Uluslararası Fen Araştırmalarında Yenilikçi Yaklaşımlar Dergisi
International Journal of Innovative Approaches in Science Research 2020, Vol. 4 (4), 153-190
https://doi.org/10.29329/ijiasr.2020.312.6


Review article

# CRISPR-Of-Things: Applications and Challenges of the Most Popular Gene Editing Tool in the Fields of Health, Agriculture and Environment

Cihan Tastan [a,*], Sulhattin Yasar [b], Muhammed Bahattin Tanyolac [c], Kenan Turgut [d], Ufuk Tansel Sireli [e], Cimen Atak [f], Kamil Haliloglu [g], Kemal Benlioglu [h], Kemal Melih Taskin [i], Nurhayat Barlas [j] & Gultekin Yildiz [k]

[a] Transgenic Cell Technologies and Epigenetics Application and Research Center (TRGENMER), Uskudar University, Istanbul, Turkey
[b] Department of Food Engineering, Faculty of Engineering, Karamanoglu Mehmetbey University, Karaman, Turkey
[c] Department of Bioengineering, Faculty of Engineering, Ege University, Izmir, Turkey
[d] Department of Field Crops, Faculty of Agriculture, Akdeniz University, Antalya, Turkey
[e] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey
[f] Department of Molecular Biology and Genetics, Faculty of Science and Letters, Istanbul Kultur University, Istanbul, Turkey
[g] Department of Field Crops, Faculty of Agriculture, Ataturk University, Erzurum, Turkey
[h] Department of Plant Protection, Faculty of Agriculture, Adnan Menderes University, Aydin, Turkey
[i] Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
[j] Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey
[k] Department of Animal Nutrition & Nutritional Diseases, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

**Abstract**

Almost all cells of any living organism contain DNA, a hereditary molecule that passes from generation to generation during reproduction. The term "genome" generally refers to the total DNA sequences in an organism. The genome consists of DNA sequences called “gene”, which plays a role in the basic biological processes involved in many phenotypic and genotypic characteristics, such as performing cellular functions, controlling numbers and species, regulating energy production, metabolism, and combating diseases. Gene editing is the process of pre-designing and modifying a particular DNA sequence in a targeted gene. The most widely used technique is CRISPR-Cas technology. For this purpose, the DNA helix is cut at a certain point, to form a double-strand break (DSB), and naturally existing cellular repair mechanisms repair the DSB. Modes of the repair mechanisms may affect the gene function. When DSB is formed, gene editing techniques can be applied to remove, insert, or replace a newly modified sequence using a synthetic donor template DNA. In developed and developing countries, CRISPR-Cas studies in addition to research and development studies are rapidly increasing. In addition to increasing population, changing weather conditions, declining farmland, increasing biotic and abiotic stresses are other important barriers to agricultural production, food, and feed supply. In this report, CRISPR-Cas applications are introduced in detail from the studies that carried out gene modifications in the fields of health, animals, plants, microorganisms, and food supply. Besides, these technologies and applications have been examined in terms of world biosafety legislation and the scientific risk assessment of the products developed using the CRISPR-Cas technique.

**Keywords:** CRISPR, Cas9, health, animal, plant, agriculture.

**Received:** 07 October 2020 * **Accepted:** 21 November 2020 * **DOI:** https://doi.org/10.29329/ijiasr.2020.312.6

* **Corresponding author:**

Cihan Tastan, Transgenic Cell Technologies and Epigenetics Application and Research Center (TRGENMER), Uskudar University, Istanbul, Turkey.
Email: cihantastan.ct@gmail.com

## INTRODUCTION

CRISPR stands for "Clustered Regularly Interspaced Short Palindromic Repeats", which was first described by Jansen et al. (2002) and accepted by the scientists working in this field. In short, it is now called a gene-editing tool. The CRISPR-associated enzyme (Cas)-9, on the other hand, is an endonuclease enzyme used to deactivate some genes and plays an active role in the DSB formation. The CRISPR-Cas system was first introduced 30 years ago by Ishino et al. (1987) in the E. coli bacterial gene responsible for the alkaline phosphatase. CRISPR was observed for the first time in an archea in 1993 as *Haloferax mediterranei* (Mojica et al. 1993). The defense system function of the CRISPR-Cas system for prokaryotes was experimentally proven in *Streptococcus thermophilus* bacteria in 2007 (Barrangou et al. 2007). In the first experiments, *Streptococcus thermophilus* was exposed to virulent phages to test that exogenous phage DNA could be added to the bacterial genome as part of CRISPR repeats, which is important for yogurt and cheese production. The different Cas proteins then function by expressing the CRISPR region to produce CRISPR RNAs (crRNAs). Using sequence homology, these crRNAs direct a Cas nuclease to a targeted exogenous genetic sequence located next to the species-specific protospacer neighbor motif (PAM) (Cong et al. 2013; Mali et al. 2013). The CRISPR-Cas complex binds to foreign DNA and dissects it to destroy the invader (Barrangou and Oost, 2013). CRISPR-Cas systems, which are found in 95% of the archaeal bacterial genomes and 48% of other genomic bacterial genomes, are extremely diverse in terms of diversity in PAM sequences and the number and type of Cas proteins. Makarova et al. (2015) classified 5 types and 16 subtypes of CRISPR-Cas systems based on shared characteristics. Synthetic biology and gene repair technologies have been developed to target and mutate damaged DNA sequences or regions that cause rare diseases. These technologies are called Zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and CRISPR. These nuclease-based technologies can provide DSBs in DNA. These breaks force the cell to repair its genome in different ways, such as non-homologous end-joining (NHEJ) and Homology Directed Repair (HDR). NHEJ causes breaks in the specific region of the genome to lose the function of the target gene or insertion of the functional gene into the target DNA region. HDR is used to replace the mutated DNA sequence with the natural phenotype-healthy gene sequence. To stimulate the HDR-associated genetic repair mechanism, a highly compatible donor DNA fragment with the target genomic region should be added. The CRISPR method is one of the fastest, cheapest and easiest methods to activate NHEJ or HDR genetic repair pathways. The CRISPR-Cas9 technique can be used for various purposes. Gene silencing (Knock-out) or knock-down of gene expression, knock-in, creation of loss of function libraries throughout the genome, gene therapy, chromosomal deletion and insertions, transcription control, epigenetic modification, DNA extraction from the chromosome can be done with CRISPR-Cas technology (Yamamoto, 2015). This method, which is thought to have high specificity and efficiency and is more economical than ZFN and TALEN, has been applied in livestock since 2013 (Petersen and Niemann, 2015). Jinek et al. (2012) after the restructuring of model organisms' in vitro

conditions and after experiments on plants and animals, the CRISPR-Cas9 technique has been used in genome editing studies (Kim, 2016). This system used for genome editing is an RNA-guided endonuclease system (Yamamoto, 2015). Also, the transfer of the CRISPR-Cas9 system to cells and organisms is provided by various transfection methods (Yamamoto, 2015).

The invention of the CRISPR-Cas system has opened a new era in the field of biotechnology. The CRISPR-Cas9 system thus serves as a unique gene-editing tool that allows scientists to cut, inactivate, or precisely alter a specific gene region in the genome of any organism. The CRISPR-Cas gene regulation system can produce hereditary, targeted mutations. Transgene-free animals and plants can also be produced by this application. After this stage, advanced practices that can be transferred to the practice of genome regulation are carried out in many fields related to health, agriculture, and the environment.

**Biomedical and medical applications**

Although there are approximately seven thousand rare diseases diagnosed in humans; few of these diseases can be treated today (European Organization for Rare Diseases (EURORDIS)). It is known that 80% of these are genetic diseases (Rare Genomics Institute). Therefore, the need for gene therapy methods for individuals affected by genetic diseases that reduce the quality of life and shorten life is indisputable. Gene therapy is considered a technique that modifies a part of a genetic structure to repair or maintain a functional gene in a genome of a person with rare genetic diseases. With the introduction of healthy human genome sequence at the beginning of the millennium, genetic disorders underlying many diseases have emerged and the development of gene therapy methods to repair these damaged DNA sequences has accelerated. For this reason, pharmaceutical and biotechnology companies are focusing their research especially in the field of gene therapy in recent years.

