseases are posing a serious threat to both human and animal health. Advances in sequencing and understanding of the genomes of parasites like *Plasmodium sp.*, *Trypanosoma sp.*, *Leishmania sp.* etc. have played an important role in the science of parasitology [88,89]. Understanding the relationship between a parasite and its host through genome sequence information had led towards development of new diagnostic methods for parasitic diseases and also helped in the development of vaccines and anti-parasitic agents. Significant advancements have been made in genome editing of parasites like *Leishmania*, *Trypanosoma cruzi* and *Toxoplasma gondii* as well as their vectors.

Leishmania sp.

Genetic manipulation of *Leishmania* sp was a problematic task till the development of CRISPR/Cas9 system. Application of CRISPR/Cas9 system in *Leishmania* sp. has taken place with the expression of Cas9 endonuclease under the control of DHFR-TS promoter and the U6 snRNA promoter and terminator were used to produce the sgRNA and subsequently generated the null mutants of paraflagellar rod-2 locus and the precision of the system was validated by the absence of off-target mutations in the genome [90]. Similarly, in a more recent study, Zhang et al. used a strong RNA polymerase I promoter and ribozymes to develop both single- and dual-gRNA expression vectors, and generated loss-of-function insertion and deletion mutations. Green fluorescence protein (GFP) sequence was introduced into the *L. donovani* genome [91]. Zhang et al. in one of their recent research, targeted the Milte-fosine transporter gene by Co-CRISPR strategy (developed by using a gRNA and Cas9 co-expression vector) and conducted serial transfections of an oligonucleotide donor in *Leishmania* sp. Their aim was to accelerate the characterization of *Leishmania* genes for new drug and vaccine development. They successfully used CRISPR genome editing to delete A2 multigene family and induced targeted chromosomal translocation in *Leishmania* sp. They reported that this CRISPR system could also be used to generate specific chromosomal translocations and those studies will provide a great help in the study of *Leishmania* gene expression [92].

5.1. Plasmodium sp

Non-homologous end joining (NHEJ) does not occur in *Plasmodium* spp, thus making it impossible to perform genome editing using template independent approach. Thus with Cas9 we need to use a homologous template to enable homology directed repair [92,93]. Though genetic engineering is the major goal to be achieved using this system, the use of Cas9 as transcriptional enhancer/blocker has also been demonstrated using a catalytically dead version of cas9, dCas9, lacking the endonuclease activity [41,42]. *Streptococcus pyogenes* Cas9 endonuclease and sgRNA produced using T7 RNA polymerase, was used to efficiently edit portions of the *P. falciparum* genome [94]. In another study in *P. yoelii*, DNA double-strand breaks in a specific locus was produced by Cas9. Genes with knockout efficiency up to 50 and 100% and allele replacement up to 22–45% were achieved using CRISPR/Cas9 system, with subsequent repair through homologous recombination, generating replaced genes of parasites at multiple nucleotides [94,95]. CRISPR/Cas9 mediated editing of *pfmdr1* ratifies that point mutation in *pfmdr1* causes enhanced resistance to ACT-451840 in fifth stage gametocytes [96].

