CRISPR-Cas Fundamentals and Advancements in Translational Biotechnology

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Deshraj Deepak Kapoor, Shilpi Yadav, and Ravi Kr. Gupta

Abstract

With the advent of genome editing technologies that allow direct targeting and editing of genome sequences across nearly every eukaryotic cell, has made it possible to uncover hidden facts and regulation of genetic diseases and many other diverse applications by developing more precise cellular models. In the past decade, genome editing technologies have advanced rapidly and emerged as highly useful technologies in various fields ranging from basic to applied research, including biomedicine. Since the development of CRISPR-Cas gene editing system, at least 45 Cas protein families now being recognised. The term CRISPR stands for **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats. CRISPR-Cas system present in bacteria can neutralise the invasion of virus by destroying the viral genome. Thus, CRISPR works as an immune system of bacteria and responsible for protecting them.

CRISPR-Cas system has two essential components; the guide RNA, which needs to match the target sequence, as well as Cas (CRISPR-associated protein), an endonuclease responsible for DNA double-strand breaks that lead to genome editing.

Scientists are using CRISPR-Cas system to correct errors in genomes and turn on or off genes in cells and organisms rapidly and efficiently. In this chapter, we have elaborated the CRISPR-Cas system history, its working mechanism and applications in various fields. We have also included the ethical issues and limitations of this advance technique.

D. D. Kapoor · R. K. Gupta (✉)
Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, Lucknow, India

S. Yadav
Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow, India

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12.1 Introduction

12.1.1 History

During 1970s to 1980s, the first genetically modified mice were created (Jaenisch and Mintz 1974), and the first genetically modified bacteria were able to produce insulin and somatostatin (Goeddel et al. 1979). An impressive modification has been made to organisms that have been very difficult, costly and time-consuming to achieve. Scientists undertook more research into genetic engineering techniques in order to overcome these challenges. It led to the development of innovative tools such as zinc finger nucleases (ZFNs), TALENs (Transcriptional activator-like effector nucleases) and now CRISPR-Cas system. Combined with the cleavage domain of the restriction enzyme Fokl, zinc finger nucleases can produce DNA double-strand breaks by using their specificity to recognise DNA as well as their robust, yet controlled, activity. In several organisms, ZFNs have been used to create site-specific modifications; however, these have not been widely used in microbes (Urnov et al. 2010). ZFNs have several disadvantages, including context dependency, design difficulty and inefficiency in multiple gene targeting. A TALEN can produce double-strand breaks due to its DNA-binding specificity combined with its cleavage domain. Despite minimal off-target effects, they are not easy to clone and have a limited capability to target multiple genes.

CRISPR-Cas system simplicity and specificity are thought to make it superior to ZFNs and TALENs when compared. ZFN and TALEN proteins need to be produced separately for each DNA target, whereas CRISPR requires only matching a sequence of the guide RNA to a target region that will lead the Cas enzyme to a specific point where double-strand breaks can be introduced. It also has the advantage of being highly efficient since changes can be made directly through the system by inserting RNAs encoding the Cas protein and gRNA. Since, we can introduce multiple guide RNAs simultaneously, the CRISPR-Cas system can produce multiple gene modifications simultaneously (Jiang et al. 2015).

The CRISPR-Cas system was first described in 1987 when an unusual repetitive DNA sequence was observed in the *E. coli* genome during the analysis of genes involved in phosphate metabolism. Bacteria began inserting 32 nucleotide spacer sequences at regular intervals between the repeats whenever they encountered phage DNA (Ishino et al. 1987). The term CRISPR was coined in 2002 by Jansen et al. The repeat sequences were later found in 90% of archaea and 40% of sequenced bacterial genomes, although their functions were unclear (Horvath and Barrangou 2010). CRISPR-Cas history was made in 2005 when it was discovered that the spacer sequences actually originate from the phage genome (Mojica et al. 2005).