Genetic modification method based on HDR in gene therapies has been used frequently to repair mutations in rare diseases (Papasavva et al., 2019). Animal models with rare diseases can be treated with CRISPR genome modification and HDR (Li et al., 2019). Genetically modified cells created in laboratory and animal models with CRISPR technology can now mimic rare diseases and help pharmaceutical companies develop drugs faster at less cost. For example, patients with mutations that deactivate a protein with the calcium pumping function known as ORAI1 have severe immunodeficiencies. Deleting the ORAI1 gene in immune T cells in the laboratory with CRISPR gene modification opens a valuable gateway to understanding the molecular mechanisms underlying this genetic disease and improving treatment (Chen et al., 2018). Similar studies may be performed with other immunodeficiency diseases with known genetic impairment. CRISPR genome modifications give rise to the hope of fully curing hundreds of genetic diseases that are no longer treatable to the public. For example, the first CRISPR gene therapy in human embryos has been shown to yield successful

results in patients with cardiac muscle disease (MYBPC3, Hypertrophic cardiomyopathy) (McNamara et al., 2017).

Today, many genetic/familial/hereditary or rare diseases are being studied for the treatment of model animals, as well as human stem cells by CRISPR gene therapy. Some of these diseases are beta-thalassemia, muscular dystrophy, Parkinson's, and hemophilia (Table 1).

**Table 1.** Modeling or Repairing Genetic Mutations Associated with Genetic Diseases with CRISPR Genome Repair Technologies: Disease modeling (M) or gene repair (R) performed on mutated target genes that cause rare or genetic diseases with CRISPR genome repair systems. Treatments were performed in an animal model (IV) or ex vivo in a laboratory (EV) (Taştan and Sakartepe, 2018).

| **Disease** | **Gene** | **Disease modeling (M) or gene repair (R)** | **In an animal model (IV) or ex vivo in a laboratory (EV)** | **References** |
|---|---|---|---|---|
| Huntington | HTT | M & R | IV & EV | Eisenstein et al. 2018 |
| Chronic Granulomatous | CYBB | R | EV | Sweeney et al. 2017 |
| Tyrosinemia | FAH | R | IV | Carter et al. 2017 |
| Myotonic Dystrophy (MD) | RAN | R | IV & EV | Raaijmakers et al. 2019 |
| β-thalassemia | HBB, a-globin | M & R | EV | Li et al. 2018 |
| Muscular Dystrophy | Dystrophin, Lama2, LGMD2B | R | IV & EV | Lim et al. 2018 |
| Sickle Cell Disease (SCD) | HBB, HbS | R | EV | Demirci et al. 2019 |
| Hemophilia B | coagulator factor IX | R | IV | Stephens et al. 2019; Wang et al. 2019; Morishige et al. 2019 |
| Neuronal Seroid Lipofuccinosis | DNAJC5 | R | IV | Yao et al. 2017 |
| Cystic Fibrosis (CF) | CFTR | R | EV | Marangi et al. 2018 |
| CRYGC-Associated Cataract | CRYGC | R | IV | Wu et al. 2013 |
| Epidermolysis Bullosa | COL7A1 | R | EV | Bonafont et al. 2019 |
| Parkinson's | Mitochondrial p13 | M | EV | Safari et al. 2019 |
| Retinal Pigmentosa (RP) | Rhodopsin | R | IV | Peng et al. 2017 |
| COQ Associated Mitochondrial Disorder | COQ | R | EV | Romero et al. 2017 |
| OTC Associated Metabolic Liver Disease | OTC | R | IV | Yang et al. 2016 |
| Primary Ciliary Dyskinesia (PCD) | TTC25 | M | IV | Wallmeier et al. 2016 |
| Familial Mediterranean Fever (FMF) | MEFV | R | EV | Alimov et al. 2019 |
| High-grade serous carcinoma (HGSC) | Trp53, Brca2 | M | EV | Walton et al. 2017 |

The first clinical trials involving CRISPR-Cas9 were initiated in 2016 and have continued to date. Excitingly, a recently approved clinical trial (ClinicalTrials.gov: NCT03655678) uses CRISPR-Cas9-modified therapeutic human hematopoietic stem cells (hHSCs), CTX001, to treat□□-thalassemia. CRISPR-Cas9 technology can transfer tumor-specific expression signals to cytotoxic immune cells and remove immunosuppressive genes from these cells that are re-transplanted to patients for cancer treatment. The therapeutic potential of CRISPR is being investigated by clinical trials (ClinicalTrials.gov: NCT02793856, NCT03044743, NCT03398967, and NCT03081715).

Despite significant ethical and legal issues, significant progress has been made in the use of CRISPR-Cas9 technology to correct pathogenic gene mutations in the human embryo. The CRISPR-Cas9 gene editing helped to repair MYBPC3 mutations associated with hypertrophic cardiomyopathy in the human embryo with a targeting efficiency of 72.2% and showed no genetic differences, non-target gene mutation, and no other abnormalities in the embryos (Ma et al. 2017).

Numerous clinical studies have been initiated on the design and biosecurity of PD1 knockout T cells using CRISPR-Cas9 technology in lung cancer patients (ClinicalTrials.gov: NCT02793856) and esophageal cancer patients (ClinicalTrials.gov: NCT03081715). PD1 gene knockout can also be combined with TCR modification and CAR-T cell therapy to prevent the development of a new tumor. For example, research into the effects of CRISPR-Cas9-mediated PD1- and TCR-knockout anti-mesothelin CAR-T cells continues for positive solid tumors (ClinicalTrials.gov: NCT03545815). Baylor School of Medicine (USA) and the Chinese People's Liberation Army (PLA) General Hospital have initiated other clinical trials to investigate the responses of T cell and B cell malignancies to CRISPR-Cas9-modified CAR-T cells (ClinicalTrials.gov: NCT03655678, NCT03745287, NCT037372832, and NCT037372832).

The last two articles describing the emergence of undesirable mutations in human cells regulated by CRISPR-Cas9 have received great interest and serious debate about the safety of this technology. In these publications, a group of Karolinska Institute in Sweden and Novartis reported that in human cells, CRISPR-Cas9-mediated modification of the target region preferentially occurs in defective cells in the p53 pathway. Therefore, it has been reported that if any of the mutated cells are used for therapeutic purposes, the risk of cancer may increase for the patient (Haapaniemi et al., 2018; Ihry et al., 2018).

The development of inhibitors or activators to increase the effectiveness of CRISPR-Cas technology will accelerate clinical trials. Also, social and ethical concerns about the implementation of CRISPR-Cas9 technology have attracted public attention. The world's first CRISPR-created Chinese twin babies caused ethical confusion (Normile, 2018). Whether human genomes can be altered in somatic cells or embryos to suit their needs is a controversial issue. For example, it is thought that due to undetected non-target effects that have not been studied extensively, it will completely alter the human genome from generation to generation and even lead to disastrous consequences. Concerns also include

those related to the mental health and social behavior of infants. To eliminate these drawbacks, major advances have been made in reducing the off-target effects of customized CRISPR genetic modifications through techniques such as artificial intelligence and machine-deep learning developed recently.

**Potential limitations and future directions of the CRISPR/Cas system in medical applications**

CRISPR-Cas applications continue to carry the current risk of side effects. In particular, situations such as the risk of creating non-target mutations and the risk of intra-chromosomal or inter-chromosome changes that can affect human health significantly cause CRISPR-Cas applications to develop slowly in the field of medicine for now. However, rapidly developing technologies in the field of CRISPR-Cas and high-budget project application programs to reduce these side effects give the impression that these risks may be eliminated in the short term. Especially by combining the field of artificial intelligence with genetic science, more effective and reliable CRISPR-Cas applications can reach a level that can be applied in the future even on the embryo (Tastan, 2019). With CRISPR-Cas applications, we anticipate that many gene therapy drugs will enter the market by 2025.

