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CRISPR-Cas: A continuously evolving technology

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ABSTRACT

The discovery of the CRISPR/Cas microbial adaptive immune system and its ongoing development as a genome editing tool represents the work of many scientists around the world. The time line of CRISPR/Cas system shows that this technology is improving continuously to remove the demerits of preceding one with the aim of development of highly efficient, specific with low off target effect and ultimately transgene free technology in light of ethical and environmental issues related with transgenic technology. Initially, CRISPR/Cas9 was developed as method of choice as it provides targeted mutagenesis under *in vivo* condition and all the homeoalleles of a gene can be targeted in same plant, especially in case of polyploid species efficiently which is difficult through other existing technology. No residual or foreign gene insertion is required and modification is permanent. Now, CRISPR/Cpf1 has been developed as more potent, efficient and simpler than CRISPR/Cas9. Different forms of Cas enzymes provide new avenues for regulation of genomic component. In view of the present devastating COVID-19 disaster the scientists used this novel technology for detection of virus in humans at an early stage of infection thus saving human lives. The evolution of CRISPR/Cas technology, their advantages, apprehensions and solution, experimental design and updates of this technology is discussed in the present review.

Keywords: CRISPR/Cas, Cpf1, Covid-19, Genome editing, Transgene free technology

CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated) is a technique that increases the efficiency and specificity of target mutagenicity. Number of scientists across the globe are working for refinements in this technology (Lander *et al.* 2016). Although principle and methodology of this technique has been well-established but still new things are being unraveled and new ideas are being added (Prajapat and Mahajan 2016). Continuous evolution of CRISPR/Cas system, different components of CRISPR/Cas system, their importance, application and apprehensions are discussed in the present review.

Identification of different components of CRISPR/Cas systems

The first incident related to CRISPR/Cas was reported by Ishino *et al.* (1987) when he was working with iap gene of *E. coli* responsible for isozyme conversion of alkaline phosphatase. He accidently cloned the CRISPR locus with five repeats of 29 nucleotide length interspaced by unique spacer of 32 nucleotides. These interrupted repeat sequences were unique at that time and thus he called it as 'unique spacer'. Sequencing technology was at newborn stage at that time and sequence databases information was not so easily available thus he was unable to establish any biological significance of this locus. The actual work started by Mojica *et al.* (1995) with archean species *Haloferax* sp. a halophyte and the CRISPR locus was observed. They identified these sequences in many prokaryotic species and were aware of similar sequences reported by Ishino *et al.* (1987) and termed the new class of repeat as SRSR (Short Regularly Spaced Repeat). The SRSR sequences were conserved within species and differ among species as supported by sequence analysis and phylogenetic analysis which suggested them about the possible role of SRSR in replicon partitioning among species during evolution. In the year 2000, they reported the presence of these sequences in most of the prokaryotic species including Archean sp. and Bacterial sp. To differentiate SRSR from other class of previously reported repeat Mojica and Jansen jointly used the acronym "CRISPR". The CRISPR term was first used by Jansen *et al.* (2002) in their report. The insilco characterization of the CRISPR loci from different species revealed a conserved leader sequence of 300-500 base pairs adjacent to the CRISPR loci, especially in those species where CRISPR/Cas was present in more than one copies. Homology search in different species showed that the CRISPR locus is

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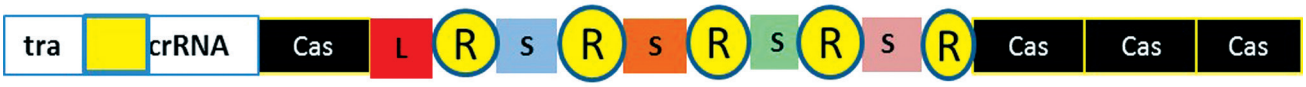

Fig 1 Different components of CRISPR/Cas system. L, Leader; R, Repeat; S, Spacer; Cas, CRISPR associated; tracr RNA, Transacting CRISPR RNA.

present in most of the prokaryotic species but not in virus and eukaryotic genome sequences. A leader sequence was located upstream to CRISPR locus form a single common genetic entity. To reveal the detailed structure of this genetic entity a larger sequence of CRISPR locus from different species were compared and it was observed that CRISPR locus was mostly flanked by a set of genes and four genes among them were highly frequent that showed homology among themselves; named as CRISPR associated genes; Cas gene 1-4 (Jansen *et al.* 2002). However, the direction and arrangement of these Cas genes (upstream/downstream) with respect to CRISPR were not fixed among species.

