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Research Review

New Generation CRISPR–Cas Technology and its Applications

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ABSTRACT

Human genome manipulation, each in vitro and in vivo, remains a subject for moral dialogue. The safety and toxicity of all clinical trials and processes still be strictly monitored. The invention of the CRISPR (clustered regularly interspaced short palindromic repeats) and CRISPR-Cas 9 adjustive immune systems has revolutionized scientists' understanding of the human genome. Recent advances in this technology have opened the doors for genome editing research in basic science to new age. The prokaryote-derived CRISPR–Cas genome editing systems have revolved our ability to control, detect, image and annotate specific polymer and ribonucleic acid sequences in living cells of various species. In CRISPR-Cas technique Genome editing is done by utilizing non-homologous end joining and homology-directed DNA repair and single-base editing enzymes. Initial successes have impressed efforts to get new systems for targeting and manipulating nucleic acids, together with those from Cas9, Cas12, Cascade and Cas13 orthologous. Additionally, to targeting polymer, CRISPR-Cas-based RNA-targeting tools are also being developed for diagnostics and new age medicines. Current review is aimed to describe the recent improvement, challenges, future prospects and innovative applications of Cas9 in a diverse field range from basic biology to biotechnology and medicine.

***Key words*:** CRISPR/Cas9, Genome editing, Off-target effect, Guide RNA

Recombinant DNA technology has marked the initiation of a new era of biology. Gene therapy has always been a promising tool to correct a variety of human diseases and defects. Discovery of the Clustered Regularly-Interspaced Short Palindromic Repeats (CRISPR) has given a potential opportunity & flexibility to molecular biologists to govern DNA molecules, making it feasible to check genes and design them to develop novel drugs. The mechanism of the CRISPR-based prokaryotic adaptive immune system (CRISPR-associated system, Cas) has created a new insight for new improved gene therapies [1].

CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) systems are a novel organism defence against foreign genetic parts, like those from bacteriophages or plasmids. Functionally, CRISPR/Cas systems act as RNA-directed nuclease complexes that are capable of targeting foreign nucleic acids in a very sequence-specific fashion. Every individual system consists of a CRISPR array (crRNA array) composed of distinctive spacer sequences (21–72 base pairs [bp]) flanked by short (23–47 bp), repetitive and typically palindromic repeat sequences, moreover as teams of preserved Cas proteins encoded adjacent to the crRNA array. Following transcription, the crRNA array is processed into individual CRISPR RNAs (crRNA) containing a spacer and a partial repeat. The spacers hybridise to complementary protein targets, triggering their degradation by Cas proteins. A distinctive feature of CRISPR/Cas systems compared to different sequence specific microorganism defences, like restriction modification systems, is that CRISPR/Cas systems are reconciling. Specific Cas proteins acknowledge foreign genetic parts and integrate their deoxyribonucleic acid as new spacer sequences into the crRNA array, ultimately permitting the microorganism to adapt and afterwards target these foreign parts [1].

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Classification

Type 1: Are the foremost frequent and widespread systems, that target invading DNA during a cascade (CRISPR-associated complex for antiviral defence) advanced driven and Protospacer Adjacent Motif (PAM) dependent manner. The PAM is a short sequence motif placed adjacent to the protospacer on the invading DNA. it's essential for recognition, cleavage and the distinction between self and non-self DNA. The absence of the PAM sequence at the CRISPR locus within the host genome

protects it from self-cleavage. In kind 1 system, pre-crRNA cleavage is carried out by Cas endoribonuclease, Cas6. Mature crRNA makes ribonucleoprotein complex with Cascade. during the interference stage, the cleavage of invading target DNA is carried out by recruiting a Cas nuclease, Cas 3 (Fig 2) [2].

