CRISPR/Cas9 – A Buzz in Plant Biotechnology

Faiza Unbreen, Ayesha Masood

Abstract— Revolutions in the field of functional genomics and crop improvement has led to many advancements in the Genome Editing Technologies .CRISPR/Cas9 is a genetic engineering technology that is dependent on the complementarity of the guideRNA (gRNA) to a specific sequence and also on the Cas9 endonuclease activity .By the aid of this technology novel plant varieties have originated with the addition of significant traits and deletion of detrimental traits. CRISPR has increased advantage over other gene knock out technologies like ZFNs and TALENs and RNAi. It is an eye-catching technology that has grasped attention of many Plant Biotechnologists as by it various genetic manipulations like gene knockout, genetic modification, genetic engineering, and also activation and repression of gene of interest. This paper highlights the importance of CRISPR/Cas9 tools with surplus and specialized applications.

Index Terms— CRISPR/Cas9, Functional genomics, Genome editing, guideRNA, Gene knockout, ZFNs, TALENs.

1 Introduction

CRISPR-Cas9 is a genome editing tool that is creating a buzz in the science world. It is faster, cheaper and more accurate than previous techniques of editing DNA and has a wide range of potential applications.CRISPR-Cas9 is a unique technology that enables geneticists and medical researchers to edit parts of the genome by removing, adding or altering sections of the DNA sequence. It is currently the simplest, most versatile and precise method of genetic manipulation and is therefore causing a buzz in the science world. The CRISPR-Cas9 system consists of two key molecules that introduce a change (mutation) into the DNA. These are:
An enzyme called Cas9. This acts as a pair of 'molecular scissors' that can cut the two strands of DNA at a specific location in the genome so that bits of DNA can then be added or removed. A piece of RNA called guide RNA (gRNA). This consists of a small piece of pre-designed RNA sequence (about 20 bases long) located within a longer RNA scaffold. The scaffold part binds to DNA and the pre-designed sequence 'guides' Cas9 to the right part of the genome. This makes sure that the Cas9 enzyme cuts at the right point in the genome. The guide RNA is designed to find and bind to a specific sequence in the DNA. The guide RNA has RNA bases that are complementary to those of the target DNA sequence in the genome. This means that, at least in theory, the guide RNA will only bind to the target sequence and no other regions of the genome. The Cas9 follows the guide RNA to the same location in the DNA sequence and makes a cut across both strands of the DNA. At this stage the cell recognizes that the DNA is damaged and tries to repair it.

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Scientists can use the DNA repair machinery to introduce changes to one or more genes in the genome of a cell of interest.Some bacteria have a similar, built-in, gene editing system to the CRISPR-Cas9 system that they use to respond to invading pathogens like viruses much like an immune system.
Using CRISPR the bacteria snip out parts of the virus DNA and keep a bit of it behind to help them recognize and defend against the virus next time it attacks. Scientists adapted this system so that it could be used in other cells from animals, including mice and humans. Over the years scientists have learned about genetics and gene function by studying the effects of changes in DNA. If you can create a change in a gene, either in a cell line or a whole organism, it is possible to then study the effect of that change to understand what the function of that gene is. For a long time geneticists used chemicals or radiation to cause mutations. However, they had no way of controlling where in the genome the mutation would occur. For several years scientists have been using 'gene targeting' to introduce changes in specific places in the genome, by removing or adding either whole genes or single bases. Traditional gene targeting has been very valuable for studying genes and genetics; however it takes a long time to create a mutation and is fairly expensive. Several 'gene editing' technologies have recently been developed to improve gene targeting methods, including CRISPR-Cas systems, transcription activator-like effector nucleases (TALENs) and zinc-finger nucleases (ZFNs). The CRISPR-Cas9 system currently stands out as the fastest, cheapest and most reliable system for 'editing' genes. CRISPR-Cas9 has a lot of potential as a tool for treating a range of medical conditions that have a genetic component, including cancer, hepatitis B or even high cholesterol. Many of the proposed applications involve editing the genomes of somatic (Non-reproductive) cells but there have been a lot of interest in and debate about the potential to edit germ line (Reproductive) cells. Because any changes made in germ line cells will be passed on from generation to generation it has important ethical implications. Carrying out gene editing in germ line cells is currently illegal in Asia and most other continents. By contrast, the use of CRISPR-Cas9 and other gene editing technologies in somatic cells is uncontroversial. Indeed they have already been used to treat human

