

1 Review

2 Applications of CRISPR-Cas9 as an advanced genome editing system in life science

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16
17 **Abstract:** Targeted nucleases are powerful genomic tools to precisely change the target
18 genome of living cells, controlling functional genes with high exactness. The clustered
19 regularly interspaced short palindromic repeats associated protein 9 (CRISPR-Cas9) ge-
20 nome editing system is one of the most useful tools in genetic engineering that is taken
21 from adaptive immune strategies for archaea and bacteria. This system has made signif-
22 icant progress in recent years, and it has been widely used in genome editing to create
23 gene knock-ins, knock-outs, and point mutations. This paper summarizes the application
24 of the CRISPR-Cas9 system in various biological sciences, including medicine, plant sci-
25 ence, and animal breeding.

Keywords: CRISPR system; *Cas* proteins; Agriculture; Animal science; Human disease

Citation: Tavakoli, K.;
Pour-Aboughadareh, A.; Kianersi,
F.; Poczai, P.; Etminan, A.; Shooshta-
ri, L. Applications of CRISPR-Cas9
as an advanced genome editing
system in life science. *BioTech* 2021,
10, x. <https://doi.org/10.3390/xxxxx>

Received: date
Accepted: date
Published: date

Publisher's Note: MDPI stays
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1. Introduction

Significant advancements have been made in biotechnology in recent years, and the branch of genetic engineering is advancing at an unprecedented pace, yielding numerous advantages. Genome editing technology has revolutionized genetic and biological re-search via the novel ability to precisely manipulate and modify the genomes of living or-ganisms, and by accelerating the study of functional genomics. In recent years, dif-ferent genome editing tools have been utilized to study simple and intricate genomes [1, 2]. Genome editing technology emerged in the 1990s, and various methods have since been developed for targeted gene editing [3, 4]. In general, three systems—each with their own advantages—have been widely used in cells and animals, including zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats [3, 5]. ZFNs are targetable DNA cleavage proteins with the ability to cut DNA sequences at any location [4]. TALENs comprise double-stranded breaks (DBs) in target sequences that trigger DNA damage response pathways that lead to repair [4]. Despite the widespread application of these tools during 2002–2012, some restrictions prevent their effectual use [4]. TALENs face problems with cloning large modules in series, reduced efficiency for screening positive targeted cells, and efficient ordered affiliate by ligase [3], whilst complexity and lack of specificity are big challenges for ZFNs [2–4]. A powerful genome editing system called CRISPR

emerged in 1987, which became known as the greatest genetic tool of the century due to its outstanding advantages [6]. CRISPR-Cas9 technologies took the lead over other previous methods, such as TALENs and ZFNs, owing to its numerous advantages, like low cost, simplicity, high efficiency, and speed. Table 1 compares their differences [7]. In this work, a short summary of the CRISPR system and its application has been reviewed.

Table 1. Main differences between three genome editing techniques

Feature	CRISPR-Cas	TALEN	ZFN
Cost	Low	High	Low
Ease of design	Simple	A little complex	Moderate
Specificity	High	Intermediate	Low
Pros	Modifies multiple PAM motif required next to target	Highly effective and Time consuming	Highly effective and Time consuming
Cons			
Multiplex genome editing	High-yield multiplexing	Few models	Few models

2. Origin, development, and mechanism of the CRISPR-Cas9 system

The CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) is an adaptive immunity systems in archaea and bacteria. This system was first reported by Yushizumi Ishino [8], but its biological application was unknown at the time [3]. According to effector proteins, this system has been categorized into two main classes with six subtypes [2, 9]. The type2 CRISPR-Cas9 system is the most widely used item in the field of gene editing with three main components: a CRISPR RNA (crRNA), an endonuclease named Cas9, and a transactivating crRNA (tracrRNA) [6]. This system consists of two components: (1) the Cas9 protein which can cleave the DNA; and (2) the guide RNA that distinguishes the sequence of DNA to be rectified. To apply CRISPR-Cas9, sequences of the intended target genome are first identified. Then, the guide RNA is tailored to recognize a particular stretch of As, Ts, Gs, and Cs in the DNA. The guide RNA is affiliated to the DNA cutting enzyme Cas9, and then this complex is presented to the target cells. Cas9 locates the target letter and cuts the DNA at that point, allowing alteration of the existing genome by either modifying or adding to the sequence (Figure 1). As such, CRISPR-Cas9 functions as a cut and paste tool for DNA editing [10–11]. Using this technology, any genomic sequence identified by a short strand of guide RNA can be exactly modified [12]. This system targeted human genome for the first time in 2013 [13–15]. To date, this system has been commonly applied to create gene editing in plants, animal, and human samples. This technique is widely used in various scientific fields, including medical science and therapeutics, as well as plant and animal sciences [16–19].