There were subsequent proposals that the CRISPR-Cas system could be used as a defence against phage attacks by bacteria and archaea. A spacer DNA similar to the DNA of phages can be added or deleted in *Streptococcus thermophilus* to increase or decrease its resistance to phage attack (Barrangou et al. 2007). Thus in 2007, CRISPR-Cas9 system was experimentally demonstrated as an acquired immune system in prokaryotes. Currently, researchers have revealed many Cas-proteins, CRISPR-associated genes, protospacer adjacent motif (PAM), CRISPR-RNA (crRNA) and transactivating crRNA (tracrRNA) which provide detail information about working mechanism of CRISPR-Cas system (Bolotin et al. 2005).

12.1.2 Mode of Action of CRISPR-Cas System

Researchers have used CRISPR-Cas system as a gene editing tool capable of making targeted genetic changes to any organism's DNA. In response to foreign DNA invasion by a phage or plasmid, CRISPR/Cas9 is most commonly used, which combines Cas9 endonuclease with a short guide RNA (gRNA) that contains two parts: a target-specific CRISPR RNA (crRNA) and a helper transactivating RNA (tracrRNA). Cas9 is guided by gRNA to specific genomic loci based on complementary nucleotides base pairing between the crRNA and target sequences (Pattanayak et al. 2013). The target DNA sequence containing the protospacer adjacent motif (PAM) on the 5′ end also have complementary sequences to the gRNA (Anders et al. 2014). A PAM sequence is necessary for the Cas enzyme to complement and distinguishes between the bacterial DNA from invaders DNA (Marraffini and Sontheimer 2010). In recent studies, researchers have shown that gRNAs derived from the fusion of guide sequence-containing crRNAs with tracrRNAs work as individual components (Jinek et al. 2012). Cas9 endonuclease binds specific sequences to induce specific double-strand breaks (DSBs), the repair of which is carried out by two distinct mechanisms:

(a) Non-Homologous End Joining (NHEJ): It is an error prone mechanism that results in random insertions or deletions during the repair procedure (Jeggo 1998).

(b) Homology-Directed Repair (HDR): A method that results in precise nucleotide edits but is less efficient, as DNA is repaired using either endogenous or exogenous templates (Komor et al. 2017; Hsu et al. 2014). For this reason, CRISPR/Cas9 can be employed to manipulate genetic sequences by inducing NHEJ or HDR. A CRISPR region is found in the bacterial genome; that helps them defend themselves against viruses.

Three main steps are involved in CRISPR-Cas system working mechanism (Fig. 12.1):

1. *Adaptation*-Short segments of viral DNA are inserted into the CRISPR sequence as new spacers after they have been processed.
2. *Processing and assembly*-In bacterial DNA, CRISPR repeats and spacers are transcribed, and this produces short RNA molecules known as CRISPR-RNAs.