disease on a small number of exceptional and/or life-threatening cases. It is likely to be many years before CRISPR-Cas9 is used routinely in humans. Much research is still focusing on its use in animal models or isolated human cells, with the aim to eventually use the technology to routinely treat diseases in humans. There is a lot of work focusing on eliminating 'off-target' effects, where the CRISPR-Cas9 system cuts at a different gene to the one that was intended to be edited. In most cases the guide RNA consists of a specific sequence of 20 bases. These are complementary to the target sequence in the gene to be edited. However, not all 20 bases need to match for the guide RNA to be able to bind. The problem with this is that a sequence with, for example, 19 of the 20 complementary bases may exist somewhere completely different in the genome. This means there is potential for the guide RNA to bind there instead of or as well as at the target sequence. The Cas9 enzyme will then cut at the wrong site and end up introducing a mutation in the wrong location. While this mutation may not matter at all to the individual, it could affect a crucial gene or another important part of the genome. Scientists are keen to find a way to ensure that the CRISPR-Cas9 binds and cuts accurately. Two ways this may be achieved are through: The design of better, more specific guide RNAs using our knowledge of the DNA sequence of the genome and the 'off-target' behavior of different versions of the Cas9-gRNA complex and second one is the use of a Cas9 enzyme that will only cut a single strand of the target DNA rather than the double strand. This means that two Cas9 enzymes and two guide RNAs have to be in the same place for the cut to be made. This reduces the probability of the cut being made in the wrong place.

2 History

In 1987 the researchers found CRISPR sequences in *Escherichia coli.* But they didn't not characterize the function of CRISP sequences. In 2000 CRISPR sequences were found to be common in other microbes. In 2002 scientists coined the name CRISPR and explained signature Cas genes. In 2007 First ever experimental evidence for CRISPR adaptive immunity was found. In 2013 First demonstration of Cas9 genome engineering in eukaryotic cell was given.

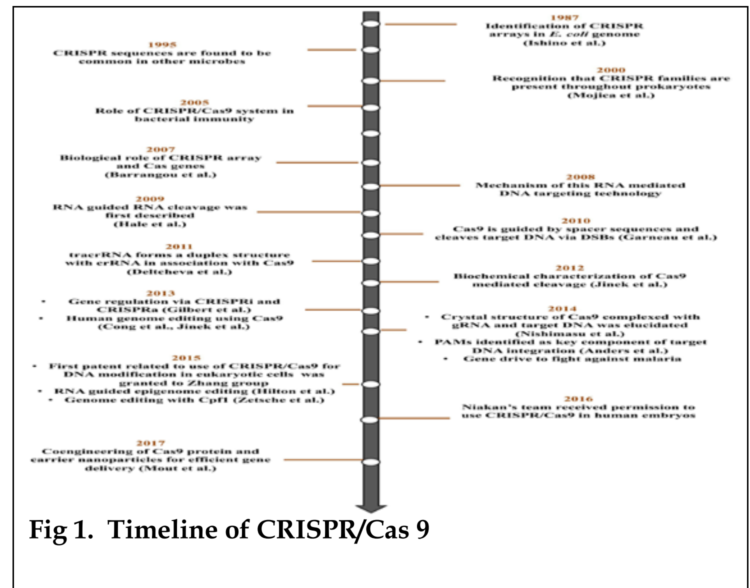

Fig 1. Timeline of CRISPR/Cas 9

3 *CRISPR* in Bacteria:

CRISPR is the part of the Bacterial immune system which detects and identifies the foreign DNA and cleaves it. The CRISPR and Cas (CRISPR- associated) proteins can identify and cleave the invading DNA in a sequence in a significant manner. A CRISPR array is composed of a series of repeats interspaced by spacer sequences acquired from invading genomes.