Domain search showed that Cas gene domain was similar to helicase domain but it was not reported. Clear function of CRISPR and Cas genes were not known until three group of scientists reported their work independently and reached to similar conclusion based on similarity between phage DNA and unique spacer sequences (Bolotin *et al.* 2005, Mojica *et al*. 2005, Pourcel *et al*. 2005). They hypothesized that the mechanism of adaptive immunity by CRISPR loci may be similar to RNAi mediated down regulation of gene expression in eukaryotes. This hypothesis was supported by homology between component of CRISPR/Cas and RNAi systems (Makarova *et al*. 2006). In *Streptococcus pyogenes,* the differential RNA sequencing and double strand RNA sequencing revealed an abundant transcript of 210 nucleotides transcribing from the opposite strand of the CRISPR associated genes and the leader-repeat-spacer array transcript. Therefore, these abundant transcripts were termed as tracrRNA (trans-activating CRISPR RNA) as they were complementary to crRNA. Complementarity between tracrRNA and crRNA play role in crRNA maturation by csn1 and RNAse III enzyme was proved by *in vitro* cleavage studies. *Streptococcus pyogenes* also contain csn1 (Cas 9 homology) that required both processed tracrRNA and crRNA for target cleavage (Deltcheva *et al.* 2011). In view of above developments Fig 1 gives an account of generalized structure of CRISPR/Cas system.