**CRISPR-Cas applications in animals**

As the human genome mapping technology progresses, the number of combinations (caused by mutations in various genes) is being tried to be identified, responsible for increasingly complex growing conditions. As in CRISPR, organisms may have multiple mutations at the same time. CRISPR-Cas can be used tissue-specific in oncological studies (Dow, 2015). This technology reduces the number of animals used, which provides a great advantage in terms of obtaining short-term embryonic stem cells (Boodman, 2015). The main uses of genetically modified (GM) animals are particularly in medical research, such as laboratory animals or disease models.

Mice, rats, zebrafish, and rabbits are commonly used as experimental animals. In some areas of research, particularly in neuroscience (such as autism, Alzheimer's, and schizophrenia), primates may be preferred to rats or mice as laboratory animals. The reason for this preference is not only because of their genetic similarity to humans but also because of the similarities in their behavior in response to the diseases revealed (Cyranovski 2014; 2016).

Although GM primates were before CRISPR-Cas (first transgene ape, 2001), advances in this area have progressed very slowly due to technical and ethical objections. A variety of primate species have been successfully modified using genome editing techniques. By creating GM Java and Rhesus monkeys in flexible countries in terms of legal legislation, it is possible to identify autism-related mutations in humans and study the behavioral characteristics of autism (Niu et al., 2014; Liu et al., 2014).

The application of CRISPR-Cas technology in the development of cellular and animal models of human diseases has accelerated. Making a desired genetic modification depends on the design of RNA molecules that direct the endonucleases to an appropriate genomic location. The discovery of CRISPR-Cas9 technology provides researchers with an advantage (high accuracy) for genome editing over alternative methods (Zarei et al., 2019). Using this technology, it is possible to construct cellular and animal models of diseases such as Huntington's disease, cardiovascular diseases, and cancer. Optimized CRISPR-Cas9 technology will facilitate access to important new cellular and animal genetic models related to innovative drug discovery and the development of gene therapy.

Gene drivers (faster propagation of traits in natural populations) allow genetic modifications to spread rapidly in sexual reproductive populations. In classical heredity, a heterozygous parent has a 50% chance of transferring a modified gene to her offspring. In gene-drive inheritance, almost 100% of the offspring inherit the changed genes. In a locus targeted by the gRNA in CRISPR-Cas-based gene driver transport, Cas and gRNA are located adjacent to each other. If this cassette is present on one chromosome, Cas9 activity can cause the gene driver to be copied to the other chromosome, increase the inheritance rate, and rapidly spread to the population.

Genome regulation in livestock can be used as an enriched method to improve disease resistance, production, or breeding and to develop new biomedical models (Proudfoot et al. 2015). These techniques can be used to improve the yield characteristics such as meat, milk, eggs, wool, and skin, increase the quality, resistance, adaptability to the environment, improve fertility yield, increase the rate of feed utilization, eliminate the factors that cause predisposition, treatment of hereditary diseases or prevent the transfer to offspring as an alternative to classical breeding methods that require a long process.

The first attempt of the CRISPR-Cas9 technique in cattle was performed by Choi et al. (2015). It was determined that embryos can develop up to the 40th day. Heo et al. (2015) used different transfer methods for the transfer of the CRISPR-Cas9 system to an embryo and pluripotent cells. Bevacqua et al. (2016) designed five different guide RNAs on PRPN (prion protein) by using the CRISPR-Cas9 technique, these RNAs were transferred with Cas9 to somatic cells at two different concentration levels for each gRNA. Gao et al. (2017) provided a single Cas9 nickase (Cas9 mutant causing single-chain fracture) and NRAMP1 (natural resistance-associated macrophage protein-1) gene in the bovine genome. It was observed that 11 calves who lived more than three months out of 20 calves had NRAMP1 gene heterozygote and were more resistant to tuberculosis compared to the control group.

The first study in goats is CRISPR-Cas9 mediated gene silencing application on four genes separately by Ni et al. (2014). Wang et al. (2015) produced one or both goat's modified genes. The results of the study showed that the CRISPR-Cas9 system is an effective gene engineering technique in livestock and therefore can be an important and applicable tool in animal breeding. Wang et al. (2016a) examined the effect of gene silencing and FGF5 gene on hair length in goats. FGF5 was silenced by the

CRISPR-Cas9 technique and a significant increase in the number of secondary follicles and hair lengths was detected. It has been shown that the modification can be passed on to subsequent generations.

Embryo development in sheep (Brooks et al., 2015), gene regulation in zygotes (Crispo et al., 2015), and the effects of β-carotene on the color of yellow fat tissue in sheep (Niu et al., 2016) were studied.

Wang et al. (2016b) have successfully used gene targeting technology in sheep by injecting gRNAs targeting Cas9 mRNA and three genes (MSTN, ASIP, and BCO2) into single-cell stage embryos. The study and the findings show that the CRISPR-Cas9 method can be used as a powerful tool for increasing animal production by targeting multiple genes responsible for economically important features at the same time.

Wu et al. (2016) select the highest activity gRNA and transferred it to the single-cell sheep embryos via the plasmid. Thirty healthy embryos from a total of 35 embryos were transferred to mothers and eight offspring were obtained. One of these offspring was reported to indicate the targeted mutation. Zhang et al. (2017) in the study of ovulation rate and offspring size of the designed gRNAs were transferred to 88 single-cell embryos, mutations occurred in 33 embryos, 12 of 33 embryos were found to be homozygous and 21 of them heterozygous. As a result of the studies, it was reported that no off-target effects were observed.

CRISPR-Cas9 gene-editing technique is used for the first time in chicken Véron et al. (2015). Oishi et al. (2016), gene silencing was performed with CRISPR-Cas9 in the genes responsible for the synthesis of potential allergen proteins in egg white.

However, some factors are limiting the use of CRISPR in plants and animals. It should not be ignored that the transferred CRISPR-Cas9 system may cause undesirable effects in different regions of the genome, various diseases, cancer, damage of regulatory regions of the genes, and the effects of genes adjacent to the target region. In the future, it is aimed to develop new methods to increase the target specificity and to reduce the non-target effect with the studies to be performed on this technique (O'Geen et al. 2015). If the system is based on DNA damage, if there is any deficiency or defect in DNA repair mechanisms, it will harm the organism instead of benefiting it.

**CRISPR-Cas applications in plants**

New breeding techniques include new technologies that allow only targeted regions to be regulated in the DNA of a plant, animal, and microorganisms. CRISPR-Cas techniques have been used more extensively to regulate plant genes compared to other genomic techniques, and are preferred for their ease of use. Therefore, in 2013, three independent groups used the CRISPR-Cas system for the first time in paddy, wheat, *Nicotiana benthamiana,* and *Arabidopsis thaliana* plants. The CRISPR-Cas9 gene regulation method has been studied to date in approximately 20 different plant species.

Today, products produced using CRISPR-Cas technology include tomatoes, white button mushrooms, paddy, citrus fruits, wheat, and cocoa plants. In the field of food and agriculture, using CRISPR-Cas technology, higher yields can be obtained from the unit area of fruits and vegetables. For example, for the suitability of plants, production of larger fruits that can ripen at the same time and products containing higher levels of vitamin C, cultivation of diseases and salt-tolerant plants, and the production of fruit and vegetables that are better attached to the stems can be given. The number of studies related to the use of this technology in agricultural production is also increasing. Most of the published articles are conceptual studies and mostly positive results are reported. Even there are studies aimed at application and sometimes commercialized products are encountered. By using CRISPR-Cas, different genes were targeted in many plant species such as soy, corn, tomato, paddy, sorghum, edible fungus, and orange (Table 2).

