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CRISPR/Cas9: A Nobel Approach for Genome Editing

Shreya[1*], Kiran Rana[2] and Ainmisha[3]

[1]Department of Genetics and Plant Breeding, IAS, BHU, Varanasi-221005, India
[2]Department of Agronomy, IAS, BHU, Varanasi-221005
[3]Division of Plant Pathology, IARI, New Delhi, India
**Corresponding author*

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A B S T R A C T

Recently evolved technique, Clustered Regularly Interspaced Palindromic Repeats (CRISPR)-CRISPR-associated (Cas9) has added new armory for the genome editing approaches. This CRISPR/Cas9 pathway of archaeal and bacterial defense mechanism against the invading genomic material utilizes a short guide RNA to direct the endonuclease Cas9 to cut the foreign genetic material and provide resistance against the same. The immunity in archaea and bacteria is developed due to the transcription of cut segment of the exogenous material which has been incorporated in host genome system as memory which is transcribed in the form of guide RNA. So by artificially synthesizing the desired guide RNA, Cas9 can be virtually directed anywhere in the genome to cause DNA double strand breaks (DSBs) and can accomplish the repair or insertion, deletion *etc* actions to edit genome of the organism in desired directions. The manifestation of this novel technique depends on the presence of PAM (protospacer adjacent motif) sequence which lies downstream to the target site. Hence here we are discussing the concept and use of CRISPR/Cas9 mechanism that can be a very efficient and indispensable tool for genetic manipulation in future.

Introduction

History

In the Year 1987 marked the begin of CRISPR while studying the mechanism underlying the isozyme conversion of alkaline phosphatase in *E. coli* by Ishino *et al.*, (1987) and they discovered several 'curious sequences' in the 3'end flanking region of the *iap* gene and described it as a set of 29 nucleotide repeats with 32 nucleotide spacing sequences.

Later short regularly repeats were reported in more than 40% of bacteria and 90% of archaea by Mojica *et al.*, (2005). These short repeats were officially named Clustered Regularly Interspaced Short Palindromic Repeats by Jansen *et al.*, (2002) and the abbreviation CRISPR began to circulate widely.

Further the presence of Cas genes, situated next to CRISPR locus were identified in prokaryotes by Schouls *et al.*, (2003). Subsequently, with the discovery of the Cas gene, Cas protein, protospacers adjacent motifs (PAM), CRISPR RNA (crRNA) and trans-activating crRNA (tracr RNA) gave the root to a genome editing mechanism. So in 2013, CRISPR/Cas mechanism of immunity in prokaryotes was established as novel

genetic manipulation armor by Cong *et al.*, (2013) and Mali *et al.*, (2013).

Clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein (Cas)9-mediated genome modification enables us to edit the genomes of a variety of organisms rapidly and efficiently. CRISPR/Cas9 is a RNA guided nuclease based genome/DNA engineering in contrast to other protein guided genome editing artificial techniques like TALENs (Transcription activator like effector nuclease) and ZFNs (Zinc finger nuclease).CRISPR/Cas was first time discovered as an acquired immune system in bacteria and archaea against foreign DNA, either viral or plasmid. The locus of CRISPR comprises of a series of conserved repeated sequences which are interspaced by unique non repetitive sequences called as spacers. During the defense mechanism in bacteria and archaea, the invading foreign DNA is cut by nuclease encoded by Cas genes and the processed small segment of invading DNA is then incorporated within the CRISPR loci as spacers in host genome. It is the spacer sequence which act as transcriptional template for producing the crRNA during the infection caused by viruses and phages. crRNA is the agent which guide the Cas to cleave the target invading sequence. There are more than 40differentCasprotein families have been reported by Haft *et al.*, (2004) playing important roles in crRNA biogenesis, spacers incorporation and invading DNA cleavage. CRISPR/Cas system is classified into many sub classes *viz.*, Type I, II and III based on the Cas gene phenology by Makarova *et al.*, (2011). Only crRNA is required by Type I and III for targeting but Type II system also requires tracr RNA, Deltcheva *et al.*, (2011). In addition there is variation in the composition of crRNA-Cas targeting complexes. Type I and III system typically consist of greater than eight subunits, Bronus *et al.*, (2008) and Hale *et al.*, (2009). In contrast Type II system requires only a single polypeptide, Cas9; Sapranaukas *et al.*, (2011) which contains a HNH nuclease domain and a RuvC like nuclease domain; Jinek *et al.*, (2012). The Cas9 is a DNA endonuclease which functions naturally via dual guide RNA (a hundred nucleotide molecule) which is constituted by fusion of a 20-nucleotide (crRNA) with a transactivating CRISPR RNA (tracrRNA); Jinek *et al.*, (2012). The 5'end of the crRNA base pair with target DNA, but the 3' end forms a ds (double stranded) stem with the tracrRNA which thereby facilitate Cas9 nuclease recruitment. These orientations are accomplished as DNA targets are identified through RNA-DNA base pairing. Hence by making change in the sequence of the guide RNA, the targets on the DNA can be altered. One more important short sequence is also essential for the effective and efficient targeting on DNA is called as protospacer adjacent motif (PAM) which is located 3' of the protospacer element; Mojica *et al*., (2009). It is the PAM sequence that enables the CRISPR-Cas immune system to distinguish between the self and non-self sequence; Yosef *et al.*, (2012). Because the PAM sequence is only present at the targets sites in the foreign DNA. Cas9 from *Steptococcus pyogenes*, which has been the focus of most studies to data, recognizes a 5'-NGG-3' PAM sequence; Jinek *et al.*, 2012 and Mojica *et al.*, (2009). Based on the complementation, the crRNA position itself at the target site on the DNA and form a RNA-DNA hetero duplex and then DNA strand of heteroduplex and its opposite stand is cleaved by the HNH nuclease domain and RuvC like domain of Cas9 and thereby generating a DSB (double stranded break) at the target site. There is always a limitation of creating double stranded break in DNA at specific sites; Carroll (2014). Methods of genome editing like TALENs and ZFNs are based on protein and the feasibility of engineering these designer enzymes to

recognize new sequences are limited in contrast to the RNA guided genome editing through CRISPR/Cas. Also compositional simplicity of CRISPR has been paramount to its successful application. Not only does it encompass only a single polypeptide, but remarkably, it retains full activity with a chimeric single –guide (sg RNA), generated by connecting the 3'end of crRNA to the 5'end of the tracr RNA; Jinek *et al.*,(2012).

Genome editing with CRISPR/Cas9

To make the genome editing and engineering process convenient, an artificial guide RNA (g RNA) is being used which contains all the attributes of crRNA and tracr RNA ; Jinek *et al.*, (2012). Many variants of CRISPR/Cas9 has been developed to recognize 20 or 24 nucleotides sequences of engineered guide RNA and 2-4 nucleotides PAM sequence at the target site. Therefore, CRISPR/Cas9 cantheoreticallytargetaspecificDNAsequence with22–29 nucleotide which is unique in most genomes. It has been reported that the CRISPR/Cas9 is tolerant to base pair mismatch between guide RNA and its complementary target sequence; Jinek *et al.*, (2012), Cong *et al.*, (2013), Fu *et al.*, (2013), Mali *et al.*, (2013) and Hsu *et al.*, (2013). For example, the CRISPR/Cas9 of Streptococcus pyogenes appeared to tolerate up to six base pair mismatches at target sites; Jinek *et al.*, (2012). The non homologous end-joining (NHEJ)-mediated error-prone DNA repair and homology directed repair (HDR)-mediated error-free DNA repair is carried out by DNA repair system of cell where DSB is triggered by CRISPR-Cas9 system. The NHEJ mediated DNA repair mechanism is very fast but it causes small deletion and insertion mutations at the target site thereby abolishing and disrupting the function of the target gene. INDELs were created at the yellow locus of Drosophila genome through CRISPR/Cas9-induced DNA cleavage following by NHEJ-mediated DNA repair mechanism resulted into frame shift mutation; Gratz *et al.*, (2013). The HDR-mediated DNA repair, more complicated than NHEJ-mediated DNA repair. HDR-mediated error-free DNA repair requires a homology-containing donor DNA sequence asrepairtemplate.Throughco-injectionofCas9, two gRNA targeting, respectively, the 5′ and 3′ sequences of the yellow locus, and a single-strand oligodeoxynucleotide template, successfully replaced the yellow locus with a 50 ntattP recombination site in Drosophila genome; Gratz *et al*.,(2013).