5.2. Toxoplasma gondii

Among the many zoonotic parasitic diseases, toxoplasmosis is one of the most important. It is caused by an obligate intracellular protozoan *T. gondii*, which is capable of infecting all warm-blooded animals including humans. It is estimated that one-third of the world's population is chronically infected with *T. gondii* [97]. The strategies to prevent or cure *T. gondii* infection are very limited [98]. The ability to genetically manipulate the genome of *T. gondii* can be of great help to the advancement towards developing effective vaccination strategies and better therapeutics. The predominant NHEJ repair pathway of *T. gondii* are carried out by two master molecules Ku80 and Ligase IV DNA, so ΔKU80 and Δ*Ligase IV DNA* mutants would have enhanced efficiency to carry out the competitive HDR. Sidik et al. [99] generated non-selection knockouts of *T. gondii* using the RNA-guided Cas9 endonuclease to introduce point mutations and epitope tags into the parasite genome. They confirmed that Δ*KU80* mutants are more susceptible to genetic edition than wild-type *T. gondii* due to the lack of NHEJ machinery for double-strand breaks repair. Later on, they conducted a genome-wide genetic assessment exploiting the CRISPR/Cas9 system to ratify the role of every single gene in human fibroblasts infection and successfully identified several novel and unique genes, responsible for the infection and also identified an invasion factor called claudin-like apicomplex, a microneme protein (CLAMP) [100]. Zheng et al. tried a knockout method that takes advantage of the CRISPR/Cas9 system to evaluate the potential of leucine aminopeptidase from *T. gondii* as a drug target. They chose a Cas9 target site in the gene encoding *T. gondii* leucine aminopeptidase and then constructed a knockout vector containing Cas9 and the single guide RNA. Phenotypic analysis was done and it revealed that knock-out of *T. gondii* leucine aminopeptidase resulted in inhibition of invasion. Both the growth and invasion capacity of knockout parasites were restored by complementation with substituted allele of *T. gondii* leucine aminopeptidase. *T. gondii* leucine aminopeptidase knockout in experiments with mouse demonstrated that it reduced the pathogenicity of *T. gondii* to some extent [101–103]. TgUAP56, a component of mRNA export machinery, was studied in *T. Gondii*, according to the functional genomic approach with CRISPR/Cas9 as an editing tool [104]. This system may be applied to disrupt selected genes by ddCas9 (fusion of ddFKBP with Cas9) to monitor mRNA export factors. Researchers exploited CRISPR-Cas9 system to perform a genetic screening and established that a given parameter participated in mRNA exportation in *T. gondii*. The results showed that, like TgUAP56, TgRRM_1330 protein led to the aggregation of mRNA in the nucleus.

5.3. Trypanosoma cruzi

Trypanosoma cruzi, the causative agent of Chagas' disease, is a protozoan parasite that infects both humans and animals. *T. cruzi* is the least understood protozoan, responsible for infection-induced heart disease, due to their complex genetics. Absences of efficient biomolecular tools for genome engineering have hindered the investigation of this organism which was overcome with the CRISPR-Cas9, as it was working efficiently in the *T. Cruzi* genome. Peng et al. [105] have targeted α-tubulin and histidine ammonia lyase (HAL) genes, and efficaciously suppressed the genes. Later on, multiplexing capacity of CRISPR-Cas9 was demonstrated by producing high-capacity repeat knockouts to decrease expression of the β-galactofuranosyl glycosyltransferase family of enzymes, by targeting the known members of the gene family with as few as three rounds of transfection [106].

5.4. Trichomonas vaginalis

Trichomonas vaginalis is the causal organism of Trichomoniasis. It is one of the major sexually transmitted disease worldwide [107]. It causes increase in the vaginal pH and toxication leading to epithelial breaks and inflammation [108]. Deciphering the pathogenicity and gene function of *Trichomonas* by CRISPR-Cas9 associated reverse genetics approach was way too problematic due to the increase in Cas9 toxicity upon *in vivo* gene expression [105] and the low transfection

efficiency in this organism [109]. The barriers in gene editing using the CRISPR/Cas9 system was overcome by the application of nucleofection resulting in 20-fold transfection efficiency and regulation of the FKBP-DD and Shield-1 expression, as lowering the level of Shield-1 expression resulted in the elevated stabilisation of FKBP-Cas9. Researchers recently reported the application of this methodology of CRISPR-Cas9 introduction in *Trichomonas vaginalis* to knockout *ferredoxin-1* and *mif* genes. In this manner, molecular methods for *T. vaginalis* genome engineering can be used to shed light on gene function pathogenesis of this parasite [110,111].

5.5. Cryptosporidium sp

Cryptosporidium sp. is one of the important pathogen causing diarrhoea after Rotavirus [112,113]. There is no effective vaccine against this parasite due to their deficient number of genes (~ 3950 genes). Recently the researchers have optimised the transfection of *C. parvum* sporozoites in HCT-8 cells in tissue culture. The advent of CRISPR/Cas9 technology has allowed detection of genes, responsible for neomycin resistance. *C. parvum* CRISPR/Cas9 system were constructed with Cas9 gene, flanked by parasite regulatory sequences, and the luciferase gene was deactivated. Restoration of luciferase activity took place when specific guide was co-transfected in *C. parvum* sporozoites.

