CRISPR/Cas9 – A highly targeted genome editing technique

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1. Introduction

Viruses are a threat to prokaryotic life, since they can invade bacteria or archaea and hijack the cell and ultimately cells die out. In order to cope up this adverse condition played by bacteriophages or phages, prokaryotes have evolved manydefence mechanisms. Jenifer Anne Doudna, an American biochemist and another French microbiologist, Emmanuelle Charpentier, discovered a molecular tool known as clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (1). They have been awarded the 2020 Nobel Prize in Chemistry for their outstanding discovery and the subsequent generation of CRISPR-Cas9 genome editing. In bacteria, CRISPR system protects them from invading viruses and plasmids via RNA-guided DNA cleavage (2). A small portion of foreign DNA is incorporated inside the CRISPR locus and which ultimately transcribed into CRISPR-RNA (crRNA), which then joins to tracrRNA to cleave the pathogenic DNA through sequence specific manner (3). This natural system of adaptive immunity of prokaryotes has been utilized by many scientists for genome engineering (4, 5). It contains the Cas9 nuclease and a single guide RNA (gRNA) consisting of crRNA and tracrRNA elements.

Recombinant DNA technology was reported for the first time in Paul Berg's laboratory in 1972 and from then genetic engineering has been improved many folds and acquired enormous success(6). Conventional genetic engineering strategy has many limitations, which face complexity in manipulating large genomes of higher organisms like plants and animals(7). It becomes very important to solve the problem associated with a large genome for precise gene editing in higher organisms. Till now there have been many genome editing tools such as transcription activator-like effector nucleases (TALENs), Zinc finger nucleases (ZFNs), CRISPR and homing endonucleases or meganucleases. CRISPR-Cas9 method has been widely utilized in genome editing purposes over other tools due to its most

flexibility and user friendly nature. Since in this method target site recognition is mediated by gRNA which do not require the need of engineering new proteins on each target site. There are six different types of CRISPR systems (I-VI) (8) and each uses a distinct set of Cas proteins along with crRNA for CRISPR interference (9). In case of type I and type III systems which employs a large multi-Cas protein complex for crRNA binding and degradation of target sequence, whereas type II CRISPR systems utilizes a single DNA endonuclease i.e., Cas9 to find specific dsDNA substrates and break each strand with a distinct nuclease domain HNH or RuvC (10, 11).Jenifer Doudna and her team reported that Cas9 recognizes the target and cleaves sequence specifically. Cas9 requires a seed sequence within the crRNA and a conserved protospacer-adjacent motif (PAM) upstream of the crRNA binding site.In this article, we will cover many aspects of CRISPR-Cas system such as historical background, the mechanism by which CRISPR-Cas9 provides adaptive immunity to prokaryotes against invading viruses, the process by which it can be utilized as a genome editing tool, their applications in plants and animals and the future perspective of genome editing.

2. History of CRISPR/Cas9-An unusual repeated sequences to Genome Editing.

CRISPR are repeating DNA sequences present in the genome of prokaryotes such as bacteria and archaea. In the year 1987 CRISPRs were first identified in E.coli by a Japanese scientist, YoshizumiIshino, who unknowingly cloned a series of repeated sequences interspersed with spacer sequences during the analysis of the gene specified for isozyme conversion of alkaline phosphatase (12). Due to the lack of sufficient data of DNA sequences, these unusual repeated sequences remain a mystery until mid-2000s. As Life Science domain has moved in the genomic era, CRISPRs were reported in Archaea in 1993 and subsequently in many more bacterial and archaeal genomes (13). Mojica and co-workers in the early 2000s observed that spacer sequences were more or less similar to sequences found in viruses, bacteriophages and plasmids and also confirmed that bacteriophages can’t infect bacteria which possess homologous spacer sequences. This observation clearly suggests that these sequences provide the adaptive immune system in prokaryotes (14). In a nutshell, when a virus invades a prokaryotic organism, the spacer sequences present in CRISPR arrays undergoes for the process of transcription and forms a short CRISPR RNA (crRNA), which ultimately guide the Cas (CRISPR associated) protein to break complementary DNA or RNA of invading viral sequences. This broken part created by Cas9 is then repaired either through nonhomologous end joining (15), generating random insertions or deletions (indels) at the site of cleavage or

can also be repaired by high-fidelity homology directed repair, generating specific modification of genome at the site of cleavage through homologous repair template (16). This great discovery was the key to turn the genome editing into its new era and scientists were able to design guide RNA to match the target specifically in the genome to modify it. CRISPR-Cas9- the DNA editing tool, can be used to introduce or removal of genes as well as they can also be used in silencing or activating genes. Therefore with the continuous efforts of many scientists, this natural phenomenon of the adaptive immune response of prokaryotes leads to the generation of a precise genome editing tool.