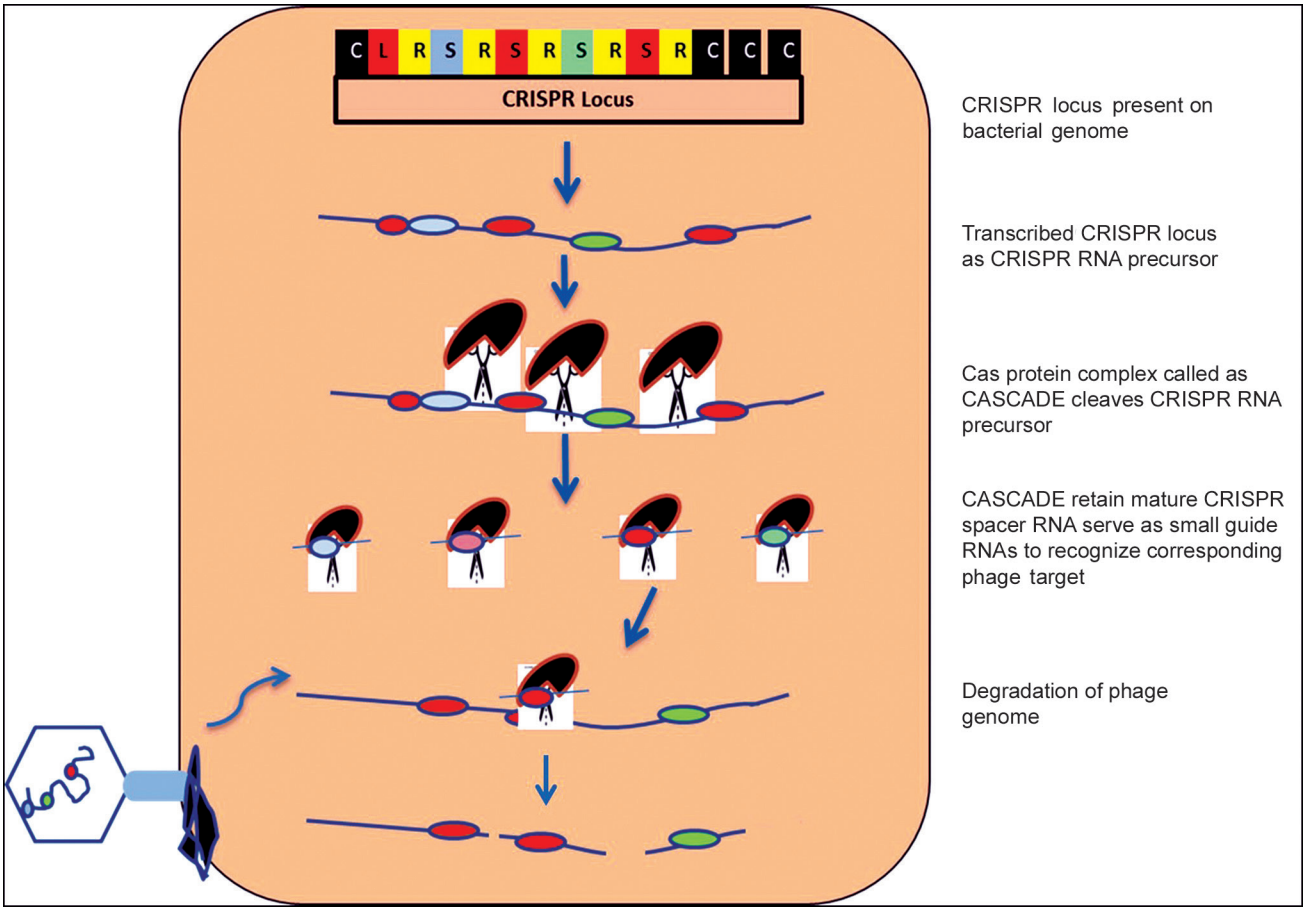

Fig 2 Mechanism of CRISPR/Cas mediated phage immunity (C, Cas; L, leader; S, spacer; R, repeats).

Identification of functional role of CRISPR/Cas system

First experimental proof of CRISPR/Cas using wet lab method was reported by Barrangau *et al.* (2007). They used two *Cas* mutated strains of *Streptococcus thermophiles* along with their wild type strain and observed their sensitivity to different phages after adding different spacer sequences to the *Cas* mutant strains. They observed *Cas5* knock out lead to high sensitivity to all the tested phages in presence of any spacer but same was not true for *Cas7* mutants for all the tested phages. It suggested the association of *Cas5* with cleavage and possible role of *Cas7* in acquiring and processing of spacer and repeat sequences. The mechanism of CRISPR/Cas system was more clearly described by using knock out experiment in *E. coli,* where function of eight Cas genes; *Cas1* (integrase), *Cas2* (endoribonuclease), *Cas3* (nuclease) and a protein complex composed of five Cas proteins: *CasA, CasB, CasC, CasD,* and *CasE* denoted as CASCADE (CRISPR-associated complex for antiviral defense) were analyzed (Brouns *et al.* 2008). It was also found that cleaved CRISPR RNA (*crRNA*) remains associated with CASCADE complex protein and act as small guide RNA which targets the phage genome (Fig 2).

Identification of CRISPR/Cas target

Till 2008, role of CRISPR/Cas in adaptive immunity by cleavage of target molecules of phage became well established but whether the target was DNA or RNA of phage molecule was still questionable. Initially, based on similarities between different components of RNAi in eukaryotic system and CRISPR/Cas, it was emphasized that CRISPR/Cas is functional analogue of the eukaryotic siRNA systems and may be involved in prokaryotic small RNA interference (Makarova *et al.* 2006). Eukaryotic RNAi system targets RNA and therefore prokaryotic defense system might work in the same way. This ambiguity between DNA/RNA in target site for CRISPR/Cas was finally solved by Marraffini and Sontheimer (2008) while working with *Staphylococcus* strain. S*taphylococcus* strain in this experiment act as recipient harbored a CRISPR spacer corresponding to nickase gene, present nearly in all staphylococcal conjugative plasmids for preventing conjugation and plasmid transformation by targeting nickase gene. To prove the target is DNA and not the RNA, they used donor bacterial strain containing self splicable intron within *nes* (nickase gene) DNA. This self splicable intron interrupted the target of CRISPR in DNA, whereas in RNA target remained intact as intron spliced out. It was observed that conjugation was not affected as intact mRNA and nickase protein were formed. Thus, it was proved that target for CRISPR/Cas system targets DNA not RNA (Maraffini *et al.* 2008). In *Pyrococcus furiosus,* it was observed that CRISPR system targets RNA instead of DNA on contrary to *Staphylococcus* strain and *Cas* protein of *Pyrococcus furiosus* contain RAMP (Repeat Associated Mysterious Protein) called as *Cmr* protein which is involved in target cleavage (Hale *et al.* 2009). The comparison of cleaved phage fragments showed that the target site for CRISPR/Cas is within protospacer adjacent motif (PAM). It was observed that the cleavage site was identical in plasmid DNA and phage DNA (Garneau *et al.* 2010).

