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CRISPR-Cas9 Technology: in Biotechnology a Breakthrough Innovation

ABSTRACT

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 is a gene-editing technology based on regularly spaced short palindromic repeats that have revolutionized biotechnology research. This system offers the potential to edit desired changes and genes in the genome in a fast, inexpensive, and simple way. Gene editing has many potential applications, including treating genetic diseases and the enhancement of yield and quality in agricultural products. The CRISPR-Cas9 system has a wide range of applications, from treating genetic diseases in medicine, improving crop yields in agriculture, obtaining disease-resistant animals, studying antibiotic resistance in microbiology, nitrogen fixation, biofuels, biosensors, greenhouse gas emissions, pesticide reduction, water management, etc. For example, this system has been used to successfully regulate mutations in intestinal organoids in the treatment of cystic fibrosis. In plants, successful results have been achieved in creating herbicide resistance in rice, powdery mildew in tomatoes, and reducing high amylopectin content in potatoes. In animals, studies are underway to provide resistance to bacteria that cause mastitis in cows. At the same time, research is underway to reduce milk allergens in goats by silencing the beta-lactoglobulin gene. However, the ethical aspects of CRISPR technology are also an important topic of debate. Given the potential risks and social implications of genetic engineering, ethical debates on this issue should continue. In conclusion, CRISPR-Cas9 is a revolutionary tool in genetic engineering and offers new opportunities for innovative applications in many fields. This article reviews studies on CRISPR-Cas9 technology, its use in medicine, agriculture, animal husbandry, and ethical debates.

Keywords: CRISPR/Cas9, biotechnology; genome editing; agriculture; environment

CRISPR-Cas9 Teknolojisi: Biyoteknolojide Devrim Niteliğinde Bir İnovasyon

ÖZ

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9, biyoteknoloji araştırmalarını devrim niteliğinde değiştiren düzenli aralıklarla yerleşmiş kısa palindromik tekrarlar temelinde bir gen düzenleme teknolojisidir. Bu sistem, genomda istenilen değişiklikleri ve genleri hızlı, ucuz ve basit bir şekilde düzenleme potansiyeli sunmaktadır. Gen düzenlemenin birçok potansiyel uygulaması vardır; bunlar arasında genetik hastalıkların tedavisi ve tarımsal ürünlerin verimliliği ile kalitesinin artırılması yer alır. CRISPR-Cas9 sistemi, genetik hastalıkların tedavisinden tarımda ürün verimliliğini artırmaya, hastalıklara dirençli hayvanlar elde etmeye, mikrobiyolojide antibiyotik direncini incelemeye, azot fiksasyonu, biyoyakıtlar, biyosensörler, sera gazı emisyonları, pestisit azaltımı, su yönetimi vb. bir dizi farklı alanda uygulamalara sahiptir. Örneğin, bu sistem, kistik fibrozisin tedavisinde bağırsak organoidlerinde mutasyonları başarılı bir şekilde düzenlemek için kullanılmıştır. Bitkilerde ise pirinçte herbisit direnci, domateste unlu mantar hastalığı ve patateslerde yüksek amilopektin içeriğinin azaltılması gibi başarılı sonuçlar elde edilmiştir. Hayvanlarda ise ineklerde mastitise yol açan bakterilere karşı direnç sağlamak için çalışmalar devam etmektedir. Aynı zamanda, keçilerde beta-laktoglobulin geninin susturulmasıyla süt alerjenlerinin azaltılması amacıyla araştırmalar yapılmaktadır. Ancak, CRISPR teknolojisinin etik yönleri de önemli bir tartışma konusudur. Genetik mühendisliğin potansiyel riskleri ve toplumsal etkileri göz önüne alındığında, bu konuda etik tartışmaların devam etmesi gerektiği söylenebilir. Sonuç olarak, CRISPR-Cas9, genetik mühendislikte devrim niteliğinde bir araçtır ve birçok alanda yenilikçi uygulamalar için yeni fırsatlar sunmaktadır. Bu makale, CRISPR-Cas9 teknolojisi, tıptaki, tarımdaki, hayvancılıktaki kullanımı ve etik tartışmalarla ilgili yapılan çalışmaları incelemektedir.

Anahtar Kelimeler: CRISPR-Cas9, biyoteknoloji, genom düzenleme, ziraat, çevre

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INTRODUCTION

The term biotechnology was first defined in 1918 by the Hungarian engineer Karl Ereky. However, the basic applications of biotechnology are actually as old as human history. Following Ereky, biotechnology has been defined in different ways by different scientists. According to one definition, biotechnology combines the principles of engineering and biological sciences to produce new and useful products from raw materials of biological origin (Verma et al., 2011).