3.1 Components of CRISPR

- CRISPR-RNA (crRNA)
- trans-activating crRNA (tracrRNA)
- Protospacer adjacent motif (PAM)

3.2 Action of CRISPR in Bacteria

The CRISPR immune system helps to protect bacteria from the viral attack through the following three basic steps:

- Adaptation
- Production of cr RNA
- Targeting

CRISPR Cas is the protection tool of bacteria from invading pathogens such as bacteriophages. It is part of prokaryotic adaptive immunity. CRISPR Cas system adjusts itself to invade any foreign nucleic acid. It works in site specific manner. CRISPR Cas system is very diverse and divided into two classes which are further grouped into six major types and sixteen subtypes. CRISPR Cas9 consists of two basic components. First is cas9 protein which performs nuclease activity that produce

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double stranded break at specific location in DNA. Second component is piece of RNA called guide RNA. It is a long, pre-designed RNA scaffold consisting of small pieces of RNA which are separated by spacers. These spacers have a length of 20 base pairs and came from the sequence of invading nucleic acid and used in future for recognition. Guide RNA works to guide the cas9 protein to attach the right site of genome. It is involved in specificity of cas9 protein.

3.3 Molecular Mechanism of CRISPR

Three major steps involved in chopping the foreign nucleic acid:
Adaptation, synthesis of crRNA, target degradation
When the foreign element attacks the bacteria short fragments of DNA or RNA(approximately 20bp in length) called protospacers are recognized and excised from invader genome and then processed and integrated into CRISPR arrays for use in future for adaptive immunity. New spacer acquisition primarily occurs at the site of double stranded breaks. Acquisition of spacers also requires presence of 2-5 protospacer adjacent motifs (PAM) in selected protospacer sequence. Cas1 and cas2 recognize and integrate PAM into CRISPR array. There is very little known about the exact mechanism of adaptation.
Once the protospacers are incorporated CRISPR array is transcribed into precursor crRNA which is further processed into guide crRNA by a special trans-activating crRNA into guide RNA. At the final stage crRNA are used as guide to specifically interfere with the invading nucleic acids. Class 1 systems use multi-protein complexes for target degradation, while in class 2 systems, a single effector protein is sufficient for target interference. Guide crRNA binds to complementary sequence of target genome and chop it into pieces. To avoid self-targeting different mechanisms are used in different types.