The details of some of these studies are given below.

- The CRISPR-Cas system has been successfully used for genome editing in rice. Wang et al. (2017) produced deletions in the dense and erect panicle1 (DEP1) gene with CRISPR sgRNAs designed to increase yield in paddy plants. In mutant plants, plant lengths were shortened and they obtained more steep and dense clusters of flowers.
- The application of CRISPR-Cas9 technology in paddy was made to increase product yield and quality. The high sensitivity of the paddy to negative environmental factors has enabled CRISPR-Cas9 technology to work on paddy rice to produce products resistant to various stress factors. New paddy cultivars have been produced as a result of mutations in the gene region controlling Abscisic acid perception, a plant growth regulator that affects plant growth and reactions to stress factors. Mutation clusters in the specific gene region resulted in a 25-31% yield increase. Bacterial blight disease caused by *Xanthomonas oryzae* in rice is a common problem. The deletion of the OsSWEET13 promoter has been shown to produce plants resistant to this disease (Jaganathan et al., 2018).
- In a study conducted in China, mutations were made in the GmFT2a gene, which integrates the photoperiod flowering pathway in soybean plants using the CRISPR-Cas system. Mutant soy plants increased vegetative growth by late flowering and this feature was transferred to future generations stably (Cai et al., 2017).
- In a watermelon study, the CIPDS gene encoding the phytoene desaturase enzyme with CRISPR-Cas was targeted and ultimately yielded albino mutant plants (Tian et al., 2016). This study showed that the CRISPR-Cas9 system will be used successfully in watermelon breeding programs.

- The CRISPR-Cas system is used for *Xanthomonas citri* subsp. bacteria resistant orange lines were used to develop. Researchers have targeted lines of increased resistance to this disease by targeting the promoter of the CsLOB1 gene, which provides susceptibility to cancer in orange (Peng et al., 2017).
- Soyk et al. (2017) used the CRISPR-Cas9 system to change the response of the tomato plant to photoperiod. To this end, they have produced various mutations in the SELF-PRUNING5G (SP5G) gene, which suppresses flowering. These mutations with CRISPR-Cas9 resulted in rapid flowering and compact growth habitus in tomato plants, resulting in early yields.
- Virus production is one of the most important problems in crop production and it is often impossible to combat. The most effective way to combat viruses that cause huge losses in production every year is to breed resistant plants. Most of the most common viral infections encountered in plant production worldwide are caused by single-chain Gemini viruses. The CRISPR-Cas9 system has recently been designed to give resistance to Gemini viruses in plants. Six different regions of the genome of the bean yellow dwarf virus (BeYDV) were targeted using the CRISPR-Cas9 system containing modified sgRNA to reduce Geminivirus replication. Thus, significant reductions in the number of copies of the BeYDV virus were observed in the genetically modified tobacco plant (Baltes et al., 2015). This technique, which is applied for the first time on Gemini viruses, can allow different sgRNAs to be collected in a single transgene and several nuclease enzymes can be directed to multiple viruses.

Ji et al. (2018) created two virus-inducible CRISPR-Cas vectors, and sugar beet peak curl virus (BSCTV) was effectively inhibited in Arabidopsis thaliana and Nicotiana benthamiana plants. Deep sequencing studies on Arabidopsis showed no off-target mutations.

- In another study, using the CRISPR-Cas system, the molecular immunity of Nicotiana benthamiana against tomato yellow curl virus (TYLCV) was increased (Ali et al. 2015). The designed sgRNAs are designed specifically for encoded and non-encoded sequences of TYLCV. SgRNAs targeting the stem-loop sequence on the origin of replication in the intergenic region of the virus genome showed a more potent effect. Thus, the virus genome was successfully intervened and the number of viral DNA copies in the plant was reduced.
- Potyviruses with single-chain RNA also cause significant losses in plant production. In the Arabidopsis plant, CRISPR-Cas9 technology has been applied to develop a genetic resistance to the radish mosaic virus (TuMV). Resistance to TuMV was achieved by the eIF (iso) 4E deletion of the host factor, which is necessary for the survival of the virus. Segregation of the CRISPR-Cas9 transgene mutation yielded TuMY resistant plants that did not carry the transgene in the T2 generation (Pyott et al., 2016).

- Soybean oil, which contains high oleic acid, was the first food product developed in the US with commercially approved gene regulation technology. In this study, by the use of the TALEN technique, fatty acid desaturase (FAD2-1A and FAD2-1B) genes were mutated and the oleic acid content in the oil was increased to over 80% and linoleic acid content was reduced to less than 3% (Demorest et al., 2016).
- Another study conducted with CRISPR technology targeted the ALS gene encoding Acetolactase synthase enzyme in paddy. Since ALS is the target of herbicides such as chlorsulfuron and Bispyribac-Sodium (BS), changes in this gene by homologous recombination have resulted in herbicide-resistant rice plants (Sun et al., 2016).
- Nitrogen fixation and nitrogen flow are other environmental problems related to agriculture. Plants cannot directly absorb most of the different nitrogen compounds in the soil. Legumes, such as beans and peas, bind-free nitrogen in the air to nodules in plant roots, linking bacteria that help make soil nitrogen more suitable for the plant. For this purpose, CRISPR technology is used for nitrogen fixation (Nicholas et al. 2015).
- Tomato is one of the most successful examples of foods with CRISPR technology. New tomato varieties have been developed from wild tomato (*Solanum pimpinellifolium*), a type of Cherry tomato. With CRISPR-Cas9 technology, a 3-fold increase in tomato size, a 10-fold increase in the number of surviving plants, and a 500% increase in lycopene (carotene pigment) deposition in fruit (Zsögön et al., 2018).
- In Turkey, ground cherry (*Physalis pruinosa*), known as golden berries, ground cherry, or southern lantern, increased the size of the plant and fruit and the number of fruit (Chen et al., 2019).
- TcNPR3 gene is blocked by CRISPR-Cas9 technique and used for resistance to disease caused by *Phytophthora tropicalis* which is seen to destroy the generation of cocoa trees (Fister et al., 2018).

This method has been used extensively to develop various properties in plants and commercial products have been put on the market.

**Table 2.** Plants obtained using CRISPR-Cas9 (Karkute et al., 2017)

| **Species** | **Targeted genes** | **Result** | **References** |
|---|---|---|---|
| Citrus | LOB1 | Orange Canker disease resistant | Peng et al., 2017 |
| Glycine max | ALS | Herbicide tolerance | Li et al., 2015 |
| Soya | Drb2a, Drb2b | Drought and salt tolerant soy | Curtin et al., 2018 |
| Solanum lycopersicum | SP5G | Rapid flowering and early crop formation | Soyk et al., 2017 |
| Solanum pimpinellifolium | SP5G, SP, CLV3, WUS | Flowering and day length neutral, high yield and compact fruit wild tomatoes | Li et al., 2018 |
| Oryza sativa | ERF922 | Disease resistance | Wang et al., 2016 |
| | Gn1a, GS3, DEP1 | Increased grain size and number, short and erect flower structure | Li et al., 2016 |
| | NRT1.1B, SLR1 | Increase in nitrogen utilization and shortening of plant height | Lu et al., 2017 |
| | ALS1 | Herbicide resistance | Sun et al., 2016 |
| | TMS5 | Temperature-sensitive line for hybrid paddy production | Zhou et al., 2016 |
| | CSA | Development of light-sensitive male-sterile paddy line | Li et al., 2016 |
| | Waxy | Glutinous rice | Ma et al., 2015 |
| | SWEET13 | Resistance to bacterial blight disease | Zhou et al., 2015 |
| | SaF, SaM, OgTPR1 | Neutral alleles and hybrid-compatible rice | Xie et al., 2017a. Xie et al., 2017b |
| Sorghum bicolor | alpha-kafirin genes | Sorghum with increased lysine content and digestibility | Li et al., 2018 |
| Triticum aestivuml | GASR7 | Grain weight | Zhang et al., 2016 |
| Zea mays | Wx1 | High Amylopectin-containing corn | Waltz E., 2016 |
| | ARGOS8 | High grain yield corn in drought conditions | Shi et al., 2017 |
| | MS26, MS45 | Male sterile plant production | Svitashev et al., 2015 |
| | ALS | Herbicide resistance | Svitashev et al., 2015 |
| | TMS5 | Temperature-sensitive line for producing hybrid maize | Li et al., 2017 |