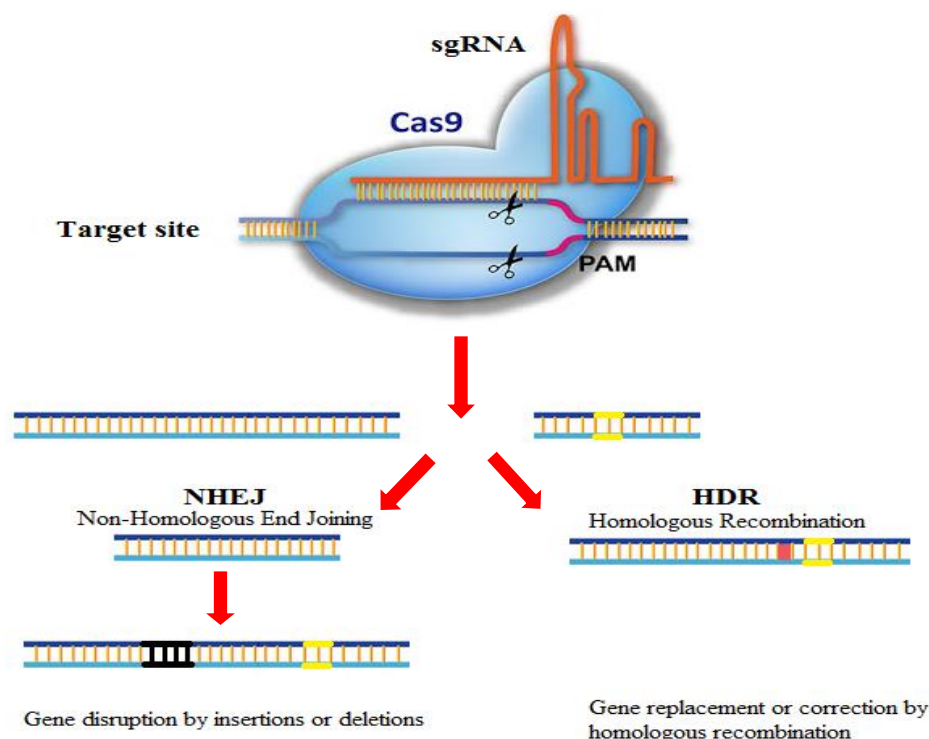


Figure 1. Schematic of CRISPR-Cas9 genome editing system. Cas9 is directed to its target site by base pairing between the single guide RNA (sgRNA) and DNA sequences. A protospacer-associated motif (PAM) downstream of the sgRNA-binding site is required for Cas9 recognition and cleavage. The Cas9-sgRNA complex cuts both strands of the target DNA, triggering endogenous double-strand break (DSB) repair. Then, the fragments resulting from DSBs are subsequently repaired either by homology-directed repair (HDR) or by non-homologous end joining (NHEJ). Repair via the NHEJ pathway frequently results in genome mutation through deletion or insertion that can lead to genome instability. In contrast, through an exogenous donor DNA template, the DSB can be repaired via the HDR pathway, which can engineer precise DNA modifications without any error.

3. Ethical issues in genome editing by CRISPR-Cas9 technology

It is well understood in the scientific community and related industries that one of the most important findings and inventions of the present century is CRISPR-Cas9 technology. However, due to the potential impact of these technologies on food safety and clinical applications, various bioethical issues have arisen [20, 21]. On other words, the rapid rise of CRISPR-Cas9 has led to new bioethical, social, and legal issues in medicine, agriculture, livestock, and the environment. Scientists think that they can extract genes that cause diseases in the population using CRISPR-Cas9 [22, 23]. Possible risks and bioethical issues related to CRISPR-Cas9 are summarized in Table 2.

Table 2. Possible risks and bioethical issues related to CRISPR-Cas9 technology

Organism	Risks	Bioethical issues	References
Bacteria	Nontarget mutations/ Gene drifts	Ecological imbalance	[20, 24, 25]
Plants	Nontarget mutations/ Gene drifts	Ecological imbalance Patenting	[20, 26]
Animals/ chimeric animals	Nontarget mutations	Ecological imbalance Patenting Animal welfare and dignity Threatening human dignity/identity	[24, 27, 28, 29, 30, 31, 32]
Humans	Nontarget mutations Side effects Cost Genetic mosaicism	Eugenics Informed consent Enhancement Accessibility Patenting Safety Incomplete or over legislation	[21, 24, 26, 33, 34, 35, 36, 37]

4. Applications of CRISPER-Cas9 technology

4.1. Human science

Nowadays, CRISPR-Cas9 is being employed to study various genetic diseases, such as hemophilia [38], Duchenne muscular dystrophy [39-43], α 1-antitrypsin deficiency [44, 45], hearing loss [46, 47], and hematopoietic [48-50] diseases. Figure 2 shows the application of this system in medical science. Hematologic diseases have previously been difficult to treat via genome manipulation. Studies in recent years demonstrated that CRISPR-Cas9 can correct genetic errors in hematopoietic stem cells that give rise to hematologic diseases [51-55]. These can then be applied to CRISPR-Cas9-based hematopoietic stem and progenitor cells (HSPCs) transplantation therapy. One of the most promising methods to treat hematopoietic diseases is editing the HBB mutation with CRISPR-Cas9, and it has been successfully carried out in patient-derived induced pluripotent stem cells (iPSCs) [56-57]. In a survey conducted by Park et al. [58] the CRISPR-Cas9 system was employed with a single stranded oligonucleotide to rectify the HBB gene mutation in HSPCs from the bone marrow of sickle cell disease (SCD) patients [58]. Final results inferred a decrease in the amount of hemoglobin and sickle cells [17].