In a recent study of *C. parvum*, the authors described methods for adaptation of the CRISPR/Cas9 system for genome editing to *C. parvum*. As the NHEJ pathway is absent in this parasite, they developed a 1-vector strategy for expression of sgRNA and Cas9 nuclease driven by C. parvum U6 RNA promoter and parasite regulatory sequences, and then co-transfected it with a DNA donor for homologous recombination. To test the system, they used a DNA template to repair the sequence of an inactive nanoluciferase (dead Nluc), thus restoring the open reading frame and the function of the reporter [114] (Table 3).

5.6. Challenges in therapeutic application of CRISPR/Cas9 system in animal model

While CRISPR-Cas9 has shown immense potential in targeted genome editing, it is still a long way from being applied therapeutically. While generation of "knock-out cells" using CRISPR-Cas9 technology has become routine practice in modern cellular biology, it is mainly restricted to *in vitro* models. Applications of CRISPR-Cas9 have been done in mice model; however its efficacy is low and is prone to complications [39,40,121]. Apart from its off-target effect, the delivery of CRISPR-Cas9 is a big challenge that remains to be overcome. The various strategies that have been employed to deliver gene editing plasmid constructs can be mainly categorised as viral and non-viral delivery methods. *In vivo* therapeutic approaches (involving injection or oral mediated entry into subject for genome editing) utilize mostly viral delivery methods. *Ex vivo* approach (isolating the target cells, modifying them and putting them back in the host organism) utilises both viral and non-viral delivery methods. Previous gene editing technologies were non-specific in nature. Clinical trials with this non-specific approach resulted in disaster with off target genotoxicity [148; 122–124]). Some of these therapies resulted in patients suffering from cancer due to off target gene insertion near tumour promoting genes.

Current CRISPR-Cas9 technology is promising in this aspect of reduced off-target effect. However, another problem that plagues this technology is its transporter. The vehicle used for such *in vivo* approaches is quite often viral mediated. Different types of viral mediated vectors are currently being used. Adeno-associated virus (AAV) (Carroll et al. [125], Platt et al. [126], Hung et al. [127]), Adenovirus (Voets et al. [128], Maddalo et al. [129]) and Lentivirus (Shalem et al. [20,21], [22–24]) are some such transporters. Each vector mentioned here has its own advantage along with severe disadvantages. The next step for their successful initiation into clinical trials would require overcoming these disadvantages. Both Adenovirus and Lentivirus have better gene

Table 3
CRISPR-Cas study in protozoal system.

Organism	Purpose	Target	Repair mechanism	References
P. falciparum	Gene knockout	*kahrp* gene, integrated GFP reporter gene, *orc1*	HDR	[115]
P. falciparum	Gene knockout	*Kharp* gene, *eba-175*	HDR	[94]
P. falciparum	Gene knockout, C-terminal tagging	PfMDR1, pFatp4		[96], [116], [117]
T. gondii	Gene knockout and knockin	Uprt, ROP18 locus	NHEJ, HDR	[106]
T. gondii	Gene knockout, C-terminal tagging and insertion of point mutations	KU80, ΔLigase IV DNA, CLAMP	NHEJ, HDR	[99]
T. gondii	Gene knockout	Leucine aminopeptidase	NHEJ	[101], [103], [102]
T. gondii	Gene knockout	TgUAP56	NHEJ	[104]
C. parvum	Gene knockout and knockin	Thymidine kinase	HDR	[118]
T. cruzi	Gene knockout	Paraflagellar rod protein 1and 2, gp72	MMEJ, HDR	[119]
T. cruzi	Gene disruption (single, multi), exogenous gene swapping	Alpha tubulin, histidine ammonia lyase	MMEJ, HDR	[105]
T. cruzi	Gene tagging	Flagellar calcium binding protein(TcFCaBP), the vacuolar proton pyrophosphatase (TcVP1), two proteins of undefined or disputed localization, the mitochondrial calcium uniporter (TcMCU), and the inositol-1,4,5- trisphosphate receptor (TcIP3R)	MMEJ, HDR	[119], [114]
L. major	Replacement of a gene tandem	Paraflagellar rod-2 locus	HDR	[120]
L. donovani	Gene knockout and C-terminal tagging	LdMT(M381T) responsible for miltefosine resistance	IHR, MMEJ, HDR	[98], [91]
T. vaginalis	Gene knockout and knockin	*Ferredoxin-1* and *mif* genes. Tv HMP23	NHEJ, HDR	[110], [111]