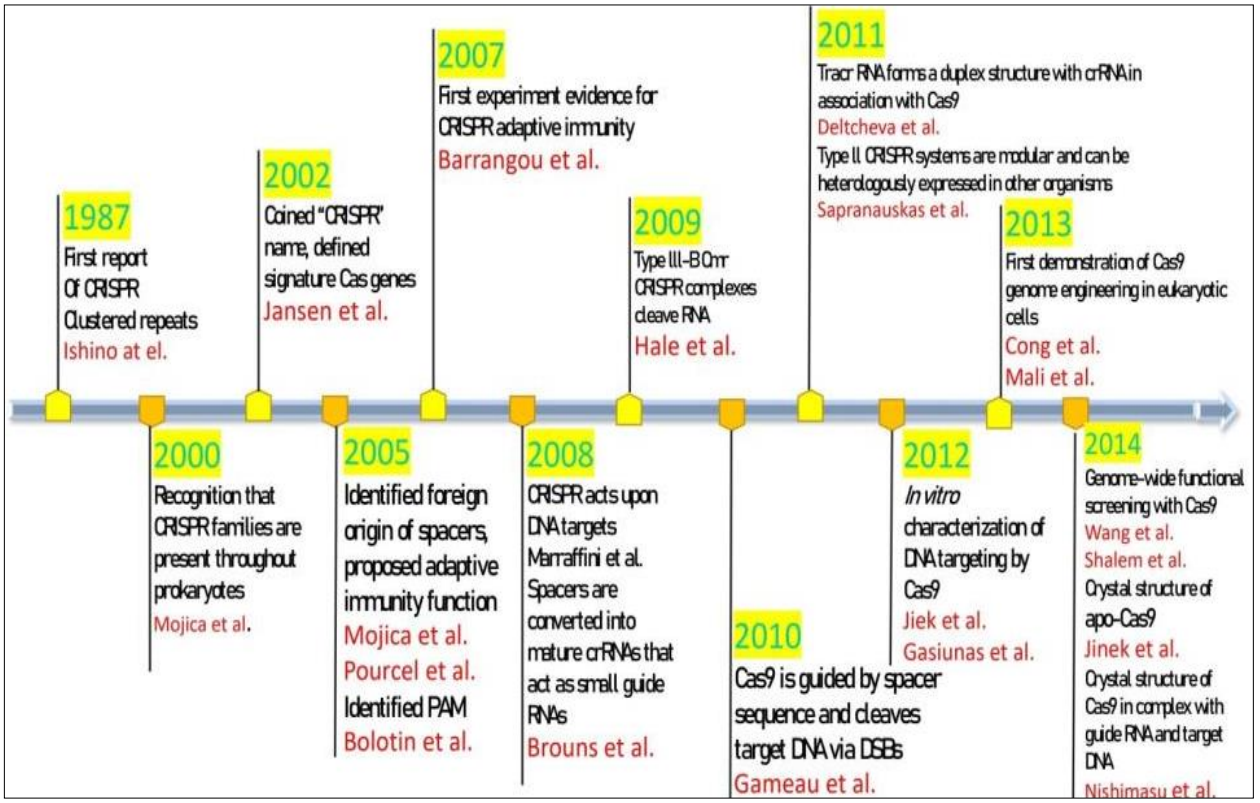

Fig 1 Timeline of history of development of CRISPR technology

Type 2: Work in a tracrRNA (a little trans noncoding RNA) and PAM dependent manner. In this system, pre-crRNA process involves the expression of a tracrRNA, that base pairs with the repeat within the pre-crRNA transcript. The ensuing duplexes are cleaved within the repeat sequences by RNase III during a Cas9- dependent reaction. The tracrRNA remains bound to the crRNA and the mature crRNA-tracrRNA duplexes are complexed with Cas9 (Fig 2). The cleavage of invading target DNA is carried out by the Cas-crRNA ribonucleoprotein complex itself [2].

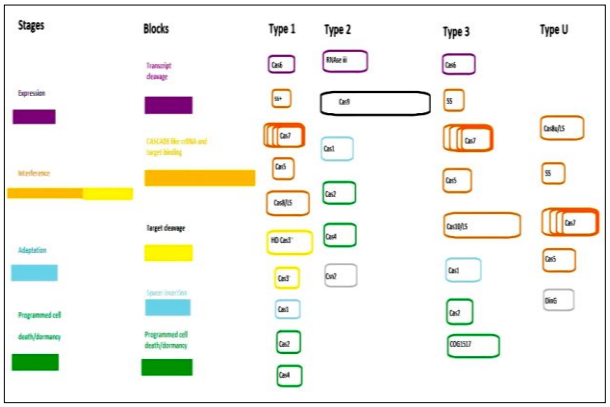

Fig 2 Types of CRISPR system

Type 3: Work in PAM-independent manner. Pre-crRNA cleavage is carried out by Cas endoribonuclease, Cas 6. Mature crRNA makes ribonucleoprotein complex with Csm in type III-A systems and Cmr in type III -B systems. The cleavage of invading target DNA is carried out either by the Cas-crRNA ribonucleoprotein complex itself (in kind III-B) or by recruiting a Cas nuclease, in all probability Cas6 (in type III-A system). the 2 forms of type III systems target either dsDNA (type III-A systems) or RNA (type III-B systems) (Fig 2) [2].