The task of plant breeders in the development of new varieties can adapt to climate and soil conditions, superior yield and quality, disease and pest resistant varieties to find out and remove the

existing varieties in this direction. For this purpose, breeders benefit from the existing variations in nature and new techniques and methods they have developed. Nowadays, many varieties have been developed by traditional breeding methods and presented to the service of agriculture. However, these methods require a long time, extra labor, and resources to develop varieties. Therefore, plant breeders are focusing on new approaches that will enable faster and easier variation. One of these breeding methods is mutation breeding (Başer et al. 2007). Mutation breeding in plants can develop varieties resistant or tolerant to biotic and abiotic stress factors. A mutation is a permanent change in the genetic structure of the organism, which can occur in both reproductive and somatic cells. Somatic mutations are very important in obtaining mutant individuals, especially in vegetative propagating plants. Because somatic cells are mutated and the organs that develop from here become mutant. Numerous studies have been conducted to promote somatic mutations with various mutagens in plants and to achieve the desired properties (Griffiths 1996; Franks et al. 2002; De Oliveira Collet et al. 2005). The frequency of spontaneous occurrence of desired mutations in plants is very low. Therefore, many breeding studies have used physical or chemical mutagens to increase the frequency of mutations (Forster et al. 2011; Spencer-Lopes et al. 2018). The use of various mutagens increases the frequency of naturally occurring mutations. For this reason, more than 3300 mutant varieties from 232 different plants have emerged in more than 70 countries since 1950 and the food safety of the developed varieties has been recorded by FAO / IAEA (Atomic Energy Agency of the United Nations) (Oladosu et al. 2016; Yamaguchi 2018; IAEA 2019).

The mutation promoted by mutagens accelerated the development of varieties with desired properties in plants. However, the use of mutagen can also cause numerous random mutations in the genome. When a mutation for the intended property is promoted, it is necessary to select beneficial mutants from the plant population. Although many varieties have been developed with this technique, selection in mutation breeding takes a certain amount of time and undesirable properties need to be eliminated by repeated selection cycles.

In recent years, genome-editing techniques have been developed that produce beneficial mutations that provide directed (or targeted) mutation to the site of the promoted mutation. CRISPR-Cas is one of these techniques, with the use of which it is possible to interrupt the genome in a particular region and to insert, remove, or modify another gene. As a result, the desired changes can be made to the targeted DNA using this technique.

Mutations in plants can result in single or double chain fractures (DSB) or base damage. Double chain fractures cause genotoxic events and cell death by causing chromosome fractures and changes. For this reason, the repair of double chain fractures and correct repair is important for the protection of the genome. There are two ways of repairing the resulting double chain fractures: homologous recombination and non-homologous end junction. In mutations, DSBs are randomly induced in the

genome and error-prone repair produces very rarely desired phenotypes (Zhao and Wolt, 2017; Pacher 2017). New gene-editing technologies, such as CRISPR-Cas, allow fast and highly sensitive gene regulation using the natural DNA repair mechanisms of plants. In principle, it is equivalent to those produced by random mutagenesis with mutations from CRISPR-Cas9 (Liu 2017; Yuan 2019). These mutations, which are targeted, compared to random mutations, are preferred because they are better defined, have a low risk of non-target effects, and are suitable for their costs (Abdelrahman, 2018). Targeted mutations in various plants have also been shown to increase genetic diversity (Yuan 2019; Zaidi et al., 2019).

The removal of negative elements in the plant genome can be considered as a promising strategy for plant breeding. Therefore, the knock-out (silencing) of genes encoding unwanted features is the easiest and most common application of the CRISPR-Cas system. To date, improved properties include yield, quality, and resistance to biotic and abiotic stresses. Many stages of crop production and hybrid breeding techniques have improved with this method (Chen et al. 2019). The speed of hybrid breeding programs has been increased with genome editing techniques and shorter cultivation time has been shortened. CRISPR-Cas-induced gene silencing method has been developed for paddy rice (tm5 lines, Zhou et al. 2016) and light-sensitive (CSA lines, Li et al. 2016) male sterile lines and is used in hybrid breeding programs. If CRISPR-Cas technology contains foreign DNA in the products obtained, it should be subject to GM regulations in terms of legislation (Zhao & Wolt, 2017).

Furthermore, productivity enhancement studies on plants using CRISPR-Cas genome editing technology have begun to have a tremendous impact in the field of the food supply. To improve food processing (eating and cooking) technology, waxy paddy rice with low amylose content was produced by transporting 3 genes (GW2, GW5, and TGW6) using CRISPR-Cas9, which has been reported to cause a significant incremental increase in grain weight (Xu et al., 2016).

Preservation of food and waste is one of the most important problems. One of these problems is the early blackening of food flasks. CRISPR technology has been used for this purpose and it has achieved great success in preventing the darkening of harvested edible mushrooms (Waltz, 2016). CRISPR-Cas9 technology in edible mushrooms is designed to prevent the mushrooms from darkening quickly and to extend their shelf life. To prevent tarnishing, an extraction process targeting the gene region of polyphenol oxidases (PPO) was performed. The study showed that the successful overhauling of one of the six PPO genes reduced blackening activity by 30%. Mushrooms produced with this application are permitted to be commercially marketed by the US Department of Agriculture (Waltz, 2016).

It is aimed to produce gluten-free wheat with CRISPR-Cas9 technology to enable people to consume wheat varieties without showing immunoreactivity due to Celiac disease in wheat which is an important food for human nutrition. This is done by altering the α-gliadin gene, the main family of

gluten-encoding genes in wheat. In these studies, 35 mutations of 45 genes from wild wheat species were produced to produce low gluten wheat types without transgene and an 85% decrease in immunoreactivity was observed.

As is known, maize is a product in which transgenic technologies are used substantially. By using the CRISPR technique, it is aimed to obtain pest resistant, herbicide-tolerant, and high yield non-transgenic genotypes. Also, positive results were obtained in important quality parameters by using this technology in the corn plant. For example, the phosphorus element is bound in the form of phytic acid in corn seed (more than 70%); thus, it cannot be digested by monogastric animals and excreted in feces. Therefore, it is an environmental pollutant in areas where animal husbandry is concentrated. In some studies, the genes responsible for phytic acid synthesis in maize (ZmIPK1A, ZmIPK, and ZmMRP4) have been reported to be destroyed by CRISPR. In another study, it has been shown that the application of the gene promoter of SDN3 to Argos8 by the CRISPR technique in maize causes an increase in maize yield during drought stress.