3. Mechanism by which CRISPR-Cas9 provides adaptive immunity to prokaryotes

On exposure to invading genetic material from bacteriophage or plasmids, a stretch of foreign DNA fragments are incorporated into the CRISPR repeat-spacer array and form new spacer within the host chromosome (17). As a result of this process, it provides a genetic record of previous infection which ultimately protects the host from future invasion of the same invader (18, 19). Subsequently, CRISPR array undergoesthe process of transcription and precursor CRISPR transcripts so formed experiences enzymatic action through endonucleolytic cleavage and gives short stretch of mature CRISPR RNAs (crRNAs) (20). crRNA possesses the spacer at its 5'end and a stretch of CRISPR repeat sequence at its 3'end. The spacer is a short portion of RNA which shows complementarity to a sequence from invading foreign genetic element. Once crRNA spacer and a complementary target sequence (protospacer) undergoes for the process of hybridization, it can trigger the destruction of invading DNA or RNA in a sequence specific manner by Cas nuclease on its second invasion (21,22)

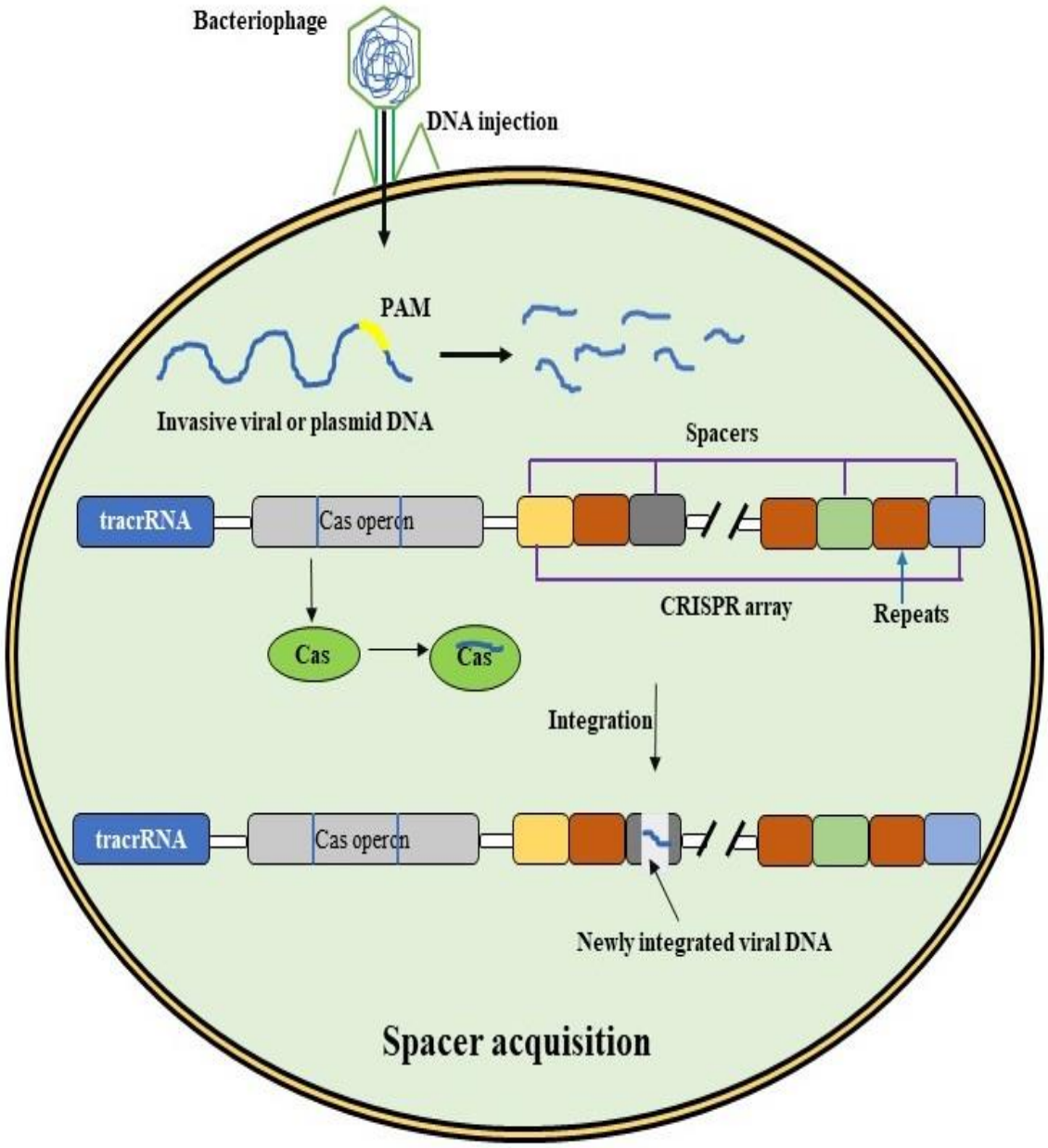

Fig. 1 (a) CRISPR-Cas system includes an array of repetitive sequences interspaced by short stretches of non-repetitive sequences (spacers), also many CRISPR-associated (Cas) genes are present. Upstream to the Cas operon transactivating CRISPR RNA (tracrRNA) gene is present which codes for a unique non-coding RNA and shows homology to the repeat sequences. Upon new phage infection, another spacer produced from the invasive genetic material is inserted into the CRISPR array by acquisition machinery (Cas operon).