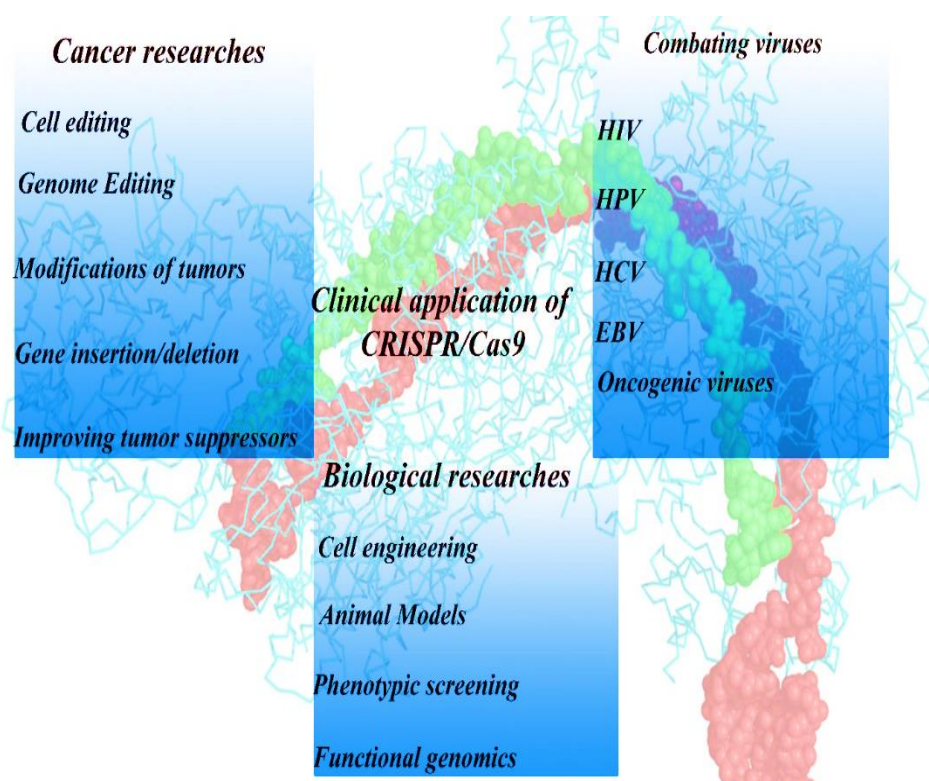


Figure 2. Exploitation of the CRISPR-Cas9 system in the field of medicine, including infectious diseases, tumors, and genetic diseases.

Since the CRISPR-Cas9 system originated from bacteria and acts as a bacterial immune system against invasive genetic agents, it has an inherent benefit in the treatment of bacterial and viral infection, and it is known as a new type antiviral therapy against various incurable viral infections. Oncogenic viruses are highly related to carcinogenesis, including: human papillomavirus (HPV), which could cause cervical cancer; Epstein Barr virus (EBV) that causes nasopharyngeal carcinoma; and hepatitis B virus that triggers liver cancer. CRISPR-Cas9 was first used to cleave HBV genomes [59]. A noticeable decrease was observed in the production of the HBV core and surface antigens in Huh-7 cells transfected with an HBV expression vector [17]. Among human genetic diseases, cancer is the most common, so it is considered as the second most common cause of death universally [6, 17]. The tumor-suppressor genes play a key role in tumorigenesis. They can downregulate cancer progression by controlling cell proliferation and differentiation. CRISPR-Cas9 technology can target tumor-suppressor genes and restore them to interdict the tumorigenesis [60]. Table 3 indicates the extensive use of the CRISPR-Cas9 technique to knockout proto-oncogenes supporting the potential of this method in various tumors.

Table 3. The application of CRISPR-Cas9 in the treatment of different types of cancer [60].

Type	Oncogene	Tumor suppressor gene	Drug-resistance gene
Breast	<i>SHCBP1, MIEN1, miR-27b, mi23b, HER2 exons</i>	<i>CPEB2, ETS1, BRCA1</i>	<i>HER2, EGFR, ER</i>
Prostate	<i>PCAR19, CECAM21, SENP1, miR-302/367, miR-1205</i>	<i>PGC1a, DEPTOR, p53, RB1</i>	<i>BLS-211, NANOGP8, NANOG1</i>
Lung	<i>IGFIR, ERBB2, RSF1, FOS, MCM4</i>	<i>MFN2, MiR-1205, GOT1, TP53</i>	<i>NRF2, MiR-1205, ER300, RSF1</i>
Liver	<i>Plxnb1, NCAPG, CDK7, IncBRM, Nf1</i>	<i>BAP1, HELLS, Tp53, Traf3</i>	<i>NF1, MED12, ERK2</i>
Colorectal	<i>KRAS, HPV16, Fut4, NRAS</i>	<i>LIMCH, PTEN, SOX15</i>	<i>miR-139-5P, ZEB1</i>
CRISPR-Cas9 function	Knockout	Activate	Promote drug sensitivity

4.1.1. Clinical trials using CRISPR-Cas9 technology

The United States clinical trials database (Clinicaltrials.gov) includes all studies that comply with the definition of "applicable clinical trial" started from or after September 27, 2007, and continued in December 26, 2007. In addition to registering for the desired trials, individuals can also register voluntarily. Registration for trials in countries other than the United States, which may meet one of the conditions in the future, is also voluntary. The United States clinical trial database contains useful data of experiments that have been used CRISPR, Cas9, Cas12, Cas13, ZFN, zinc finger, gene edit, gene modification, and genome edit techniques [61]. In the United States, the Food and Drug Administration (FDA) evaluates new treatments for safety and efficacy through clinical trials in patient volunteers. Preliminary tests check for safety and side effects. Subsequent trials test the effectiveness and compare new treatments with standard treatments. Current trials using CRISPR-based therapies are still in their infancy. This means that even if the treatments are safe and effective, they are likely to be several years away from FDA approval and are widely available to patients. New possibilities in precision medicine have arisen with the advent of CRISPR technology. Current trials are underway for blood disorders, cancers, eye diseases, chronic infections, and protein folding disorders. All current CRISPR clinical trials have been performed to edit specific cells or tissues without affecting sperm or eggs, meaning that no DNA changes can be passed on to future generations [62, 63].