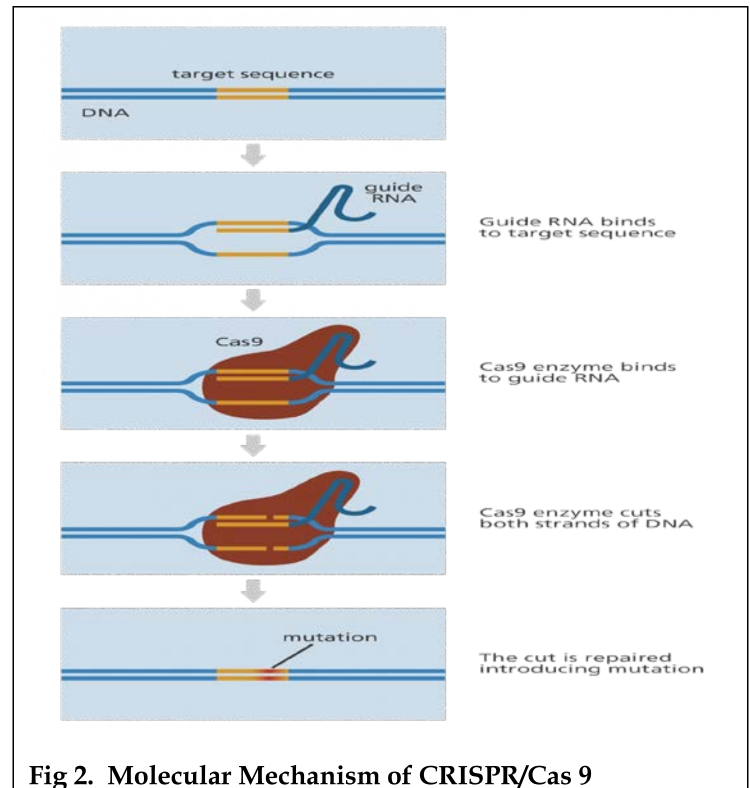

Fig 2. Molecular Mechanism of CRISPR/Cas 9

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5 CRISPR CAS9 AS GENOME EDITING TOOL:

CRISPR Cas9 is used as programmable genome editing tool. Cas9 protein remains inactive in the absence of guide RNA. This could be done by simply changing the nucleotide sequence of crRNA which binds to complementary DNA target. Design a stretch of 20 base pairs that matches a gene that you want to edit. Make sure that nucleotide sequence is found nowhere else in the genome. RNA and cas9 cut the DNA at that site. Once the DNA is cut, cell's natural repair mechanism kicks in and works to introduce mutations or other changes to the genome. Two types of repair mechanism involved, one is non-homologous end joining which result in deletion or insertion. In second method gap is filled with sequence of nucleotides. Scientist can supply the DNA template of their choosing, thereby writing-in any gene they want, or correcting a mutation.

6 APPLICATIONS

- Viral genome can be removed by changing immune cells.
- Genes can be added in embryos. For example Kang and colleagues added the CCR5Δ32 allele into early human 3PN embryos

- We can add or remove the sequence that is causing the disease.
- We can edit the genes of mismatched human or even non-human mammals as potential organ donors.
- Gene editing through CRISPR can reduce the risk of rejection and immune responses when using mismatched organs/cells/tissues.
- Drug development can be done efficiently.
- Disease models can be prepared.
- Ecological vector control can be done for example mosquito sterilization.
- Biofuels
- In Agriculture industry the modification of crop strains or animals can be done effortlessly.

7 CRISPR Cas9 as Genome Editing Tool

7.1 Somatic cell therapy

- No new ethical concerns/issues
- Regulations available to govern these applications
- Therapies being developed
- Most likely to be largest area of clinical development using CRISPR technology
- Research permitted

7.2 Germ line therapy

- Inconsistent and variable guidelines and regulation
- Considered for some indications because it would alter the genome in all cells and become heritable
- Development of therapies may be restricted
- Ethical concerns
- Limited research permitted
- Development of therapies may be restricted

. 8 Ethical Concerns

- International Summit on Human Gene Editing was held in December in 2015 in which all aspects of human germ line editing, regulation, consequences and potential applications were discussed.
- In April, 2016 NAS/NAM Meeting was held in which scientists discussed the concerns over germ line editing.
- EU CT Directive does not allow the germ line editing.
- NIH RAC wouldn't review proposals on germ line editing.
- Eugenic practices were also prohibited by the:
- Convention on Human Rights and Biomedicine (1997)
- Oviedo Convention
- Article 3(2) of the Charter of Fundamental Rights of EU

Conclusion

Ethicists, regulators and developers should discuss collaboratively at an early stage and throughout the development pathway.Rare genetic diseases, cardiovascular indications and mutation-driven malignancies will be key areas of development.The challenging and complex diseases can be cured with raised safety level and long term efficacy. The applications 0f CRISPR may be limitless because the potential is very vast.CRISPR/Cas9-based gene editing of tissues, organs and cells will be a rapid area and exciting way of development in the coming years.It is the flexible approach and is essential to the regulatory pathways.

Acknowledgment

The authors wish to thank https://www.yourgenome.org/facts/what-is-crispr-cas9 for their contribution in this Research.

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