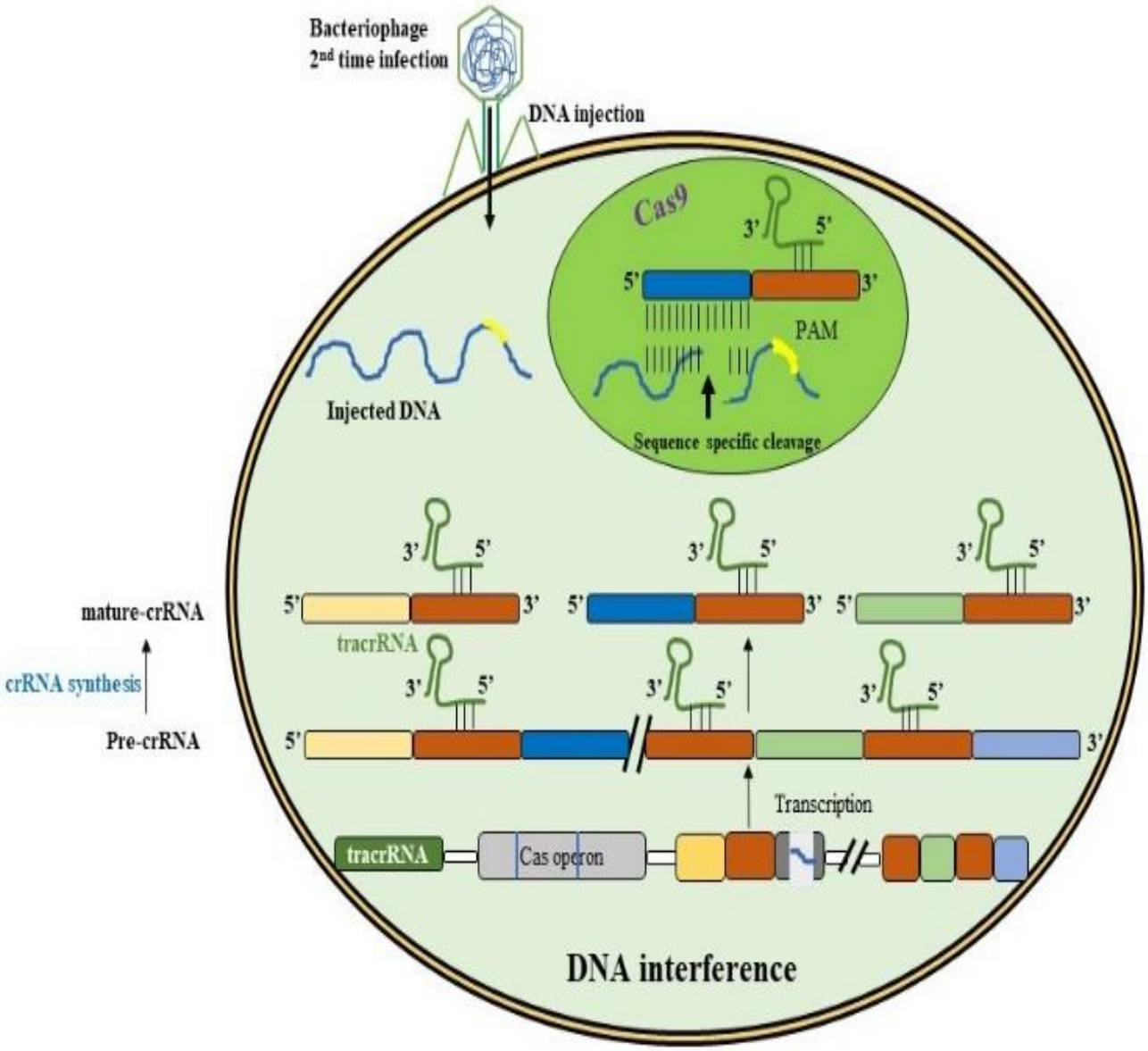

Fig. 1 (b) After integration of the new spacer, it can be co-transcribed with all other previously located spacers into a long precursor CRISPR RNA (pre-crRNA) which contains repeats and spacers. The tracrRNA is also transcribed and binds to the pre-crRNA repeats for crRNA maturation. At the time of interference, the Cas9 endonuclease along with crRNA-tracrRNA make large complex and directs the cleavage of foreign DNA.

Fig.1 Steps involved in the process of CRISPR-Cas9 adaptive immune system.

4. Mechanism of CRISPR-Cas9 mediated Genome Editing

The sgRNA or crRNA-tracrRNA complex mediates the Cas9 endonuclease to any portion of the genome through a uniquely designed 20nts guide RNA sequences and ultimately Cas9 introduces a double strand break (DSBs) in the targeted genomic DNA. The DSBs can be repaired either by error prone nonhomologous end joining (NHEJ) or error free homology directed repair (HDR). During the course of repair of DSBs, NHEJ can produce random insertions or deletions (indels) at the site of cleavage or DSBs can also be repaired by HDRs, generating desired modification of genome at the cleavage site which provides the basis for performing specific gene modification.

Cas9 has two distinct nuclease domains, one HNH domain which cleaves the DNA strand complementary to the gRNA sequence (target strand) and the other is RuvC domain which cleaves the DNA strand opposite to the complementary strand (23, 24)