In the first use of an *ex vivo* CRISPR-based therapy to treat a genetic disease, researchers treated a patient with beta thalassemia in Germany in February 2019. Twelve more patients have since been treated, and seven of them have been followed for at least three months. None of the patients needed blood transfusions in the months after treatment. The first patient with SCD was treated with the same therapy in Nashville, Tennessee in July 2019. This patient, Victoria Gray, has shown remarkable progress. Preliminary results for other patients are also promising: All patients treated for SCD or beta

thalassemia have shown normal to near-normal hemoglobin levels, where at least 30% (sickle cell disease) or 40% (beta thalassemia) of hemoglobin is fetal hemoglobin. In bone-marrow samples taken from Gray, an additional SCD patient, and five beta thalassemia patients, researchers found cells with the expected genetic edit that allows them to make fetal hemoglobin. This indicates that the edited cells have successfully taken up residence in the bone marrow. The only immediate side effects associated with the treatment resulted from the administration of chemotherapy [64]. In 2016, a lung cancer patient became the first person in the world to be treated with a CRISPR therapy: this patient was injected with PD-1 edited T cells in a Chinese clinical trial. This and an American clinical trial using CRISPR-based immunotherapies for cancer have been completed. Several other clinical trials using CRISPR-based immunotherapies, mainly to treat blood cancers, are ongoing [65]. Table 4 shows some clinical trial research based on CRISPR-Cas9.

Table 4. Clinical trials with genome editor of CRISPR-Cas9

Delivery vector	Phase	Target gene and effect	Disease	Ex vivo/In vivo	Intervention	Sponsor organization	NCT number
AAV vectors	I	Removal of alternative splice site in CEP290	Leber congenital amaurosis 10	In vivo	ZFN-mediated removal of intronic alternative splice site in retinal cells	Allergan and Editas Medicine, Inc.	NCT03872479
Electroporation (mRNA)	I/II	β TCR α , TCR β , β -2 microglobulin (B2M) knockout	B-cell leukemia	Ex vivo	CD19-CAR modified T cells with CAR delivered by lentivirus and Cas9 knockout B2M and TCR to create universal T cells	Chinese PLA General Hospital	NCT03166878
	I	TCR α , TCR β , PD-1 knockout	Various malignancies	Ex vivo	Modified T cells with Cas9-mediated deletions and lentiviral transduction of NY-ESO-1 targeted TCR	University of Pennsylvania	NCT03399448
	I/II	Disruption of the erythroid enhancer to BCL11A gene	β -thalassemia	Ex vivo	Ex vivo modified hematopoietic stem cells	CRISPR Therapeutics	NCT03655678
	I/II	Disruption of the erythroid enhancer to BCL11A gene	Sickle cell anemia	Ex vivo	Ex vivo modified hematopoietic stem cells	Vertex Pharmaceuticals Incorporated and CRISPR Therapeutics	NCT03745287
	I/II	Creation of a CD19-directed T cell	Refractory B-cell malignancies	Ex vivo	CD19-directed T-cell immunotherapy	CRISPR Therapeutics	NCT04035434
	I	disruption of HPK1	Refractory B cell malignancies	Ex vivo	CD19-CAR modified T cells with CAR delivered by lentivirus and Cas9 knockout of HPK1	Xijing Hospital	NCT04037566

Table 4. continued

Delivery vector	Phase	Target gene and effect	Disease	Ex vivo/In vivo	Intervention	Sponsor organization	NCT number
Undefined, likely electroporation	I	Programmed cell death protein 1 (PD-1) knockout	Hormone refractory prostate cancer	Ex vivo	Modified T cells	Peking University	NCT02867345
	I/II	Programmed cell death protein 1 (PD-1) knockout	EBV-positive, advanced stage malignancies	Ex vivo	Modified T cells selected for those targeting EBV positive cells	The Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School	NCT03044743
	II	Programmed cell death protein 1 (PD-1) knockout	esophageal cancer	Ex vivo	Modified T cells selected for those targeting EBV positive cells	Hangzhou Cancer Center	NCT03081715
	n/a	CCR5 knockout	HIV	Ex vivo	Modified CD34 ⁺ hematopoietic stem cells	Affiliated Hospital to Academy of Military Medical Sciences	NCT03164135
	I/II	Cas9-mediated creation of CD19 and CD20 or CD19 and CD22 CAR-T cells	Leukemia	Ex vivo	CAR T cells to CD19 and CD20 or CD19 and CD22	Chinese PLA General Hospital	NCT03398967
	I/II	Cytokine-induced SH2 protein (CISH) knockout	Metastatic gastrointestinal epithelial cancer	Ex vivo	Modified tumor-infiltrating lymphocytes	National Cancer Institute	NCT03538613
	I	Programmed cell death protein 1 (PD-1) and TCR knockout	Mesothelin positive solid tumors	Ex vivo	CAR T cells to mesothelin with added PD-1 and TCR knockout	Chinese PLA General Hospita	NCT03545815
	I	CD7 knockout in CD7 CAR T cells	T-cell malignancies	Ex vivo	CAR T cells to CD7 and knockout of native CD7 to prevent self targeting	Baylor College of Medicine	NCT03690011