General mechanism

The general mechanism of CRISPR works in three distinct phases (Fig 3).

1. Adaptation phase: Adaptation is the first step in the process of developing a weapon against foreign invading DNA (viral or plasmid DNA). It involves picking up a photospacer (apiece of invading DNA) and integrating it between two adjacent repeats in CRISPR locus. The invading DNAs are directionally integrated, as new CRISPR spacers, into a CRISPR array that is separated by repeat sequences, thus by creating a memory of invading genetic elements (Fig 3) [2].

2. Expression and maturation phase: Once the desired sequence (i.e., protospacers) from the invading DNA is integrated into the loci of CRISPR, the CRISPR locus is transcribed into a pre-CRISPR RNA (pre-crRNA). CRISPR loci are transcribed from an upstream promoter located in the AT rich leader sequence. The pre-crRNA is then processed into mature crRNAs, each containing a transcribed spacer sequence joined to the partially repeat sequence (Fig 3) [2].

3. Interference phase: The crRNA forms a complex with Cas proteins. The crRNA of the crRNA-Cas complex makes base pairing with the protospacer of the invading DNA. Finally, crRNA-directed cleavage of invading DNA

occurs by Cas proteins at protospacer, a site complementary to the crRNA spacer sequence (Fig 3) [2].

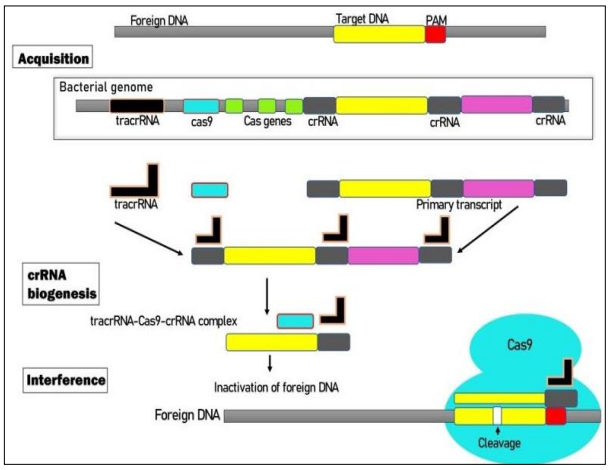

Fig 3 General mechanism of CRISPR

Applications of Cas9 in research, medicine, and biotechnology

Cas9 may be used to facilitate a wide type of targeted genome engineering applications. The wild-type Cas9 nuclease has enabled economical and targeted genome modification in several species that are recalcitrant using ancient genetic manipulation techniques [3].

Cas9 as a therapeutic molecule for treating genetic disorders

Although Cas9 has already been widely used as a research tool, a very exciting future track of action is that the development of Cas9 as a therapeutic technology for curing genetic disorders [3].

For a monogenic recessive ailment due to loss-of-function mutations (such as cystic fibrosis, sickle-cell anaemia, or Duchenne muscular dystrophy), Cas9 may be accepted to correct the causative mutation. For dominant-negative disorders in which the affected gene is haplo sufficient (such as transthyretin-related hereditary malady or dominant types of retinitis pigmentosum), it's going to also be achievable to use NHEJ to inactivate the mutated gene to realize therapeutic profit. For allele-specific targeting, one might design guide RNAs proficient of identifying between single-nucleotide polymorphism (SNP) variations in the target gene, like when the SNP falls within the PAM sequence. Some monogenic diseases also result from duplication of genomic sequences. For these diseases, the multiplexing efficiency of Cas9 is also exploited for deletion of the duplicated parts. as an example, trinucleotide repeat disorders might be treated mistreatment 2 synchronous double strand break to cut the repeat region [3].