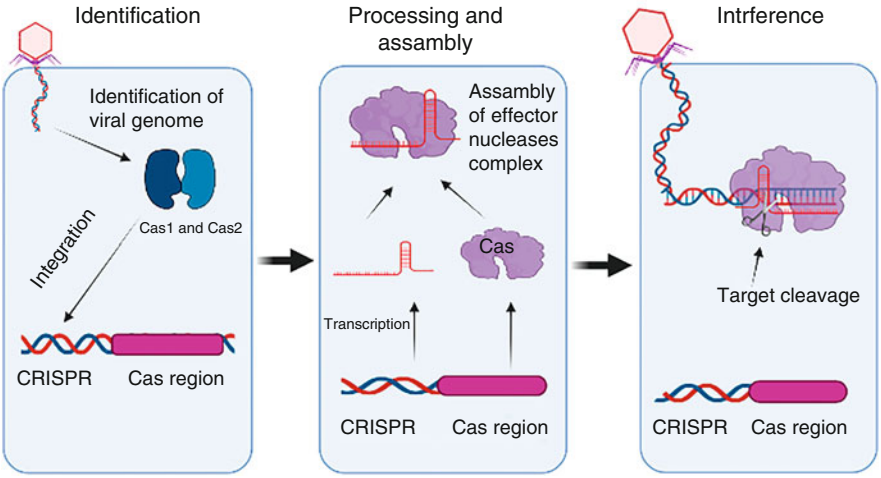

Fig. 12.1 Represents the working mechanism of CRISPR-Cas system in bacteria

3. *Targeting/Interference*-The machinery of bacteria is guided by CRISPR-RNAs to destroy the viral genetic material, since CRISPR-RNA sequences are copies of viral DNA acquired through adaptation; the sequences are exact matches of the viral genome, thus serving as an ideal guide.

12.2 Applications

The highly efficient and cost-effective CRISPR-Cas technology has many potential benefits and applications range vastly in translational biotechnology, from the introduction of point mutations to deletions, insertions, multiple-gene knock-downs and chromosomal rearrangements (Xue et al. 2014; Zhu et al. 2017). It has also potential applications in public health, species conservation, agriculture and basic research such as the ability to manipulate genetic sequences can be utilised to combat diseases such as malaria, dengue fever, Chagas and Lyme diseases. CRISPR could be used to analyse disease genes in viable human embryos and assist in immunotherapy, organoid engineering and development and identifying disease targets. In addition, it can be used to cure HIV, Haemophilia, Cancer, Duchenne muscular dystrophy, Amyotrophic lateral sclerosis, Sickle-cell anaemia, Cystic fibrosis and infertility. Some of the most potential applications of CRISPR-Cas system have been discussed below.

12.2.1 Genome Screening

Short hairpin RNAs (shRNAs) for RNA interference (RNAi) have been used in recent years to perturb transcript levels (Paddison et al. 2004). In this approach, the

gene expression was incompletely abrogated and there were significant off-target effects which led to unexpected results in transcriptional analysis (Jackson and Linsley 2010). In some studies, Cas9, pooled guide RNA libraries and next-generation sequencing (NGS) have been used to adapt CRISPR for genome-scale screening (Schumann et al. 2015). CRISPR-Cas9 modified genomes can be used for genome-wide screening either by examining 20,000 genes or by studying one gene or signalling pathway in particular. Screening CRISPRs generally involves loss-of-function assays, which utilise indel-prone NHEJ repair or sequence repression. Certain applications also require the use of gain-of-function screens, which use endogenous HDR and CRISPR activation methods (Klann et al. 2017).

12.2.2 Cell Therapy

CRISPR-Cas technology has undergone a radical shift primarily linked to stem cells and immune cells (Chen et al. 2013). The treatment of cancer and autoimmune diseases using ex vivo gene-edited T cells has shown promising results (Bikard et al. 2013; Ren et al. 2017). An example is chimeric antigen receptor T Cells. By electroporation of this chimera with Cas9 ribonucleoproteins (RNPs), it is possible to target other receptors such as CXCR4, CCR5, PD-1 and CD7 on human cancer cells named as CAR-T cells therapy (Schumann et al. 2015). CRISPR-Cas9 technology has been approved for the treatment of muscle-invasive bladder cancer, castration-resistant prostate cancer, metastatic renal cancer and metastatic non-small cell lung cancer.

CRISPR/Cas9 was considered for the treatment of Cystic Fibrosis (CF) (Schwank et al. 2013). A successful correction of the most common mutation responsible for CF in intestinal organoids was achieved using adult intestinal stem cells. Study showed that the function of the CF transmembrane conductor receptor (CFTR) was restored once the mutation had been corrected.