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Table 4. cotinued

Delivery vector	Phase	Target gene and effect	Disease	Ex vivo/In vivo	Intervention	Sponsor organization	NCT number
Undefined, likely electroporation	I	Programmed cell death protein 1 (PD-1) knockout	Metastatic non-small cell lung cancer	Ex vivo	Modified T cells	Peking University	NCT02793856
	I	Programmed cell death protein 1 (PD-1) knockout	Stage IV bladder cancer	Ex vivo	Modified T cells	Peking University	NCT02863913
	I	Programmed cell death protein 1 (PD-1) knockout	Metastatic renal cell carcinoma	Ex vivo	Modified T cells	Peking University	NCT02867332
	I	Correction of the hemoglobin subunit β globulin gene	β -thalassemia	Ex vivo	Ex vivo modified hematopoietic stem cells	Allife Medical Science and Technology Co., Ltd	NCT03728322
	I	Programmed cell death protein 1 (PD-1) knockout	Mesothelin positive solid tumors	Ex vivo	CAR T cells to mesothelin with PD-1 knockout	Chinese PLA General Hospital	NCT03747965
Long-term clinical follow-up post-intervention	I/II	Cytokine-induced SH2 protein (CISH) knockout	Metastatic gastrointestinal epithelial cancer		Modified tumor infiltrating lymphocytes	Masonic Cancer Center, University of Minnesota	NCT04089891
	Follow-up	Disruption of the erythroid enhancer to BCL11A gene	β -thalassemia and severe sickle cell anemia		Ex vivo- modified hematopoietic stem cells, 15-year follow-up study	Vertex Pharmaceuticals Incorporated and CRISPR Therapeutics	NCT04208529
Plasmid delivery	I	E6 and E7 oncogene of HPV16 and HPV18 deletion	HPV-related malignancy	In vivo	Plasmid in a gel containing a polymer to facilitate delivery	First Affiliated Hospital, Sun Yat-Sen University	NCT03057912

4.1.2. Clinical trials of eye based on CRISPR-Cas9

Clinical trials of in vivo genome editors have also begun. To date, these experiments have focused on accessible tissues such as the cervix, eyes, and liver. AAV has been used to deliver Cas9 to the eye as a treatment for congenital amaurosis type 10 LBA (LCA10). LCA10 is an ideal candidate for the treatment of Cas9 because the most common LCA10 causes a mutation in the intron of the CEP290 gene and creates a new binding site that changes the mRNA to create an early stop codon [66]. The first Cas9 clinical trial for blindness, treatment of LCA10, has already been registered (NCT03872479) (Table 4). This treatment uses two sgRNAs that together cause partial loss of the intron or inversion of this partial intron, each of which leads to the normal expression of the CEP290 protein in the patient's cells. In addition, the eye is easily accessible, and subretinal injections in mice and mammals result in stable gene editing at a level expected to be usable in humans [66].

4.1.3. Limitations of CRISPR-Cas-based gene therapy

There are many projects related to CRISPR-Cas9 for disease research, and recent results and reports summarize the benefits of CRISPR-Cas9 [67–68]. The safety and efficiency of CRISPR / Cas9-based gene therapies should be evaluated and modified before using these therapies in patients [69]. One of the common limitations of CRISPR-Cas9 is that not all mutation sites play a protospacer adjacent Motif (PAM) role, such that target detection depends entirely on this role. In addition, editing at off-target genomic sites, delivery vehicle, immunogenicity, and DNA damage response are other challenges of using CRISPR-Cas9 for gene therapy. The limitations of CRISPR-Cas9 technology are mainly related to extra-target DNA fracture formation, extra-target mutations, and PAM sequence dependence. One of the major concerns in genome editing through the CRISPR-Cas9 system are off-targets. The CRISPR-Cas9 system has a higher risk of developing off-target mutations in human cells compared to the zinc finger nuclease (ZFN) and TALE nuclease [70, 26, 28, 63]. Dysfunction of some genes and sometimes even cell death can be caused by mutations outside the target site. Selecting a target locus with as few potential off-target sites in the bioinformatics analysis phase increases the efficiency of the CRISPR-Cas9 system. Hydrolysis of one DNA strand at the target site is the result of combining Cas9-D10A with sgRNA [71].

4.2. Plant Science

Success in plant breeding depends on trait variability as well as overall genetic variation among plant populations. Recently, yield performance and several quality-related properties have been engineered by genome editing [72]. CRISPR-Cas9 has proved useful in crop improvement to increase disease resistance, improve yield, improve nutrition, and aid domestication. These advantageous genetic modifications and others will be discussed in this section.

4.2.1. Plant disease resistance

Plant diseases have typically been controlled by the development of pathogen-resistant varieties and by applying agrochemicals. However, agrochemicals can cause contamination of the environment and negatively impact human health. Plant pathogens are also constantly evolving and can become unresponsive to these controls. Therefore, to create pathogen resistance in plants, resistance genes from wild species were introduced via breeding and established genetic transformation technologies involving large genomic regions [73]. Nonetheless, given the lack of specificity of the large genomic regions being introduced, other less-desirable traits may also be introduced

using these approaches to create elite cultivars. Conversely, the CRISPR-Cas9 system provides a more accurate approach to genetic modification. Since first introduced to the field it has proven to overcome several agricultural challenges including biotic stress resistance, fungal and bacterial disease resistance, and viral resistance [74]. For instance, CRISPR-Cas9 was employed to knockout the mitogen-activated protein kinase-5 (*Os-MPK5*) gene to enhance disease resistance in rice [75]. Plant viruses invade a wide range of plants and affect the fertility of crops. As shown in Table 5, the CRISPR-Cas9 system provides a suitable context to develop resistance against both DNA- and RNA-based plant viruses [74].

Table 5. Proven viral resistance in plants introduced via CRISPR-Cas9 against DNA and RNA viruses [74].