Types of CRISPR/Cas system

Based on different Cas system cleavage in different bacterial species and phylogenetic analysis the CRISPR/Cas system was classified into three subtypes; Type I, Type II and Type III (Makarova *et al.* 2011). Cas9 protein characterized further and it was found that it is a single multidomain protein in *S. pyogenes* that targets DNA for cleavage require both crRNA and tracrRNA in type II system to introduce a double strand break at specific sites in the target molecules (Jinek *et al.* 2012, Gasiunas *et al.* 2012). The mechanism of the three subtypes of CRISPR/Cas was described by Sorek *et al.* (2013).

Comparative study of all the three types revealed that Type II CRISPR/Cas system may act as efficient system for target engineering in heterologous system (Table 1). Gene of interest can be targeted through transient expression of crRNA, tracrRNA and Cas enzymes in the host. The hybrid formation between crRNA and tracrRNA take place in the host which targets the specific genic region of DNA. Targeted region form RNA-DNA hybrid is cleaved at particular sites, *i.e.* protospacer adjacent motif (PAM) by enzyme CAS9. The cleaved site of host DNA is repaired by inherent repair system of cell, i.e. non-homologous end joining (NHEJ) and homology directed repair system (HDR). HDR repairs the host DNA in presence of donor segment with desired modification.

The application of this technology was employed in both the animals and plants using CRISPR/Cas type II system. Human and mouse cell among animals and *Arabidopsis* (dicot) and rice (monocot) among plants was firstly edited using this technology respectively (Cong *et al.* 2013, Feng *et al.* 2013). The method of genome editing through CRISPR/Cas was optimized by Shan *et al.* (2014) for application in plant system. A wide application of this technology is targeted mutagenesis. Transient expression or preassembled introduction of crRNA-tracrRNA-Cas9 complex provides transgene free approach to gene/genome editing methodology (Woo *et al.* 2015).

Experiments design associated with CRISPR/Cas based genetic engineering system

The efficiency of gene and genome editing tool depends upon the perfect experimental design that requires proper selection of target site within gene/genome which must be specific. Generally, 20 nucleotides against target region, three additional nucleotides against PAM region and 19-22 nucleotide as guide RNA, i.e. link to 5' of crRNA (a total of 39–42 nucleotide) must be used as sequence guide RNA (Deltcheva *et al.* 2011).

Steps to be followed in designing sequence guide RNA given below

- A suitable region from the target genome is chosen using online "CRISPR Design Tool" to predict sgRNA.

Table 1 Comparison of three sub types of CRISPR/Cas system

Molecules required	TYPE I	TYPE II	TYPE III
PAM	5'	3'	NOT known
crRNA	YES	YES	YES
tracrRNA	NO	YES	NO
Acquisition of protospacer	Cas1 & 2	Cas1 & 2	Cas1 & 2
crRNA maturation	Cas5d/Cas6e/Cas6f	Cas9/RNaseIII	Cas6
Target interference	Cas3, Cas8, Cas7 (crRNA effector complex)	Cas 9	Cmr 1-6 / Cas 10/ Cas 7 (crRNA effector complex)
Target molecule	DNA	DNA	DNA/RNA
Examples	Bacteria and archaea	Mostly bacteria	Mostly archaea

- Comprehensive experimental investigation of mismatching bases between the sgRNA and its target DNA (mismatch tolerance) is done.
- Position dependent: 8–14 base pairs on the 3′ end of the guide sequence are less tolerant for mismatch than bases on 5′ end.
- Quantity dependent: Generally, more than three mismatches are not tolerated.
- Guide sequence dependent: Some guide sequences are less tolerant for mismatches than others.
- Concentration dependent: Off-target cleavage is highly sensitive to the transfected amounts as well as relative ratios of Cas9 and sgRNA51.

The genome engineering in heterologous system requires the expression of both the sequence guide RNA (sgRNA) and Cas9. The expression of sgRNA and Cas9 may be facilitated either through the same vector or separate construct. The constitutive promoter T7/U6 is being used for the expression of the sgRNA and Cas9. The nuclear localization signal (NLS) must be attached with the Cas gene to target the localization of CRISPR/Cas system to the nuclear genome.