Table 4
Comparison between ZFN, TALEN and CRISPR-Cas.

Properties	ZFN	TALENs	CRISPR/Cas 9
Component involved in sequence recognition	Protein-DNA	Protein-DNA	RNA-DNA
Targeting efficacy	Less specific and efficient	Moderately specific and efficient	Highly specificity and efficiency
Cost effectiveness	Very expensive	Expensive	Cheap
Off target mutagenesis	Variable	Low	Moderate
Designing parameter	Protein	Protein	RNA
Viral delivery	Easy	Moderate	Moderate

delivery efficiency but Adenovirus has been shown to induce immunogenic responses against both viral transporter and Cas9 (Voets et al. [128]; Maddalo et al. [129]). Adenovirus based delivery used in clinical trials resulted in severe immunological response, in case of one patient resulting in death ([130]; Christopher A. 2018). Furthermore, genetic targeting, though precise in its approach, induced some off target effects and undesired downstream mRNA splicing events [20–24]. In AAV, immunological response and off-target effects are quite reduced. However, the delivery efficiency is quite low in case of AAV. *In vivo* approaches using non-viral methods have also been done and they have shown some therapeutic promise. One such method is liposome/lipid nanoparticle mediated transfer of Cas9 and guide RNA along with homology arm containing DNA. However, they have certain disadvantages as well; chief among them is the transportation mode being vulnerable for cargo degradation ([131,149]). Due to this degradation it results in a lower efficacy. Nanoparticle mediated entry of CRISPR-Cas9 vectors have also been developed and have been shown to be effective. However there is also a non-specific inflammatory response [132,133].

Ex vivo approach employs a wide variety of techniques ranging from microinjection, electroporation, hydrodynamic delivery and cell permeable peptides. Microinjection and electroporation techniques have been used extensively to produce knockout organism by modifying zygotes of these organism [39,40,134,135]. While above mentioned techniques have yielded success there are also certain limitations associated with them. Microinjection is a difficult and time consuming technique while electroporation cannot be applied to all types of cell [134,136]. Cell permeable peptides (CPP) are also utilised but are limited to only *in vitro* use [137,138]. Genome editing is a rapidly growing technology which is at the threshold of being transferred from bench-to-bedside, thus reflecting its potential as an innovative genetic manipulation tool. CRISPR-Cas9 technology is promising and would require further developments to make it highly specific for editing a genome to reduce the risk of undesired mutagenesis. Also the delivery vehicle should allow for safe and efficient transport to the target. An all-purpose delivery method has yet to emerge which will successfully overcome both of these problems.

5.7. Genome editing in mitochondria

Maternally inherited mitochondrial DNA (mtDNA) consist of several dozens of genes for proteins, tRNAs and rRNAs which are responsible for metabolic, bioenergetic and homoeostatic process. The targeted genome editing in mitochondria (mt) is challenging task and more complicated compare to nuclear DNA editing. Scientists have tried to successfully edit Mt genome using the gene editing techniques like recombinant Restriction enzymes, ZFNs, TALEN and CRISPR-Cas9. Each technique has its success and limitation [139]. Mammalian Mt lacks efficient double strand break repair mechanism (NHEJ or HR), hence introduction of double strand break leads to degradation of mtDNA. Bacterial Restriction enzymes modified by an N terminal Mitochondrial targeting sequence (MTS) to direct its transport to the mitochondria was used to manipulate mtDNA heteroplasmy [140,141]. But the success of restriction enzymes to manipulate the Mt DNA is very limited.