Virus	Type of nucleic acid	Involved protein	Plant under attack	References
Beet severe curly top virus	DNA	Cas9	Capsicum	[76]
Bean yellow dwarf virus	DNA	Cas9	Oat	[77]
Turnip mosaic virus	RNA	Cas13	Cruciferous plants, Chinese cabbage, turnip, mustard, radish	[78]
Tomato yellow leaf curl virus	DNA	Cas9	Invading a number of seeds, including tomato	[79]
Tomato yellow leaf curl virus	DNA	Cas9	Invading a number of seeds, including tomato	[80]
Yellowing virus	RNA	Cas13	Cucumber	[81]
Zucchini yellow mosaic virus	RNA	Cas13	Cucumber	[81]
Papaya ring spot mosaic virus	RNA	Cas13	Cucumber	[81]

As the latest genome editing tool, CRISPR-Cas9 plays a key role in developing bacterial resistance in plants. Several reports have been published on the use of CRISPR-Cas9 to counteract crop bacterial diseases (Table 6). For example, in rice, CRISPR-Cas9 mutagenesis of the ethylene-responsive factor (ERF) transcription factor gene *OsERF922* improved rice blast resistance. In another example, researchers achieved resistance to citrus bacterial canker (CBC) caused by *Xanthomonas citri* subsp. in Duncan grapefruit by modifying the effector-connection element in the promoter of the *Lateral Organ Boundaries-1* gene [82].

Table 6. Three examples of exploitation of CRISPR-Cas9 to counteract crop bacterial disease

Plant	Targeted area in gene	Disease	References
Rice	Mutagenesis of the ERF Transcription Factor Gene <i>OsERF922</i>	Blast	[73]
Duncan grapefruit	Effector-binding element in the promoter of the Lateral Organ Boundaries 1 gene	Citrus bacterial canker (CBC)	[82]
Wanjincheng oranges	(<i>CsLOB1G</i> and <i>CsLOB1</i> –) alleles	Citrus bacterial canker (CBC)	[83]

4.2.2. Yield of crop plants

Cereal crops play a key role in human life as food primarily supplying energy. Due to high demand for them, plant breeders have always sought ways to mass produce and generate products with high quality. With the CRISPR-Cas9 system, it is possible to modify crop characteristics and improve their tolerance to adverse climate conditions and universal threats like drought, salinity, or frost [75]. For instance, to increase tolerance to salinity stress in rice, the *O. sativa* response regulator-22 gene (*osRR22*) was knocked-out to achieve approximately 65% mutation in T0 lines [75]. CRISPR-Cas9 was also described to hold potential for enhancing traits like drought or herbicide tolerance in maize [84]. As such, three sgRNAs were designed to target the *ZmTMS5* gene in maize and generate mutations in protoplasts, which resulted in edited plants showing bi-allelic modification [75]. Over the last decade, some studies have demonstrated the application of CRISPR based genome editing in plants and few studies reported the utilization of genome editing for improving biotic and abiotic stresses for crop improvement (Table 7).

Table 7. Application of CRISPR-based genome editing method in plants for biotic, abiotic, and nutritional traits.

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Biotic stresses					Abiotic stresses				
Crop	Method	Target gene	Stress/trait	Reference	Crop	Method	Target gene	Stress/trait	Reference
<i>A. thaliana</i> / <i>N. benthamiana</i>	NHEJ	dsDNA of virus (A7, B7, and C3 regions)	Beet severe curly top virus resistance	[85]	Maize	HDR	ARGOS8	Increased grain yield under drought stress	[93]
<i>A. thaliana</i>	NHEJ	eIF(iso)4E	Turnip mosaic virus (TuMV) resistance	[86]	Tomato	NHEJ	SIMAPK3	Drought tolerance	[94]
<i>N. benthamiana</i> Bean	NHEJ	BeYDV	Yellow dwarf virus (BeYDV) resistance	[87]	<i>A. thaliana</i>	NHEJ	UGT79B2, UGT79B3	Susceptibility to cold, salt, and drought stresses	
<i>N. benthamiana</i>	NHEJ	ORFs and the IR sequence sDNA of virus	Tomato yellow leaf curl virus (TYLCV) and Merremia mosaic virus (MeMV)	[88]	<i>A. thaliana</i>	HDR	MIR169a	Drought tolerance	[95]
Rice	NHEJ	OsERF922 (ethylene responsive factor)	Blast Resistance	[89]	<i>A. thaliana</i>	NHEJ	OST2 (OPEN STOMATA 2) (AHA1)	Increased stomatal closure in response to abscisic acid (ABA),	[96]
Rice (IR24)	NHEJ	OsSWEET13	Bacterial blight disease resistance	[90]	Rice	HDR, NHEJ	OsPDS, Os-MPK2, Os-BADH2	Involved in various abiotic stress tolerance	[97]
Bread wheat	NHEJ	TaMLO-A1, TaM-LO-B1, and TaM-LOD1	Powdery mildew resistance	[91]	Rice	NHEJ	OsMPK5	Various abiotic stress tolerance and disease resistance	[98]
Cucumber	NHEJ	eIF4E (eukaryotic translation initiation factor 4E)	Cucumber vein yellowing virus (CVYV), Zucchini yellow mosaic virus (ZYMV), and (PRSV-W)	[92]	Rice	NHEJ,HDR	OsMPK2, OsDEP1	Yield under stress	[99]

4.2.3. Genome modification for nutritional improvement

As populations grow, the demand for nutritious crops increases. The CRISPR-Cas9 system has made it possible to improve crop value and food quality through enhancing their nutritional status. For example, lycopene is a plant nutrient that is widely found in tomato with antioxidant properties and advantageous therapeutic traits. With the success gained in expanding the amount of lycopene content in tomato, it is expected that CRISPR-Cas9 technology may also play a key role in enhancing the micronutrient content of plants [74]. Other examples are presented in Table 8.