**CRISPR-Cas applications in microorganisms**

CRISPR, a new gene regulation technique in the world, has many kinds of research and applications on the potential to treat or prevent human diseases. However, medicine is not the only science that CRISPR opens. This powerful genetic engineering tool also helps scientists develop new technologies to protect or improve the environment from human damage, and to improve the quality and quantity of agricultural products. When environmental science is considered, CRISPR has emerged as a new method in the issues summarized below.

a. Biofuel production: plants, algae, and cyanobacteria naturally convert carbon dioxide and sunlight into by-products. The sugars, fats, or alcohols produced in this way are all potential alternative fuel sources. Scientists have shown that CRISPR can be used effectively in the production of some important biofuel products in some cyanobacterial species (Wendt, 2016) and algae (Nymark et al., 2016). Bacteria can contribute to biofuel production by degrading plant cell walls as a result of their metabolic functions. Besides, some species can produce some biofuel precursors from waste products in waste, such as methane production. For example, the CRISPR system is applied to bacteria capable of producing biofuels naturally, yielding effective results (Nagaraju et al. 2016). These biofuel precursors enable the bacteria to grow together in the bioreactor (Liao, 2016). CRISPR is an important tool in biofuel production due to its precise and easily applicable properties.

b. Bioplastics: Biofuels are not the only petroleum-based products that can be replaced by synthetic biology. Today, it is very important in the world and an environmental problem is oil-derived plastics. Recent studies have shown that some yeasts and bacteria can naturally produce plastic-like

substances. The CRISPR system is also known to contribute to the production and extraction of these substances (Luengo et al., 2003).

c. Bioremediation: Microorganisms can be designed to assist in the production of plastic-like materials as well as in the biodegradation of plastics. As is known, some species of bacteria and fungi can break down plastics naturally (Karl and Travis, 2012). CRISPR can be used to increase the activity of genetic pathways in this regard (Holkenbrink et al. 2018). There are many areas where the CRISPR technique can be applied to biological improvements. One of them can be used to purify heavy metals, pesticides, and soil contaminating compounds and purify water (Dvorak et al. 2017).

d. Biosensors: The biosensor function is used in conjunction with bioremediation applications. In bio-detection, the presence of specific molecules can be detected with probes and sensors. Plants and microorganisms respond to the chemicals in their environment with the help of natural biosensors. This detection and response function can be shaped and improved by genetic engineering. In this regard, plants can be used to detect and locate biological weapons and pathogens. With the CRISPR technique, the complex set of functions for detecting and locating such signals has become easily applicable (Cook et al. 2014).

CRISPR-Cas9 / Cas12a in bacteria is a well-studied CRISPR nuclease designed and optimized for a wide range of applications, particularly in the fields of bioengineering and synthetic biology. As cell plants, bacteria can use simple and inexpensive raw materials such as renewable biomass and even waste for the basic cell metabolism and biosynthesis of high value-added chemicals. Although conventional and molecular methods based on recombination are available, they are time-consuming and labor-intensive. Currently, CRISPR-Cas9 / Cas12a based biotechnology has greatly facilitated genetic manipulation on the model and non-model bacteria for high regulatory efficiency and specificity (Mougiakos et al. 2018). Table 3 presents examples of CRISPR biotechnology in some bacteria (Yao et al. 2018).

Bacteria are predominantly adapted to human beings and animals due to their life desires and cause disease, especially in warm-blooded people. Although they cause disease in plants, the number of plant pathogenic fungi is quite low. Since the discovery of Fleming's antibiotics, antibiotics have been the most effective means of combating bacteria and treating the diseases they cause. Unconscious and widespread use of antibiotics has brought with it the problem of resistance to antibiotics. For example, every year in the United States, 5 million people are infected by antibiotic-resistant bacteria. The use of antibiotics against plant bacterial diseases in many countries around the world is prohibited because it may cause resistance problems. One of the most important negative effects of excessive and incorrect antibiotic use today is that they eliminate beneficial microflora (prebiotics and probiotics) in the body. As a result, it brings other diseases. In recent studies, it has been shown that the antibiotic resistance

gene can be destroyed by the CRISPR-Cas technique in resistant bacterial populations (Pursey et al. 2018).

**Table 3.** CRISPR applications in bacteria (Yao et al. 2018)

| **Bacteria** | **Applications** |
|---|---|
| E. coli | uridine, adipic acid, β-carotene and isopropanol production |
| E. coli | production of lycopene, isoprene, 4-hydroxybutyrate, malate, butanol, naringen, malonyl-CoA, and mevalonate |
| Streptomyces coelicolor | Secondary metabolite production |
| S. ablus | Silent biosynthetic gene cluster activation |
| S. viridochromogenes | Silent biosynthetic gene cluster activation |
| S. lividans | Silent biosynthetic gene cluster activation |
| S. coelicolor | Secondary metabolite production |
| S. hygroscopicus | Production of 5-oxomylbemisine |
| S. rimosus | Oxytetracycline production |
| S. venezuelae | Silent biosynthetic gene cluster activation |
| Clastoridium tyrobutyricum | Butanol production |
| C. saccharoperbutylacetonicum | Butanol production |
| C. ljungdahlii | Ethanol production from synthetic gas |
| C. pasteurianum | Butanol production from waste glycerol |
| C. cellulolyticum | Biofuel production from lignocellulose rich biomass |
| C. cellulovorans | Solvent production (acetone, butanol and ethanol) |
| Corynebacterium glutamicum | γ–aminobutyric acid, 1,2-propanediol production |
| C. glutamicum | Glutamate production |
| C. glutamicum | Production of L-lysine, L-glutamate and homo-butyrate |
| Bacillus subtilis | Production of L-valine and β-cyclodextrin glycosyltransferase |
| Bacillus subtilis | Production of hyaluronic acid and N-acetylglycosamine |

Bikard et al. (2014) reported that it destroys virulent strains with the help of RNA-guided Cas9 nuclease given by the help of bacteriophage Staphylococcus aureus, an important human and animal pathogen, but it has no effect on non-virulent ones. As is known, there is no such selectivity in antibiotics. The researchers succeeded in reducing the population of S. aureus, which survived on the skin of a live mouse in vivo, from 50% to 11%. Similarly, Citorik et al. (2014) have demonstrated that resistant E. coli populations can be destroyed by targeting E. coli beta-lactam and quinolone resistance genes using plasmid and phagemid vector systems with the aid of RNA-driven nuclease targeting specific DNA sequences. The investigators have reported that this function works effectively in an in vivo infected Large Wax Moth (Galleria mellonella) model.

One of the most important issues to be emphasized here is that technology to remove bacterial populations carrying antimicrobial resistance genes with CRISPR-Cas technology can create several legal and social problems. It is reported that especially in the use of this technique, caution should be

exercised in terms of environmental problems, and some legal regulations and usage strategies are needed (Pursey et al. 2018).

The CRISPR technique can be used as a method of detecting pathogens in living things quickly, precisely, and reliably. For example, the CRISPR-Cas system has been used to detect specific strains of Zika and Dengue viruses in patients' body fluids. This RNA-targeted CRISPR-Cas13 enzyme has been successfully used to detect virus RNA and DNA in liquid biopsy specimens by isothermal amplification (Lau, 2018). Similarly, the dCas9 system was used to detect Mycobacterium tuberculosis DNA causing tuberculosis. Generally, sgRNAs designed to bind to two different sites in the pathogen DNA of dCas9-NFluc and dCas9-CFluc were used in one of the two fragmented parts of the gene encoding the dCas9 firefly luciferase enzyme (Fluc). When dCas9-NFluc and dCas9-CFluc bind close together in the target region, luciferase enzyme function is activated and bioluminescent signals have been detected (Lau, 2018).

Recent studies have shown that this system can be a universal nucleic acid detection method and even single base-containing mutations can be detected by considering Cas9 protein binding and truncation as a programmable RNA model (Zhou et al. 2018).

Fungal pathogens are filamentous microorganisms that cause disease in humans, animals, and predominantly plants, causing significant losses in post-harvest and stored products and foodstuffs. It is estimated that the losses caused by plant diseases caused by fungi are 30% (Munoz et al. 2019). The use of fungicides against diseases is one of the most effective means of struggle and carries important risks in terms of environment and human health. For this purpose, biocontrol agents and non-virulent strains of pathogens are used in biological control. These biocontrol agents can also help control diseases by activating the plant defense mechanism. The CRISPR-Cas technique for genome regulation is an effective alternative to combating diseases caused by fungi (Song et al. 2019). Table 4 summarizes the CRISPR techniques and the results obtained against fungal agents.