Another definition of biotechnology is the use of living organisms or products derived from them to improve human health or the environment. Biotechnology is important not only for its beneficial aspects, but also for its risks, which need to be handled with care (Verma et al. 2011).

There are several sub-fields of biotechnology:

• Medical biotechnology: Includes topics such as drug development, gene therapy and disease diagnosis.

• Agricultural biotechnology: Aims to produce more resilient and productive crops.

• Environmental biotechnology: Provides solutions to reduce pollution and make sustainable use of natural resources.

• Food biotechnology: The use of biotechnology in food production.

• Industrial biotechnology: Covers biotechnology applications in industrial production.

• Marine biotechnology: Focuses on the use of biological materials from the sea.

Traditional biotechnology uses the natural biological processes of living organisms, such as bread, cheese and other foods produced by fermentation. Modern biotechnology uses advanced scientific methods such as genetic engineering, DNA technologies, monoclonal antibody production and biological therapies. These technologies offer significant innovations and opportunities in the fields of health and the environment. As a rapidly developing field, biotechnology has the potential to provide solutions to future problems (Gupta et al., 2017).

Many scientists have made great efforts, sacrifices and revolutionary contributions in the development of molecular biotechnology. One of the most prominent of these developments was realised in 1978 by Genentech, a US-based pharmaceutical company. The company isolated the gene that codes for human insulin and transferred it to Escherichia coli bacteria. These bacteria, working like biological factories, produced human insulin and this molecule was made available to diabetics. Shortly after this important development, in 1980, Genentech's shares soared on the New York Stock Exchange. The shares experienced one of the fastest jumps in stock market history, rising from $35 to $89 in 20 minutes. This surge was driven by high expectations of the potential applications of biotechnology. These include the production of micro-organisms that can replace chemical fertilisers, and the development of genetically engineered pest-resistant and nutrient-enhanced crops. However, this process has also given rise to ethical, social, legal and religious debates (Tarım, 2004).

New techniques for editing plant genomes have been developed that can target specific chromosomal regions. These techniques are implemented using sequence-specific and customisable nucleases. These nucleases create a double-stranded DNA break at the target region. The cell recognises this break as DNA damage and activates the appropriate enzymatic mechanisms to carry out the repair. When this repair is done by homologous recombination (HR), the broken region is repaired using information from a DNA template. This template can be a homologous chromosome, a sister chromatid or user supplied DNA. Custom templates can contain modifications such as nucleotide changes or transgenes and can be incorporated into the target site by HR. Another repair mechanism, non-homologous end joining (NHEJ), is usually used in somatic cells and although correct repair is often achieved, small deletions or rare insertions can occur. In addition, some mutations can result in the removal of a few amino acids in the coding sequences, but this can alter the function of the gene without affecting the reading frame. Similarly, insertions or deletions in promoter regions can affect gene expression by disrupting the structure of regulatory sequences. To generate targeted mutations, SSNs must be able to recognise specific DNA sequences in complex genomes (Songstad, 2017).

A number of basic tools have been developed to edit plant genomes. These include homologous recombination (HR), zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), pentatricopeptide repeat proteins (PPR), the CRISPR/Cas9 system, RNA interference (RNAi), cisgenesis and intragenesis. In addition, sequence-directed editing techniques and oligonucleotide-based mutagenesis methods offer the possibility of modifying the genome at the level of a single nucleotide. In recent years, innovations such as adenine base editors (ABEs) have been developed. ABEs use a combination of Cas9 nickase and a deoxyadenine deaminase (TadA) enzyme with reduced catalytic activity to convert AT base pairs to GC base pairs (Mohanta et al. 2017).

CRISPR-Cas9 Technology

In 1989, Francisco Mojica joined the University of Alicante for his Ph. D and became involved in the study of an archaeal microbe, Haloferax mediterranei, which is extremely salt-tolerant in the marshes of Santa Pola (Doudna and Gersbach, 2015).

Mojica delved deeper into the microbe's DNA, and in his initial research he discovered a palindromic structure of 30-base-long repeating sequences separated by about 36 bases. These sequences were unlike any known microbial repeat group. Fascinated by this unusual structure, Mojica dedicated himself to solving the puzzle. Over time, he found similar structures in other halophilic archaea, as well as in close relatives such as H. volcanii. A search of the literature suggested that such structures might also be present in eubacteria; in particular, a group of researchers had reported a similar structure in Escherichia coli. However, this observation had not attracted much attention. Mojica thought that the structure might have an important biological function because it was repeated in different microorganisms and published a paper describing it. He initially called them 'short regularly spaced repeats' (SRSRs), but later changed the term to 'clustered regularly interspaced palindromic repeats' (CRISPRs) (Lander, 2016).