12.2.3 HIV Treatment

HIV can also be treated with CRISPR/Cas9, though antiretroviral therapy is effective for treating HIV, there is no cure currently as the virus has been permanently incorporated into the host genome. It is possible to target HIV genome activity by using CRISPR/Cas9 technology. It inhibited the expression of the HIV gene and replication in the cells that are latently infected with HIV, without causing any toxic effects on the cells. Alternatively, cells can be immunised against HIV. As a result, this may prove to be a good therapeutic advancement in the quest to eradicate HIV. After further refinement, these findings may enable gene therapies or transplantation of genetically altered bone marrow stem cells or inducible pluripotent stem cells to eradicate HIV infection (Hu et al. 2014).

12.2.4 Editing of Human Zygotes

CRISPR/Cas9 for human germline editing appears to be at an infancy, based on the limited number of studies. However, human germline editing holds great promise for curing many genetic disorders which are lethal to human. The current accuracy of embryo mapping is limited due to off-target effects, embryo mosaicism and lack of access to the embryo. CRISPR/Cas9 germline studies in China revealed significant technical issues and made it apparent that further research on human zygotes is needed as well as comprehensive deliberation before any clinical use should be considered (Kang et al. 2016; Tang et al. 2017). CRISPR-Cas9 has achieved the highest success in human germline editing to date with intracytoplasmic sperm injection (ICSI) (Ma et al. 2017). CRISPR-Cas9 was further demonstrated to be effective in removing genetic mutations from embryos, but not in correcting the mutations in an established embryo (Tang et al. 2017).

12.2.5 Agriculture

Using CRISPR-Cas tools to edit the genomes of plant species has revolutionised agricultural science and provided new opportunities for crop improvement. Genetically engineered plants become more resistant to microbial pathogens (Dong and Ronald 2019). A biotechnological approach using CRISPR-Cas9 is able to alter the genetic code in a stable, permanent and heritable manner in order to reach a specific goal in agriculture.

The causative agent of citrus canker disease, *Xanthomonas citri* is one of the most important citrus pathogens, which severely reduces yields (Peng et al. 2017). One of the target genes present in plant was altered by the CRISPR-Cas9 system to provide resistance against *X. citri*. The promoter of *XLOB1* (lateral organ boundaries) gene has been mutated, which in turn results in the loss of the ability to acknowledge and respond to bacterial effectors hence showed increase resistance against infection (Jia et al. 2016) (Fig. 12.2).

CRISPR technology is not restricted to gene editing. It is possible to use CRISPR-Cas9 to activate (CRISPRa) or repress (CRISPRi) sequence-specific genes without altering the genome (Qi et al. 2013). In order to achieve this, deactivated Cas9 enzymes were developed (dCas9) that do not possess the catalytic domain for cleaving DNA but retain their ability to bind to target sequences (Gilbert et al. 2014). Cas9 has the ability to repress transcription by binding to target genes and preventing RNA polymerase activity. In prokaryotic genomes, CRISPRi works effectively, but is less effective when applied to eukaryotic genomes (Gilbert et al. 2013).

In a similar manner, CRISPRa has been made possible by pairing dCas9 with transcriptional activators, such as viral proteins (VP)16/VP64 or p65 and targeting them to gene promoters, resulting in gene transcription upregulation (Konermann et al. 2015; Perez-Pinera et al. 2013).