Advantages of CRISPR-Cas9 over other genome editing mechanisms

There are several advantages of using this new technique of genome editing over method like TALENs and ZFNs. Being a protein guided artificial genome editing mechanism TALENs and ZFNs needs a time consuming and complicated protein engineering, selection and validation. Whereas, the CRISPR/Cas9 needs merely a short programmable guide RNA for targeting DNA and moreover the designing and production of guide RNA is relatively easy and cheap too. CRISPR/Cas9 system is efficient enough to induce genetic manipulation through repair, insertion, deletion, recombination *etc.* at several sites in genome independently when there is use of several guide RNA with different target sites in plants and animals; Cong *et al.*,(2013). Due to its simplicity this mechanism could be a useful tool to disrupt/abolish multiple genes, to investigate the gene family and to generate transgenic with multiple mutations; Wang *et al.*, (2013) and Yang *et al*., (2013).

Applications

Within a few years of its discovery, CRISPR/Cas9 system has been used widely and it has reached to a wide range of hosts to target important genes of human (Mali *et al.*,

2013), bacteria (Fabre *et al*., 2014), zebra fish (Hwang *et al.*, 2013), plants (Guo *et al*., 2014).

Challenges

Being a very effective, useful and easy method of genome editing, the mechanism of CRISPR-Cas9 have some serious issues regarding use of it *viz.*, guide RNA production, delivery method of CRISPR/Cas9,dependence on PAM site and off-target mutations as well.

It is very much difficult for RNA polymerase II for synthesis gRNA due to PTMs (post translational modifications). The *in vivo* gRNA production is accomplished by using RNA polymerase III, U3 and U6 snRNA promoters. There is also lack of commercial availability of RNA polymerase III also limits the application of U3- and U6-based gRNA production. The delivery of the CRISPR/Cas9 into the organism is plasmid based injection techniques. More focus should be given to the delivery system to make it more efficient for different type of cells and tissues; Gratz *et al.*, (2013). Without the PAM sequence the CRISPR/Cas9 cannot accomplish the editing process because it is the 2-5 ntPAM sequence which is required for the guide RNA to bind to the target site. Without the PAM sequence the CRISPR/Cas-9 cannot accomplish the editing process. Different Cas9 orthologs identified the different PAM sequence, such as NGG PAM from *Streptococcus pyogenes;* Jinek *et al.*, (2012); Deltcheva *et al.*, (2011), NGGNG and NNAGAAW PAM from *Streptococcus thermophiles*; Gasiunas*et al.*, (2012), Garneau *et al.*, (2010), Karvelis *et al.*, (2013) and NNNNGATT PAM from *Neisseria meningitides*; Hou *et al.*, (2013), Zhang *et al.*, (2013). There is a high risk association of off-target mutations with the use of CRISPR/Cas-9 system of genome editing in contrast to the TALENs and ZFNs; Fu *et al.*, (2013). The organisms having large genome size often contain such DNA sequences that are identical or highly homologous to the target site. Under such condition CRISPR/Cas9 also cleaves non target DNA sequences resulting into off target mutations which may even cause loss in the expression of vital genes. So there much focus should be given to increase the specificity between the guide RNA and target DNA sequences to nil or minimize the off target mutation; Cong *et al.*, (2013), Fu *et al.*, (2013), Hsu *et al.*, (2013) and Xiao *et al.*, (2014).

In conclusion, CRISPR/Cas9 is an ideal genome editing tool because of its simplicity, effectiveness and versatility. Due to its user friendly nature it is gaining its popularity as one of the most potential and precise genome editing tools in the field of molecular biology. This novel technique of genome edition was first done in *Drosophila melanogaster*; Bibikova *et al.*, (2002) and Bibikova *et al.*, (2003) but due to its adaptability and success rate it has proved its power and potentials in curing the human diseases and improving the crop quality and productivity as well. In coming future CRISPR/Cas-9 system of advanced genome editing technology can be viewed as very significant genome manipulation technique in humans, animals and plants as well.

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