In 2000, initial observations showed that microorganisms such as Streptococcus thermophilus, Mycobacterium tuberculosis, Clostridium difficile and Yersinia pestis add new spacer sequences to the CRISPR region after viral attack. These DNA sequences were found to be compatible with specific parts of the virus genome, and changes in the spacer sequences can affect the bacteria's resistance to viruses. These findings confirm that CRISPR sequences play an important role in the immune system of bacteria (Vidyasagar, 2018).

To understand how life works, researchers need to alter the genetic make-up of cells. In the past, this process was slow, complex and sometimes impossible. But with CRISPR/Cas9 genetic scissors, the code of life can now be changed in just a few weeks. Emmanuelle Charpentier discovered a previously unknown molecule, tracrRNA, while studying bacteria such as Streptococcus pyogenes. Charpentier's research showed that tracrRNA is part of CRISPR/Cas, the bacteria's ancient immune system, which cuts the DNA of viruses and neutralises them. Charpentier published his findings in 2011. That same year, she began working with biochemist Jennifer Doudna. Together, they recreated the bacterium's genetic scissors in a test tube, making them more accessible. They enabled these scissors to cut DNA at a predetermined point. This discovery opened up the possibility of modifying the genetic code of life (Charpentier and Doudna, 2020).

Mechanism of the CRISPR System

CRISPR-Cas systems are an immune mechanism used by bacteria and archaea and consist of CRISPR arrays and CRISPR-associated (Cas) genes. In the operation of this system, Cas genes, which are usually located near CRISPR arrays, are responsible for the production of proteins involved in the immune response and play an important role in CRISPR activity. CRISPR arrays are formed by inserting short pieces of foreign genetic material, such as the virus that infects the bacteria, between repeated sequences. This mechanism allows certain parts of the viral DNA to be inserted into the CRISPR site along with the repeat sequences (Gök and Tunalı, 2016).

The CRISPR-Cas immune system works in three basic steps: adaptation, expression and targeting. The first step, adaptation, involves the integration of short interval sequences from the invading DNA into the CRISPR site. This process starts with the recognition of protointerval adjacency motifs (PAMs) in the foreign DNA (Barrangou and Marraffini, 2014). PAM motifs prevent the system from targeting its own DNA, while mutations allow the phage to escape immunity. In the second step, sequences at the CRISPR locus are transcribed into precursor CRISPR RNAs (pre-crRNAs), which are converted into small crRNAs by Cas enzymes. In the final step, targeting,

the crRNA pairs with the invading DNA and Cas proteins cut the target DNA, preventing replication of the invading genetic material (Savic and Schwank, 2016; Barrangou and Marraffini, 2014).

Applications of CRISPR Technology

Since the discovery of CRISPR/Cas9 by Charpentier and Doudna in 2012, the use of this technology has expanded rapidly. CRISPR/Cas9 has contributed to many important discoveries in basic science and has enabled the development of crops resistant to pests, mould and drought in agriculture. In medicine, clinical trials of new cancer treatments are underway. CRISPR/Cas9 has revolutionised the life sciences and brought significant benefits to humanity (Charpentier and Doudna, 2020).

The Role of CRISPR in Medicine

Transfusion-dependent β-thalassemia (TDT) and sickle cell disease (SCD) are monogenic disorders that are common worldwide and cause significant health problems. Approximately 60,000 cases of TDT and 300,000 cases of SCD are diagnosed worldwide each year. These diseases are caused by mutations in the haemoglobin β-subunit gene and lead to severe complications such as ineffective erythropoiesis, haemolysis, anaemia, organ damage and painful crises. Current treatments aim to manage symptoms with transfusions and iron chelation, but these approaches do not correct the underlying genetic defect. However, CRISPR-Cas9 gene-editing technology offers a revolutionary approach to treating these diseases. By targeting the enhancer region of the BCL11A gene in erythroid cells, this technology increases γ-globin expression and reactivates fetal haemoglobin production. In clinical trials, patients with TDT and SCD treated with CRISPR-Cas9 have shown positive results, including improved haemoglobin levels, transfusion independence and reduced complications. These developments show that CRISPR-Cas9 is a promising tool for the treatment of genetic diseases (Frangoul et al. 2021).