Advantage of CRISPR system over other existing techniques of genetic engineering

There are various classical techniques for genetic engineering such as Homologous recombination; RNAi mediated technique and SSN (Sequence Specific Nucleases) etc. The merits/demerits/features of each protocol of various methods of genome editing are summarized in Table 2.

Application of CRISPR/Cas9 system in plants

CRISPR/Cas has now become the method of choice for site directed mutagenesis. One major advantage of this technology is the mutation under *in vivo* conditions and types of target modification can easily be traced using PCR amplification of targeted loci followed by sequencing. Cas9 contain two catalytic domains RuvC and HNH. HNH domain cleaves the complementary strand in the tertiary complex, whereas RuvC nicks the non-complementary strand (Belhaj *et al.* 2015). Intact Cas9 cleaves both the strand of genomic DNA which is repaired by host cell

Table 2 Comparisons of different methods of genome/gene editing

Features	Homologous recombination	RNAi mediated methods	SSN – sequence specific nucleases	CRISPR/Cas
Target molecules	DNA	RNA (only expressed gene can be targeted)	DNA	DNA
Type of modification	Knockout	Knockdown	Allelic	Allelic
Effect	Switch off target	Leakage of gene expression	No leakage	No leakage
Efficiency	Low	High	Medium	High
Construct design	Difficult	Easy	Difficult	Easy
Target integration	Yes	Yes	Less chance by TE	Less chance by TE
Marker required	Yes	Yes	No (sequencing-based approach for screening)	No (sequencing-based approach for screening)
Time required	High	Less	Less	Less
Throughput	Low	High	Medium	High
Specificity	Medium	Low	High	Medium
Transgenic	Yes	Yes	No	No
Constrains	Complex integration	Never permanent switch off	DNA methylation	Off target effects

itself as a result different type of indel mutations occurs. The modification of any one of the nuclease domains leads to nick in the target DNA. Cas9 can be targeted to any site using complementary crRNA therefore different types of genome modifications may be possible at DNA and RNA level (Mitchell *et al.* 2014, Bortesi *et al.* 2015).

CRISPR-Cas system in view of COVID 19 disaster in India

Corona virus disease, 2019 (COVID 19) has created a devastating situation around the world regarding human health. The disease causes the respiratory illness with symptoms similar to flu, viz. cough, fever, and in severe cases leads to death because of respiratory failure. The major issue with the COVID 19 is early diagnosis which may enable the quarantine of infected person. This can be possible by tracking their expression at RNA level. QPCR is most widely used technology for detection of any viral disease but it requires much time. A Cas13 enzyme which is a Cas9 alternative can target RNA instead of DNA has been used in CRISPR based SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) technique for the detection of COVID-19. It can target even small copy number of viral RNA within an hour (Kellner *et al.* 2019). A similar technology has been developed in the India with CRISPR/Cas technology in which the viral RNA is first converted in to DNA, amplified and then Cas9 complex is used to detect genetic material of virus. The COVID-19 testing kit is much simpler and cheaper and is based on paper-strip test which uses the cutting edge CRISPR-Cas9 technology (Srivastava 2020).

Conclusions

The CRISPR/Cas system has lot of advantages over the existing methods of genome engineering (Table 2) but it still facing certain issues such as ambiguities in PAM selection and their sequences that often results in to lowered efficiency of the modification and off-target cleavage. The off-target cleavage mostly occurs due to mismatch tolerance between target site and crRNA. Off target activities can be reduced by choosing specific target of gene; predict cross match to other region of genome using BLAST tools thereby reducing the chances of mismatches between sgRNA and target DNA. Generally, more than three mismatches are not tolerable. However, this method cannot be applied to those species for which genomic sequence is not available. Another issue is the public acceptance of this technology as transgene free approach. Introduction of CRISPR/Cas in the target is cumbersome like transgenic and genome modification through this approach transgenic or not is still an enigma. Different possible ways of producing transgene free genome modification are summarized below (Voytas *et al.* 2014).

- Integrated nuclease removed by cre-lox recombination systems.
- Express construct as episomal plasmid, carryout editing without integration in to the host cell followed by segregation in subsequent generation.
- Introduction of preassembled ribonucleoprotein complex, no risk of integration.
- Transient delivery of construct using viral vectors.

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