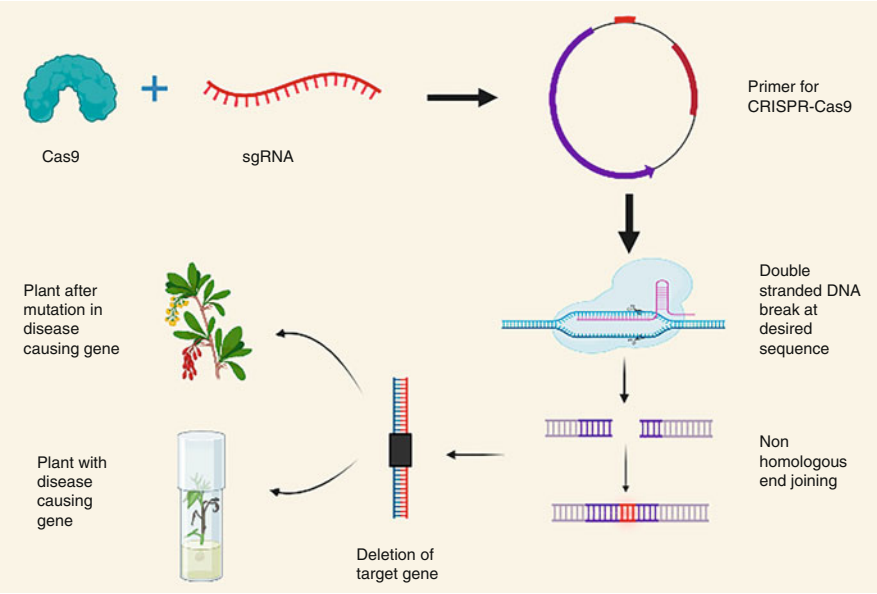

Fig. 12.2 Application of CRISPR-Cas9 system to edit the disease-causing gene in citrus plant

12.3 Limitations and Ethical Issues

There are incredible promising applications of CRISPR-Cas9 technology for the betterment of human life. But there are still some challenges to overcome. In vivo gene editing is highly challenging due to difficulties in the delivery of nuclease-encoding genes and guide RNAs to the appropriate cell types. As a means of safe delivery of cas9 nuclease genes and guide RNAs, a suitable vector must be used. In addition, there are potential off-target effects in the genome; non-intentional changes to the genome will have long-term effects on patients, including cancer.

CRISPR-Cas9 may have the greatest impact on human and its environment due to its potential applications and findings (Mulvihill et al. 2017). From an ethical perspective, use of CRISPR technology did not have the ethical issues pertaining to gene therapy and genetic engineering. In general, gene editing ethics can be divided into two groups: One which aims to correct defective genes (gene therapy) and the other one which aims to enhance physiologically normal genes (genetic enhancement). CRISPR technology does not violate the ethical issues if the gene editing is limited to the somatic cells. However, the genetic engineering of germline cells which could be inherited to the next generation should be carefully ethically reviewed (Sykora 2018). One of the major controversies about CRISPR technology emerges from its potential application in human embryos. Although group of scientists believe that experiments on human embryos after 14 days are ethically unacceptable, and no authority, whether it is the government, a law enforcement

agency, a panel of experts, a court, or a religious group, is allowed to decide the status of an embryo (Charo 1995). Patent holders stand to make a profit from CRISPR applications. Gene therapy and other CRISPR-based products will most likely to be initially expensive. As a result, it is ethically questionable whether the high price-tag will limit access to CRISPR products to a special class of people in the society. CRISPR was mostly developed and characterised through grants from government funds, so taxpayer money was used to fund much of the research and development (Chen et al. 2015) and it is ethically wrong to deny these individuals for the potentially life-saving benefits of this technology.

12.4 Conclusions

Offering the most versatile and powerful genome editing system, CRISPR-Cas technique has opened a new horizon in genome engineering and allowed us to uncover the amazing molecular secrets hidden within the living system. There are still challenges to overcome. Developing resistance against plant pathogens using CRISPR could prove a promising approach to conquer the breeding barriers. We can investigate the gene regulation of human diseases at DNA, transcriptional and translational level by this revolutionary technology. As with every powerful tool, there are also potential risks involved. It is imperative that well-controlled, reproducible experiments and clinical trial research should be conducted in order to make truly informed decisions regarding ethically contentious areas. In the present, this is problematic since many international laws discourage research of this type or ban it outright; they also inhibit research from being funded. Due to this, it is difficult to determine the risks and benefits of a technology. Overall CRISPR-Cas system has been exploited for the benefit of human health in every aspect from curing of diseases to improvement of food and we hope in future it will also help in other untouched areas for the betterment of human and animal life.

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