One study aimed to correct the Q61K mutation in the NRAS gene, which causes malignant melanoma (skin cancer), using CRISPR/Cas9 genome editing. Using the SK-MEL-30 cell line carrying the Q61K mutation, guide RNA (gRNA) sequences were designed and integrated into plasmid vectors. These genetic constructs were transferred into cells by electroporation and analysed using techniques such as fluorescence microscopy and flow cytometry. Using the HDR-assisted repair mechanism, knock-in and knock-out procedures were performed in the targeted regions and the efficiency of these procedures was verified by real-time PCR and end-point analysis methods. The results of the study show that the Q61K mutation can be successfully corrected using CRISPR/Cas9 and that this approach provides an innovative and effective method for treating cancer mutations. The study provides an important foundation for gene editing technologies and directs future research in this field (Duran, 2018).

This study investigates the efficiency and specificity of the CRISPR/Cas9 system for gene editing in human triple-nucleus (3PN) zygotes. The accuracy and off-target effects of gene editing in human embryos are critical to the success of clinical gene therapy and embryo editing studies. The study showed that CRISPR/Cas9 enables genetic editing in 3PN zygotes, and that editing occurs mainly by error-prone NHEJ, with limited linear editing by HDR. In addition, off-target effects and genetic mosaicism were observed in edited embryos. These findings suggest that although CRISPR/Cas9 has potential for genetic therapy, off-target effects should be further investigated in clinical applications (Kose et al. 2020).

Innovative Practices in Agriculture

This paper focuses on the development of herbicide-tolerant carrot genotypes using a combination of CRISPR/Cas9 and cytidine base editing techniques. CRISPR/Cas9 technology offers genetic editing by targeting specific regions on DNA, while cytidine base editing offers the ability to modify specific bases in DNA. These two powerful biotechnology tools were used to make carrot plants resistant to herbicides. Although herbicides used in agriculture are effective in controlling weeds, they can have adverse effects on plants. Therefore, the development of herbicide-tolerant plants is of great importance in terms of increasing agricultural productivity and reducing environmental impact. The aim of this study is to increase the yield of carrot genotypes developed using these biotechnological methods by providing a more resistant and sustainable production against herbicides (İpek et al. 2024).

Researchers at the University of Florida used CRISPR-Cas9 technology to reduce the bending angle of sugar cane leaves while increasing dry biomass, internodes and branch number. This change allowed the plants to access more sunlight, increasing biomass production. The research has demonstrated the potential of genome

editing to increase sugarcane productivity. The study particularly highlights the importance of sugarcane for biofuel production (Brant et al. 2024).

A research team from the Chinese Academy of Agricultural Sciences also used the CRISPR-Cas9 system to create mutations in GmFT2a, an integrator in the photoperiod flowering pathway of soybean. The modified soybean plants showed late flowering, resulting in an increase in vegetative size. The mutation was found to be stably inherited in the next generation (Cai et al. 2017).

A New Era with CRISPR in Industry

To investigate the biological feasibility of decaffeinating coffee plants using CRISPR-Cas9 gene-editing technology and to examine the impact of this technology in a social context. The aim was to stop caffeine production by regulating some genes involved in the caffeine biosynthesis pathway. The study used a methodology in which the CRISPR-Cas9 system was used to create mutations in target genes and this genetic editing was transferred to plants by Agrobacterium tumefaciens-mediated transformation. The results showed that the regulation of these genes is promising for the production of decaffeinated coffee. In particular, regulation of the DXMT gene can lead to the accumulation of theobromine, which can affect the bitterness of coffee, while regulation of XMT can completely stop caffeine production. However, it has been stressed that decaffeinated coffee plants produced using CRISPR-Cas9 technology have a significant impact on commercial success (Leibrock et al. 2022).

CRISPR/Cas9 technology is playing an important role in energy science. This gene-editing tool makes it possible to modify the genomes of microorganisms used in biofuel production more quickly and precisely. In particular, microorganisms such as Yarrowia lipolytica can be modified using CRISPR/Cas9 to make them more efficient at converting sugars into lipids and hydrocarbons. This approach allows biotech products to be produced in a more sustainable and economical way than synthetic methods. In addition, biofuel and chemical production processes are being developed using CRISPR/Cas9 in bacteria such as Clostridium autoethanogenum, which has great potential for renewable energy production (Kaboli and Babazada, 2018).