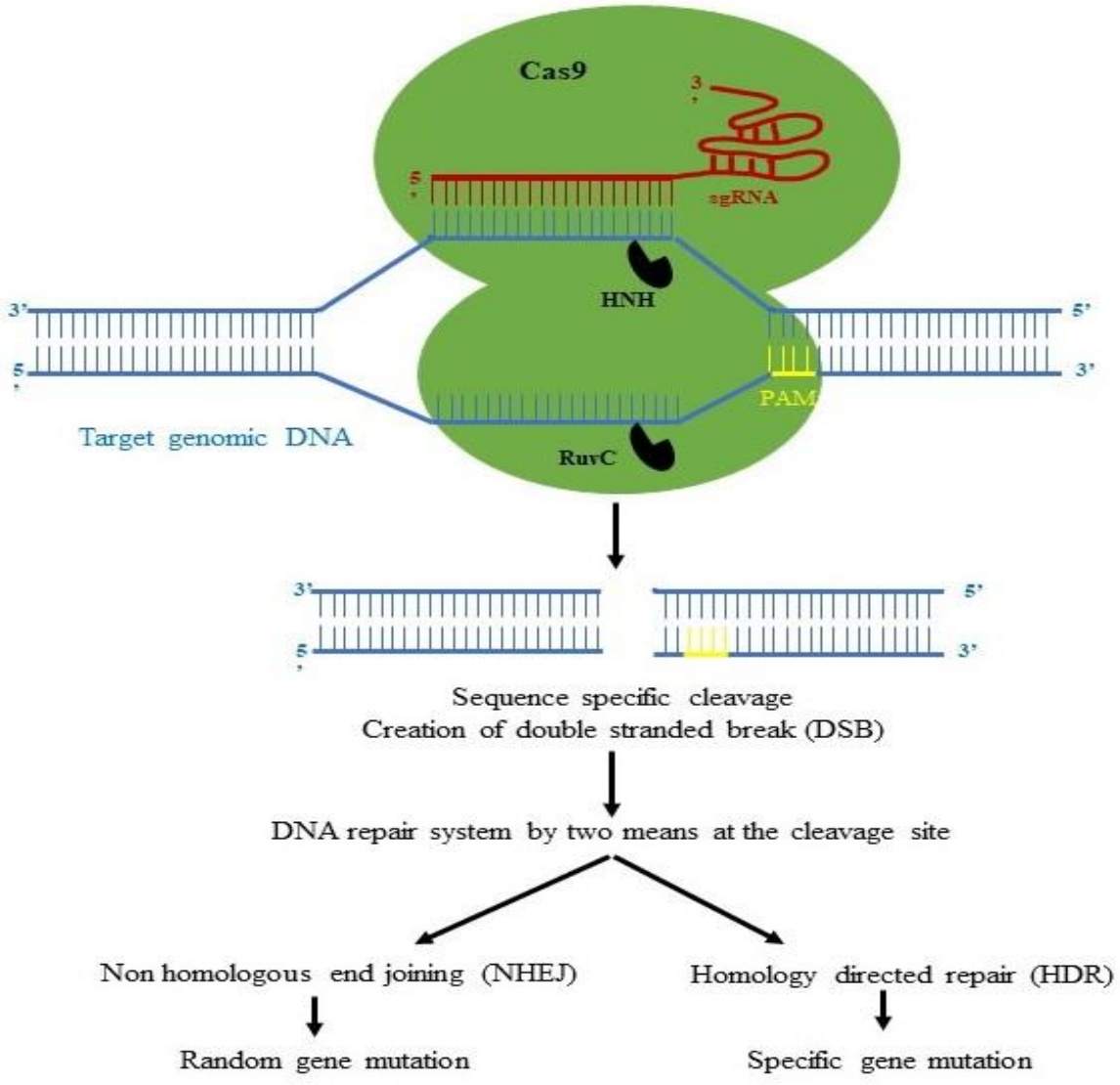

Fig. 2 Mechanism of CRISPR-Cas9 mediated genome editing. Cas9 endonuclease cleaves the targeted genomic DNA on a sequence specific manner. The gRNA which guide the Cas9 complex over the genomic DNA and where it finds the complementary sequence and upstream to it PAM must be present, Cas9 cleaves the DNA and produces DSBs. These DSBs can be repaired in two different ways, one in which there is error prone repair system (NHEJ), where it randomly incorporates nucleotides and fills the gap and as a result indel mutations, frameshift mutations, etc. occurs and all these are the examples of random mutations. On the other hand, DSBs can also be repaired by HDRs, where precise gene modification can be done by self-designed donor DNA and accordingly the gap can be filled by these sequences.

5. Application of CRISPR-Cas9

CRISPR-Cas9 method of gene editing has been adopted in many crops for multiple traits including increase in yield, biotic and abiotic stress tolerance. If any gene is playing a negative role in the immunity or the resistance against any type of pathogenic attack, any kind of abiotic stress, with the help of CRISPR-Cas9 that particular gene can be turned off. This method of precisely targeted gene editing showed tremendous improvement in many field of life sciences, including plants as well as animals. CRISPR-Cas9 based genome editing also has been utilized to improve disease resistance. Though till now, lots of genome edited plants have been created but in the following table some of the specific examples are included.

Table 1. Few specific examples of genome edited plants.