**Table 4.** Fungal pathogens and CRISPR applications (Song et al. 2019)

| **Species** | **Cas9 enzyme (selective marker, promoter)** | **Gene editing** | **Effective (%)** | **Reference** |
|---|---|---|---|---|
| T. reesei | Codon-optimized Cas9, ura5, pdc/cbh1 | NHEJ/HDR | 93/4.2–45 | Liu et al., 2015 |
| P. oryzae | Codon-optimized Cas9, Bar, tef1 | NHEJ/HDR | 36.1–80.5 | Arazoe et al., 2015 |
| Aspergillus | pFC332, pyrG/argB/hph/ble, gpdA | NHEJ | | Nodvig et al., 2015 |
| N. crassa | Codon-optimized Cas9, Bar, Trpc | NHEJ/HDR | | Matsu-Ura et al., 2015 |
| A. fumigatus | Human codon-optimized Cas9, hph/ble, amy | NHEJ | 25-53 | Fuller et al., 2015 |
| P. sojae | Human-optimized codons Cas9, G418, Ham34 | NHEJ/HDR | | Fang and Tyler 2016 |
| U. maydis | Codon-optimized Cas9, ip, Otef (modified tef1)/hsp70, | NHEJ | 50-90 | Schuster et al., 2016 |
| A. fumigatus | Human codon-optimized Cas9, pyr4/hph, niiA/gpdA | HDR | 95-100 | Zhang et al., 2016 |
| A. oryzae | Codon-optimized Cas9, pyrG, amyB | NHEJ | 10–20 | Katayama et al., 2016 |
| P. chrysogenum | Human codon-optimized Cas9, amdS, xlnA | NHEJ/HDR | 100 | Pohl et al., 2016 |
| C. albicans | Codon-optimized Cas9, Nat, ENO1 | NHEJ/HDR | | Min et al., 2016 |
| A. fumigatus | pFC332, pyrG, TetON | NHEJ/HDR | | Weber et al., 2017 |
| A. niger | pFC332, pyrG/hph, tef1 | NHEJ/HDR | 37.5–100 | Kuivanen et al., 2016 |
| U. maydis | Codon-optimized Cas9, ip, Otef (modified tef1)/hsp70 | NHEJ | 50–90 | Schuster et al., 2018 |
| M. thermophila | Codon-optimized Cas9, Bar, tef1 | NHEJ/HDR | 15–95 | Liu et al., 2017 |
| T. atroroseus | pFC330, hph, tef1 | NHEJ | | Nielsen et al., 2017 |
| A. carbonarius | pFC332, hph, tef1, | NHEJ/HDR | 27 | Weyda et al., 2017 |
| A. niger | pFC332, hph, tef1 | NHEJ | | Kuivanen et al., 2017 |
| Ganoderma lucidum | Codon-optimized Cas9, ura3, gpdA | NHEJ | | Qin et al., 2017 |
| B. bassiana | Codon-optimized Cas9, gfp/ura5/bar, gpdA | NHEJ/HDR | 5–50 | Chen et al., 2017 |
| A. alternata | pFC332, pyr4/hph, gpdA | NHEJ | | Wenderoth et al., 2017 |
| S. bambusicola | Codon-optimizedCas9, hph, TrpC | NHEJ | | Deng et al., 2017a |
| S. bambusicola | Codon-optimizedCas9, hph, TrpC | NHEJ/HDR | 32 | Deng et al., 2017b |
| Nodulisporium sp. | Codon-optimized Cas9, Bar, TrpC | NHEJ/HDR | | Zheng et al., 2017 |

| | | | | |
|---|---|---|---|---|
| Mucor circinelloides | SpCas9, pyr4 | NHEJ | 100 | Nagy et al., 2017 |
| Fusarium graminearum | Codon-optimized Cas9, fludioxonil, gpdA | NHEJ/HDR | 1–10 | Gardiner and Kazan 2018 |
| Leptosphaeria maculans | Human codon-optimized Cas9, Ip/G418/hph, act1 | NHEJ | | Idnurm et al., 2017 |
| A. niger | pFC332, hph, tef1 | NHEJ | | Kuivanen and Richard 2018 |
| C. albicans | SpCas9, Nat/Phloxine B | NHEJ | 53–98 | Shapiro et al., 2018 |
| Aspergilli | pFC332, argB/pyrG, tef1 | HDR | 15–90 | Nodvig et al., 2018 |
| B. dermatitidis | pFC332, hph, tef1 | NHEJ | 22–73 | Kujoth et al., 2018 |
| A. niger | Codon-optimized Cas9, hph/amdS, glaA | NHEL/HDR | 33.3–100 | Zheng et al., 2018 |
| C. albicans | Codon-optimized Cas9, Nat, ENO1 | HDR | 25–100 | Vyas et al., 2018 |
| P. sojae | Human-optimized codons Cas9, G418, Ham34 | NHEJ | | Miao et al., 2018 |
| F. oxysporum | pFC332, hph | NHEJ/HDR | 20–53.8 | Wang et al., 2018 |
| Ustilaginoidea virens | Codon-optimized Cas9, G418, pdc/cbh1 | NHEJ | 60-90 | Liang et al., 2018 |
| Cordyceps militaris | Codon-optimized Cas9, 5-FOA/blpR, tef1 | NHEJ/HDR | 87.2–84.3 | Chen et al., 2018 |
| Cryptococcus neoformans | Codon-optimized Cas9, Ntc, tef1 | HDR | 96.5–100 | Wang 2018 |
| Sclerotinia sclerotiorum | Codon-optimized Cas9, hph, tef1 | NHEJ/HDR | 38–100 | Li et al., 2018 |
| Aspergillus novofumigatus | pFC332, pyrG, tef1 | NHEJ | | Matsuda et al., 2018 |
| A. niger | pFC332, argB/pyrG, tef1 | HDR | 100 | Leynaud-Kieffer et al., 2019 |
| A. oryzae | Codon-optimized Cas9, pyrG, amyB | HDR | 50–100 | Katayama et al., 2019 |
| A. alternata | pFC332, hph, tef1 | NHEJ | | Igbalajobi et al., 2019 |
| A. niger | Codon-optimized Cas9, pyrG | NHEJ/HDR | 100 | Kuivanen et al., 2019 |
| M.r circinelloides | SpCas9, pyr4 | HDR | 100 | Nagy et al., 2019 |
| L. maculans | Human codon-optimized Cas9, hph, act1 | NHEJ | | Darma et al., 2019 |
| A. gossypii | Human-optimized codons Cas9, G418, tef1 | NHEJ | 44–85 | Jiménez et al., 2019 |
| Duddingtonia flagrans | pFC332, hph, tef1 | NHEJ | | Loubna et al., 2019 |

In the world, 18% of the losses in agricultural products are caused by animal pests, 16% by microbial diseases (70-80% of which are fungal diseases), and 34% by weeds (Moore et al. 2019). Weeds rank first, followed by animal pests. The most effective struggle against both groups is carried out with the use of pesticides and herbicides, and today, for this purpose GM plant species are widely used for commercial purposes. However, GM plant and plant breeding are subject to legal regulations in many countries and GM plant cultivation is prohibited by law in our country. The emergence of resistant weeds (such as *Amaranthus palmeri*) and resistant insect populations in areas where GM varieties are grown, especially tolerant to herbicides and to combat cry proteins against certain pests, is a problem in agricultural production.

Animal or plant species transported from one region to another can damage natural ecosystems. There are several different genetic strategies to destroy invasive species (Harvey-Samuel et al. 2017). One of these is gene drivers in which a gene that reduces adaptation to nature is spread to the population, and some CRISPR-based gene regulation strategies are used for this purpose (Mac-Farlane et al. 2017).