CRISPR Revolution in Animal Genetics

CRISPR-Cas9 gene editing technology is being used to improve hornlessness in dairy cows. Horned cattle pose a safety risk to both other animals and farm workers. Instead of cattle whose horns are physically removed using traditional methods, cows have been developed that are genetically rendered hornless using CRISPR. This method both prevents animal suffering and makes the production process safer. However, the classification of gene-edited cows as GMOs requires regulatory hurdles and safety testing. However, recent regulatory changes in the US suggest that these barriers may be overcome and that gene-edited cows may become more common (Sandøe et al. 2021).

The aim of this study is to use CRISPR/Cas9 technology to correct the single nucleotide change that causes IARS (Isoleucyl-tRNA synthetase) syndrome in Japanese Black cattle. The CRISPR/Cas9 system is designed to make the correct nucleotide substitution by targeting the mutated region. The genetic modification was transferred into bovine fetal fibroblast cells using donor DNA containing the AcGFP cassette, followed by embryo production by somatic cell nuclear transfer (SCNT). The result showed that the mutation was correctly repaired and no additional DNA was identified. These results suggest that genome editing technology could be an effective tool for improving livestock productivity and restoring genetic diversity (Ikeda et al. 2017).

To create mutant dogs with muscle hypertrophy by editing the myostatin (MSTN) gene in beagle dogs using CRISPR/Cas9 technology. MSTN is a gene that regulates skeletal muscle mass. Mutations in this gene can increase muscle development. First, the MSTN gene was disrupted using Cas9 mRNA and sgRNA, and then embryos were transferred to female dogs by microinjection. By introducing mutations in the MSTN gene, the researchers were able to successfully produce genetically modified dogs with marked changes in muscle structure. These results support the potential of CRISPR/Cas9 technology to generate new canine models for biomedical research (Tian et al. 2023).

Atlantic salmon is an important commercial aquaculture species. In this study, we aimed to improve the salmon genome through gene editing and induced mutations in the pigmentation-related tyr and slc45a2 genes using the CRISPR/Cas9 system. Microinjection experiments showed that low incubation temperatures and

physical characteristics of salmon eggs had limiting effects on mutation efficiency. Analyses showed that mutation rates in target genes were low compared to zebrafish and similar to tilapia. DNA analysis from fin clips was also suggested as a practical method for assessing gene editing efficiency. Phenotypic effects of mutations were observed and associated with loss of pigmentation. This study demonstrates that CRISPR/Cas9 technology can be successfully applied in salmonids and can be used as an important tool for genetic improvement in aquaculture (Edvardsen et al. 2014).

ETHICAL DISCUSSIONS

CRISPR-Cas9 technology has great potential for the treatment of genetic diseases. This technology can be applied in two different categories: somatic and germline gene editing. While somatic editing only affects the individual being treated, germline editing can make permanent changes that can be passed on to subsequent generations. However, technical problems such as off-target effects and mosaicism make it difficult to use this technology safely. Although promising, germline editing raises ethical questions. Reduced genetic diversity and practices such as 'designer babies' can lead to social inequalities. The first editing of a human embryo in 2015 fuelled ethical debates in the field. Globally, the use of CRISPR technology is regulated differently in different countries. While some countries prohibit germline editing, others allow its use for research purposes (Tosun and Kesmen, 2022).

CRISPR technology has the potential to bring huge benefits in areas such as food security, health and biotechnology, but it raises ethical and safety concerns. Applications such as food modification and gene drives could benefit the undernourished, but there is a risk that these benefits could increase inequality through limited access. In addition, gene drives can permanently alter species, which may have uncertain effects on the balance of ecosystems (Brokowski and Adli, 2019).

While the commercialisation of CRISPR technology is accelerating progress in genetic engineering, it raises questions about patent rights and intellectual property. The patenting of these technologies should be shaped not only for commercial interests, but also for the general benefit of society. Patents should be organised in such a way as to ensure the protection of innovation, while at the same time not preventing large sections of society from benefiting from these innovations. Otherwise, powerful technologies such as genetic engineering may deepen inequalities and ignore the public good (Mulvihill et al. 2017).

To improve the safety of the CRISPR-Cas9 system, several innovative approaches have been developed to reduce off-target effects (OTE). At the forefront of these is the design of more specific and targeted sgRNAs. These designs aim to minimise the likelihood of off-target mutations. Modifications to the Cas9 protein are also aiding this process; for example, Cas9 nicase mutants increase the sensitivity of editing by making only single-strand breaks in the target region. In addition, tools such as optogenetics and transposons can be used to make gene editing more controlled and safer. These strategies increase the safety of genetic interventions, allowing treatments to be delivered more effectively and with less risk (Kose et al. 2020).

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