Table 8. Application of CRISPR based genome editing method in plants for nutritional traits.

Crop	Method	Target gene	Stress/trait	Reference
Rice	NHEJ	25604 gRNA for 12802 genes	Creating genome wide mutant library	[103]
Maize	NHEJ	ZmIPK1A ZmIPK an-dZmMRP4	Phytic acid synthesis	[104]
Wheat	HDR	TaVIT2	Fe content	[105]
Soybean	NHEJ	GmPDS11 and GmPDS18	Carotenoid biosynthesis	[106]
Tomato	NHEJ	Rin	Fruit ripening	[107]
Potato	HDR	ALS1	Herbicide resistance	[108]
Cassava	NHEJ	MePDS	Carotenoid biosynthesis	[109]
Rice	NHEJ	25604 gRNA for 12802 genes	Creating genome wide mutant library	[103]
Maize	NHEJ	ZmIPK1A ZmIPK an-dZmMRP4	Phytic acid synthesis	[104]
Wheat	HDR	TaVIT2	Fe content	[105]
Soybean	NHEJ	GmPDS11 and GmPDS18	Carotenoid biosynthesis	[106]
Tomato	NHEJ	Rin	Fruit ripening	[107]
Potato	HDR	ALS1	Herbicide resistance	[108]
Cassava	NHEJ	MePDS	Carotenoid biosynthesis	[109]

4.2.4. Medicinal plants

Medicinal plants may have significant effects in the treatment of various diseases, and they have always been used in traditional medicines. Plants carry bioactive, non-nutrient and, biologically active compounds, such as coumarins, terpenoids, tannins, saponins, flavonoid, phenols, and cardiac glycosides. The health benefits of these phytochemicals are well-known. Since the genomes of some medicinal plants have been fully sequenced, it is predicted that CRISPR-Cas9 could be utilized to modify targeted genes in herbal plants, survey the synthesis of efficacious compounds, elect characteristics for increased yield, and elevate research on biosynthetic pathways and regulatory mechanisms [76].

4.3. Animal breeding

Due to population-growth driven increasing demand, the production of animal-derived food products, especially milk and meat, has increased worldwide. Providing these products is vital for the health and fitness of people [110]. Genome editing technology has made it possible to make precise changes in the animal genome to improve productivity and disease resistance [7]. One of the genes that was first genetically

modified in farm animals was *myostatin*. Changes in this gene can drastically improve economic efficiency of meat production [7]. Farm animals that have been genetically engineered thus far include: pig, cattle, sheep, goat, and channel catfish [7]. Nonetheless, because of its global economic value, multiparous nature, and comparatively short generation time, pig is the most genetically modified livestock to date [111]. CRISPR-Cas9 plays a key role in improvement of livestock as the most prominent gene-editing technology today [111] (Figure 3).

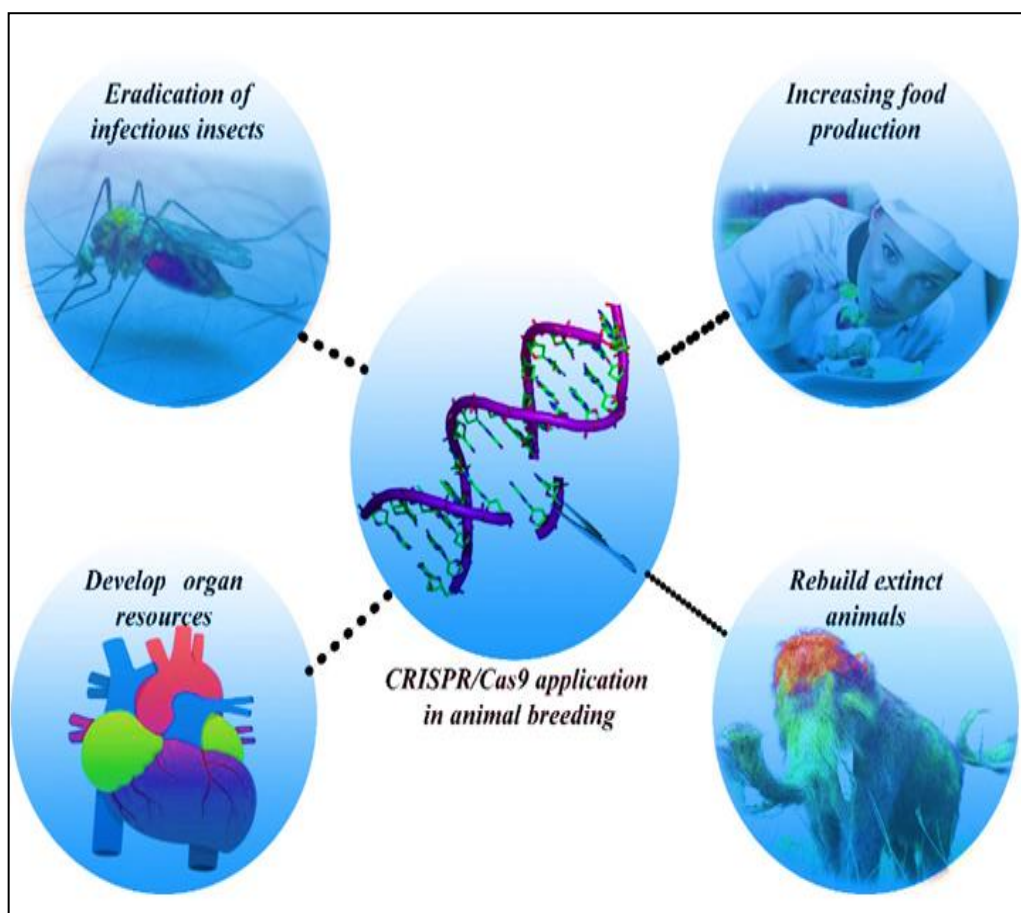


Figure 3. Exploitation of CRISPR-Cas9 in animal breeding and animal models [112].