As is known, in the sexual reproductive system of eukaryotic organisms, a set of chromosomes is provided from each parent and assembled into new progeny. If one of the chromosome sets contains a "gene driver", it cuts the homologous chromosome that lacks the gene driver and copies itself into this chromosome. In this way, a genetic system is created which is capable of sustaining gene drivers themselves and nearby genes for many generations. For example, in normal sexual reproduction, progeny carry two alleles of each gene and have an equal chance of spreading into progeny. Gene drivers thus ensure that this inheritance is transmitted to all offspring. If this gene is a gene that suppresses fertility in females, the population is confronted with extinction over time. Studies have shown that a gene driver carried out by the CRISPR-Cas technique is rapidly transferred to offspring. This study showed that a 1% release was sufficient for a 99% change in the natural population after 9 generations.

In this study, Esvelt et al. (2014) emphasized the need to take some precautions, and Gantz and Bier (2015) introduced a series of measures that initiated discussions after the studies of the CRISPR-mediated gene drive technique with vinegar fly Drosophila melanogaster. In recent years, CRISPR employees have stated that the following precautions should be taken (Akbari et al. 2015).

**Legislation in CRISPR-Cas Applications**

Given the debate about CRISPR, the US National Academy of Sciences and the National Academy of Medicine have launched a new initiative to help politicians, researchers/clinicians, and the general public to understand the human gene regulation technology and its consequences to make rational decisions about this technology. An important part of this plan is the International Summit on Human Gene Regulation, organized jointly by the US Academies, the Chinese Academy of Sciences, and the Royal Society of England in December 2015.

The International Summit has brought together experts to discuss a variety of issues, including basic research, human germline regulation, legislation on methods, and national/international impacts. At the end of the program, the organizing committee issued a statement summarizing its decisions. This explanation divides human gene regulation into three categories: basic/preliminary clinical research, clinical somatic regulation, and clinical germline regulation, with different recommendations for each category.

The committee stated that basic/preclinical investigations on both somatic and germline regulation should only be continued with appropriate legal arrangements. These cells used in embryo/germline regulation should not be used to create a pregnancy. Ideally, this balance will enable researchers to better understand the underlying biology behind such changes. However, some scientists fear that it would be controversial to replace even non-implanted embryos, as seen in previous discussions of embryonic stem cell research. Although in vivo non-hereditary gene arrangements have been considered relatively favorable, clinical germline modifications are highly controversial. These concerns include not only health but also profound social and ethical issues. Some scientists do not advocate a complete ban on germline regulation, but a periodic review of the possibility of germline regulation as scientific knowledge and social views evolve. Issues that are of concern at the international level on the clinical aspects of human genome regulation;

- Unexpected effects of genetic regulation,
- Incorrect or incomplete regulation risks,
- Non-target deleterious mutations and other variants/reduction in the suitability of interaction with the environment,
- Irreversibility of genetic regulation in a population,
- Non-traceability when regulations enter the population (maybe negatively associated with reproduction),
- Effects on genetically modified individuals and future generations,
- The potential for permanent genetic improvement affecting the social structure.

Although some countries allow genome regulation research in early human embryos, it is known that such research is not permitted in research institutions in Canada. In contrast, Canadian researchers and politicians argue that regulations should be standardized in shaping international norms for responsible execution of human germline regulation and to ensure that such research improves human health and welfare globally (Bubela et al. 2017).

Clinical applications of genome regulation technologies are based on 30 years of preclinical research. This type of research is organized in the same way as all genetic studies. The most important

issue to be noted here is that the benefits of the research should outweigh the risks and the use of animals in preclinical research should be minimized. An example of an ongoing investigation is the use of CRISPR-Cas9 as a more effective method for producing genetically modified animal models, such as mice whose gene expression has been silenced to help us understand human diseases.

CRISPR-Cas technology is used in the food industry as well as in other fields. For example, it is a promising technology for the detection and destruction of food microbiology, providing resistance to pathogens and developing vaccines and antimicrobial agents against viruses. Besides, official control, monitoring, and health control of biotechnological products are very important. For the first time in this respect, food inspection strategies were determined by the International Food Biotechnology Council in 1990 and their practices continue to the present day. The World Health Organization (WHO) and the Food and Agriculture Organization (FAO), working together, have introduced world-recognized legal regulations for food safety in applications using recombinant DNA technology.

Today, it is controversial whether the legal regulations for products based on recombinant DNA technology will also apply to CRISPR-Cas technology. Some of the concerns in particular; overlooked DNA sequences due to errors in amplification or sequencing analysis, low reproducibility of CRISPR detection and validation assays, experimental variations in DNA sequences, inconsistencies in the alignment of different length insertions, and complex problems resulting from them.

The wheat grain contains gluten proteins responsible for the unique viscoelastic properties of wheat-derived foods. However, they also trigger some pathologies in sensitive individuals. Among these, the alpha-gliadin family is the main group of proteins associated with the development of celiac disease and non-coeliac gluten sensitivity, affecting more than 7% of the European population. In bread wheat, alpha-gliadins are encoded by many genes. Traditional mutagenesis and plant breeding have failed to achieve low immunogenic wheat varieties for celiac patients. There is ongoing work on new varieties of CRISPR-Cas technology, which reduce the amount of alpha-gliadin in the seed precisely and effectively.

New breeding technologies, such as CRISPR, are not covered by the definition of GM in legal regulations in many countries. In the US, the legal status of CRISPR-Cas-induced mutations is exempt from GM legislation. The US Department of Agriculture (USDA) has released the production and marketing of plants regulated by the CRISPR-Cas technique. To date, 5 products manufactured with CRISPR technology in the USA have been allowed to be manufactured without being subject to legal regulations. One of them is the commercial product obtained by silencing the gene causing darkening in edible fungi by the CRISPR-Cas technique. Amylopectin-rich corn, developed by inactivation of an endogenous gene, Wx1, was exempted from GM regulatory requirements. Similarly, the regulation of the drought-tolerant soybean produced by the inactivation of the Setaria viridis (known as hedgehog)

homology of the Zea mays ID1 gene by *S. viridis*, oil-enhanced ketchup plant produced by Yield10 Bioscience, and drb2b genes with late flowering time was also regulated.

In Europe, in October 2016, the French State Council asked the European Court of Justice whether CRISPR-Cas and other similar technologies should be considered under EU SE legislation. On 25 July 2018, the European Court of Justice decided that these technologies, including CRISPR, would be included in Decree 2001/18 and considered genetically modified organisms (GMOs). Discussions on this issue are ongoing.

In Australia, Japan, Norway, Israel, Argentina, Brazil, and some other countries, risk assessments of products obtained using CRISPR-Cas9 technology, known as gene regulation, are subject to legal regulations. The news that this technology can also be applied to human genetics in China has led to considerable controversy. It is also suggested that new methods are being developed in China as an alternative to CRISPR-Cas technology. Chinese scientists say that the so-called LEAPER (leveraging endogenous ADAR for programmable editing of RNA) technology is safer and has fewer side effects than DNA-modifying technologies such as CRISPR-Cas. As a result, while the discussions of CRISPR technology continue in the scientific world, research continues unabated. The world of science is an important indicator that shows the extent to which the developments in this area will reach.

**Conclusion**

This review presents a wide application potential of CRISPR-Cas technology as CRISPR-of-Things (CoT). CoT is a new generation of gene editing biotechnology and has a high potential for use in humans, plant, animal, and microorganisms. Using this technique, it is possible to remove, insert, and divide a fragment from DNA in the genome of an organism. In this technology, virulent and pathogenic microorganisms can be utilized and used. Therefore, reservations about the risk assessment of products obtained using this technology should be made. On the other hand, some issues require units and laboratories carrying out research and application studies with CRISPR-Cas technology to comply with biosafety rules. The commercial products obtained with CRISPR-Cas technology does not contain foreign DNA, it cannot be distinguished from those obtained using conventional plant breeding methods (including those obtained with physical and chemical mutagens). Products whose genome has been regulated in this way can be subjected to safety assessments.

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