This is due to the lack of sequence information regarding target sites present in WT or mutant DNAs. Also, since each endonuclease can cut a specific sequence, the presence of such sequences near target sites limits its application.

To overcome the limitations of REs for targeted mitochondrial genome editing ZFN & TALENs were used. In 2008, first attempt was made to use conventional pair of hetrodimeric ZFNs to target point mutation in mt, 8993T > G, but unfortunately it failed. Alternative approach was made designing a single-chain ZFN with two Fok1 endonuclease domains joined by 35aa linkers, that recognizes mt DNA target sequence of 12bps. This was effective but raised some concern regarding toxicity [142]. More success was achieved after adding MTS-epitope tag-MES at the end terminal of ZFN which resulted in reduction in mt DNAs and a shift towards WT mtDNA [143]. Despite promise, there are limitations of ZFN usage for targeting mt DNA. Due to the short size of the mt genome, it is unable to work on all target sites due to insufficient Zinc Finger module.

Scientist have designed mitochondrialy targeted TALENs (mito-TALENs) to cleave the specific sequences in mt-DNA. Mito TALENs effectively reduced the level of targeted pathogenic mt DNAs in the cell lines and cybrid cells [144,145]. Mito TALENs employing gain of T or gain of C design principals have the capacity to revert many clinically relevant mt-DNA point mutations [145]. Mito TALEN was also used successfully to prevent the transmission of pathogenic mt-DNA in man. Potentially TALENs may be more effective than ZFNs but their bulky size limit their use; even though shorter repeat sequences and optimized mitochondrial targeting by comparing efficiencies of various MTSs could not provide full success.

To use the CRISPR–Cas9 system to manipulate the mitochondrial genome, gRNA would need to be imported inside mitochondria. But, there is conflicting opinion as to whether RNA can be imported into mammalian mitochondria. In 2015, Yunjong Lee [146] published a report claiming the use of CRISPR–Cas9 to shift mtDNA heteroplasmy in mammalian cells. However these data are controversial and other contradictory report has been published [147]. As the effective import into the mitochondria is a debatable issue it would not be incorrect to conclude the genome editing of Mt DNA may be beyond the reach of CRISPR-Cas9 system.

5.8. Conclusion and future directions

The CRISPR-Cas9 system has undergone one of the fastest developments in the field of research in recent times. From being established as a bacterial adaptive immune system to its being improved into the most efficient and simple-to-design genome editing tool. Observed to be functional in prokaryotic, mammalian and plant systems, CRISPR-Cas9 system has shown the potential to have wide applications ranging from medication using gene therapy to crop improvement. Its contribution in studying the functional parameters at DNA and protein levels has been enormous. It has further accelerated research in its own domain by allowing easy modifications at the DNA level. On-going research is focussed on extracting all possible information using this technology. The system serves as yet another that nature holds some of the simplest solutions to some of the most complicated problems.

Abbreviations

PEG   Polyethylene glycol
CaMV35S Promoter of the 35S gene of the Cauliflower Mosaic Virus
2x35SCaMV   35S promoter with duplicated enhancer
CaMV35SPDK Hybrid promoter, 35S enhancer fused to the maize C4PPDK basal promoter
OsU3,U6 O. sativa U3 or U6 promoters
ZmUbi, U3 Z. mays ubiquitin or U3 promoters
TaU6   T. aestivum U6 promoter
AtUBQ1,U6   A. thaliana ubiquitin1 or U6 promoter
PcUBI4-2 P. crispum polyubiquitin promoter
TaU6   T. aestivum U6 promoter
MpEF   M. polymorpha elongation factor 1α promoter
MpU6   M. polymorpha U6 promoter

Author contribution

D. Gupta and OB have contributed equally in preparing the manuscript.

MKS, DM, DD, AG, KTA and RG have prepared individual sections.

SS, KA, DC, VR and SR did critical review of the manuscript.

D. Ghosh conceived, compiled and edited the manuscript.

Declaration of Competing Interest

The authors declare that there was no conflict of interest.

Acknowledgement

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