This method creates desired changes by either enhancing frequency of favorable alleles or by clearing deleterious alleles [7]. CRISPR-Cas9 has not only helped to increase animal products but also made many contributions to the field of biomedicine by producing transgenic and cloned animals [110]. Genome editing tools, like CRISPR-Cas9 and PiggyBac transposon, could be useful in immunology and vaccine development due to their low risk to human health, such as preventing the transition of viruses [110].

4.3.1. Modification of Pigs for Xenotransplantation Research

Xenotransplantation or heterologous transplant, is the transplantation of living organs, tissues cells, and even living cells from one species to another. Serious attempts of xenotransplantation were first reported in the scientific literature in 1905, when slices of

rabbit kidney were transplanted into a child with chronic kidney disease [113]. After then, several subsequent attempts to use organs from pigs, primates, and lambs were reported in the first two decades of the 20th century [113]. Among tested animals, pigs (*Sus scrofa domestica*) are currently the best candidates for organ donation due to their large phylogenetic distance from humans. Indeed, this phylogenetic distance decreases the risk of cross-species disease transmission [114]. Indeed, in addition to this issue, the short gestation period of pigs is the reason that they were considered as an animal model in clinical trials [115]. The achievement of porcine endogenous retroviruses (PERVs) inactivation and the insertion of porcine RSAD2 gene into porcine Rosa26 locus is one of the most promising advancements in pig-to-human xenotransplantation research. Niu et al. [16] generated pigs in which all copies of PERVs were inactivated by CRISPR-Cas9 technology. Other examples regarding application of CRISPR technology can be seen in the literature, for example, Ryzek et al. [115], Niu et al. [116], Denner [117], and Hryhorowicz et al. [118].

4.3.2. Application of CRISPR-Cas9 technology in insects

To date, most CRISPR-Cas-based genome editing exploration uses *Streptococcus pyogenes* Cas9 nuclease, which can detect the PAM sequence NGG [119]. Insects are the most abundant organisms in the natural world. As such, continuous research efforts have enriched the body of insect genomic information, for species such as *Drosophila melanogaster* Meigen, *Tribolium castaneum*, *Bombyx mori* L., *Apis mellifera* L., *Nasonia vitripennis* Walker, *Acyrtosiphon pisum*, and *Plutella xylostella* L. [120].

CRISPR-Cas9 applications in insects have been reported increasingly, mainly in *Drosophila melanogaster*, *Bombyx mori*, and *Aedes aegypti*. This technology is not only becoming more popular in functional genomics studies [121], but it is also being exploited as a tool for the potential control of pest insects and vector-borne diseases [121, 122]. In insect studies, *Drosophila* has been considered as one of the best insect models for the study of eukaryotic biology, including human development and disease due to its ability to have a wide range of genetic tools, including gene targeting by homologous recombination. For this reason, the ability of the CRISPR-Cas9 technique to change targets in parallel has been exploited in this species. In 2013, Graz et al. [123] began the CRISPR-Cas9 discovery adventure for *Drosophila* by deleting a 4.6 kb fragment of the yellow gene with two gRNAs targeting the 5' and 3' ends of the source, respectively. Researchers are also exploring the application of CRISPR-Cas9 in other insect species (Table 9).

Table 9. Applications of CRISPR-Cas9 in insects

Species	Targeted genes	Strategy	Germline transmission rate (%)	G1 mutation rate (%)	Reference
<i>Drosophila spec.</i>	yellow, whit	mRNA INJ	0–79	0–34.5	[124]
	CG4221, CG5961, Chameau	mRNA INJ with donor	8.1–26.7	2.7–10.4	[125]
	yellow	DNA INJ with donor	5.9–20.7	0.25–1.37	[123]
	yellow	Rapid INJ with donor	8–53	15	[126]
<i>Bombyx mori</i>	BmBLOS2	mRNA INJ	95.5	35.6	[127]
	th, re, fl, yellow-e, kynu,ebony	DNA INJ	5.7–18.9	ND	[128]
<i>Aedes aegypti</i>	ECFP	mRNA INJ + DNA INJ	0	5.5	[129]
<i>Daphnia magna</i>	eyeless	mRNA INJ	18–47	8.2	[130]
<i>Tribolium castaneum</i>	eGFP1	mRNA INJ + DNA INJ with donor	55–80	71–100	[131]
<i>Papilio xuthus</i> , <i>P. machaon</i>	abdominal-B,ebony, frizzled	mRNA INJ	18.33–90.85	ND	[132]

NIJ and ND indicate injection and not determined, respectively.

5. Conclusion

The CRISPR-Cas9 system is a new and purposeful method in genome editing, which has surpassed other methods due to its outstanding advantages in recent years. This system has greatly contributed to all fields of life science, including medicine, plant breeding, and animal breeding, also expanding researchers' understanding of the basis of gene diversity and gene editing. Given its current central role in this landscape, on-going research will likely focus on improving this technology further to enhance its specificity and efficiency.

Author Contributions: A.P. and A.E. conceived the idea; K.T., F.K. and A.P. wrote the manuscript and prepared the figures; L.S., A.E. and K.T. collected information and prepared the Table. A.P. and P.P. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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