Plant	Repair Method	Target gene	Trait	Reference
A. thaliana	NHEJ	Viral DNA (A7, B7 and C3 region)	Beet severe curly top virus resistance	(25)
Rice	NHEJ	Ethylene responsive factor	Blast Resistance	(26)
Tomato	NHEJ	SIMAPK3	Drought tolerance	(27)
Cucumber	NHEJ	eIF4E (eukaryotic translation initiation factor 4E)	Cucumber vein yellowing virus	(28)
Bread wheat	NHEJ	TaMLO-A1, TaMLO-B1 and TaMLOD1)	Powdery mildew resistance	(29)
Maize	NHEJ	Wx1	High amylopectin content	(30)
Orange	NHEJ	CsLOB1 promoter	Citrus canker resistance	(31)
Mushroom	NHEJ	PPO	Anti-browning phenotype	(32)
Soybean	NHEJ	GmPDS11 and GmPDS18	Carotenoid biosynthesis	(33)
Potato	NHEJ	Wx1	High amylopectin content	(34)

5.1. Direct application of CRISPR-Cas9 system in crop improvement

a) Increase in crop yield: Crop yield depends on many factors, but one of the best way is to maintain the cytokinin level.
b) Quality improvement: Grain with lower level of amylose content has high demand in the market due to better eating and cooking quality.

Many reported applications of CRISPR-Cas9 in crop improvement are disease resistance, herbicide resistance, fixation of hybrid vigour etc.

5.2. Therapeutic potential of CRISPR-Cas9 in animals

The CRISPR-Cas9 system has tremendous therapeutic potential for treating many genetic diseases in which the reason for dysfunction is known. Therapy based on genome editing can restore the gene function. In case, if a gene is identified which causes the disease, that particular gene can be easily knocked out. In a certain case, if a disease is caused by a virus, the cleavage of viral DNA can also be done with the help of CRISPR-Cas9 system.

Some of the reported direct applications of CRISPR-Cas9 system in disease improvement are as follows:

a) CRISPR-Cas9 in pulmonary and gastrointestinal diseases: Mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene was corrected using CRISPR-Cas9 in cystic fibrosis patients (35).
b) CRISPR-Cas9 in viral diseases: There are many deadly viruses that can cause diseases in humans and ultimately the patient dies. Certain viral infections can be reduced to some extent by vaccination, while in many cases it is not possible.
   Human papilloma viruses (HPV), the human immunodeficiency virus (HIV), hepatitis B virus are more deadly and require more attention. Viral oncoproteins E6 and E7, which are directly linked to a malignant phenotype that causes cervical cancer (36) and which takes the second place worldwide for the cause of this cancer (37). There is also report of much better treatment by CRISPR-Cas9 (36, 37, 38).

The practical implications of this system are increasing every single day and it provides scientists to think in many ways for curing different diseases in humans.

6. Future Perspectives of CRISPR-Cas9 in Genome Editing

The most important direction of this system will be the establishment of functions of all the genes present in an economically important organism or any organisms of interest including prokaryotic organisms, plants as well as animals. In consideration of plant species which have agronomic importance, keep the interest of scientists as it can directly increase the economical value in the society. The manipulation of agronomic traits depends on the precise engineering of complex metabolic pathways and which require the expression of many genes. In these types of experiments CRISPR-Cas9 can be used, as it has the potential of multiplexing, the simultaneous editing of multiple target sites (39). Despite the tremendous

potential that lies within the CRISPR-Cas9 technology, further investigation is needed to make the system an applicable and safe tool for therapeutically useful approaches. The most challenging part that needs to be taken carein the future includes off-target cleavage by Cas9. Off target effects are a major concern for genome editing purposes (40). There are also many approaches to prevent off-target cleavage by Cas9 such as injection of purified Cas9 directly into a cell instead of expressing the recombinant protein in the target cell (41,42). In an overall scenario, CRISPR-Cas9 genome editing technique will lead the postgenomic era and gain the enormous attention of most scientists in the field.

7. Conclusion:

CRISPR-Cas9 as the genome editing tool is becoming popular in the field of functional genomics, therapeutics and in crop improvement purposes. CRISPR-Cas9 is gaining overall importance over other genome editing tools due to its most flexibility, user friendly, simplicity, efficiency, high specificity and also for the process of multiplexing. Finally, this CRISPR-Cas9 based genome editing will become a more popular tool and will be the essential technique to obtain suitable plants as well as for the fixation of many genetical diseases in animal.

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