Rapid generation of cellular and animal models

To generate cell models, Cas9 can be easily presented into target cells by transient transfection of Cas9-carrying plasmids and suitably structured sgRNAs. In addition, Cas9's multiplexing efficiencies offer an encouraging methodology to studying common human diseases – such as diabetes, cardiovascular disease, schizophrenic disorders, and autism – which are governed by more than one gene. Large-scale genome-wide association studies (GWAS), for example, have recognized haplotypes that show strong connotations with disease risk. However, it is usually difficult to predict that genetic variants are closely associated to the haplotype or genes in the region are accountable for the constitution. With Cas9 scientist can study the outcome of each individual variant or view the results of the manipulation of each individual gene on an isogenic background by processing stem cells and separating them rendering to the chosen cell type [3].

To produce transgenic animal models, Cas9 protein and transcribed sgRNA were injected straight into fertile zygotes to accomplish hereditary gene manipulation in one or more alleles in mouse and monkey-like models [3]. By avoiding the standard step of targeting Embryonic Stem cells in generating transgenic lines, the time to production of mutant mice and mice can be decreased from over a year to just a few weeks. Such advances could permit large-scale and cost-effective *in vivo* causal studies in placental models and combined with highly precise edits to elude unsupported non-target causes. Furthermore, effective multiplex targeting in a cynomolgus monkey model was recently reported, signifying the potential to create precise additional modeling for advanced human diseases for example medical disorders using primate models. Cas 9 can be directly loaded into body for manipulation of body tissues, thus escaping the need for embryonic modification, but as a therapeutic use for gene therapy [3].

Current applications of CRISPR-Cas in agriculture

Virus-resistant plant development: One of the foremost common infectious agent illness that notably decrease plant harvest worldwide is due to Gemini viruses (from the Gemini viridae Family). Transgenic Gemini virus-resistant plants were accidently developed using RNAi-mediated gene silencing, although with a low success rate [4]. As an added economical system, CRISPR-Cas9 system with changed sgRNA was accustomed target six completely different regions of the bean plant disease virus (BeYDV) order so as(fig4). To decrease Gemini virus replication through a transgenic plant model. Important reduction in copy variety of BeYDVs was detected in plants that were cured with CRISPR-Cas9 utilizing four engineered sgRNAs (gBRBS+, gBM3+, gBM1−, and gB9nt+). This technique that was operated for the primary time on Gemini virus could allow sgRNAs to be assembled during a single transgene and to guide many nucleases against various viruses and satellites bestowing a very important benefit in combating diseases infected by Gemini viruses [4].

Fig 4 A simplified description of key steps for the improvement of plant properties through the CRISPR technique

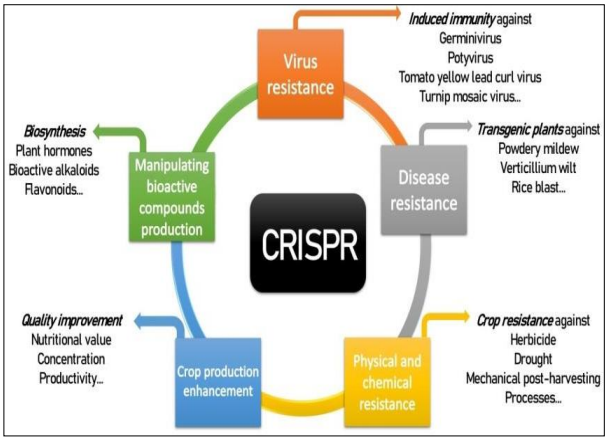

Fig 5 The schematic representation of some of the most important applications of CRISPR in food and agricultural science

CRISPR application in biotherapy: Techniques based upon CRISPR therapies are actively being developed in several laboratories. Currently there are too many preclinical studies in many countries [5].

Gene editing therapies are often divided into 2 broad categories: *ex vivo* and *in vivo*. For cells taken from modified patients in research laboratories and then transplanted into patients, *ex vivo* gene editing is favourable in attaining sufficient strength and safety. Curing genetic mutations in human hematopoietic stem and root cells (HSPC) can be a encouraging strategy to treat various genetic ailments of the hematopoietic system. in particular, impaired human erythroid enhancer BCL11A stimulates fetal hemoprotein synthesis, which is a promising strategy for the cure of -thalassemia and erythrocyte disease [5]. 2 clinical studies supporting this theory began in late 2018 with the drug prescribed by Vertex. In this part 1/2 study, they used spCas9 to manipulate the erythroid-specific BCL11A enhancer in autologous CD34+ HSPCs [5]. While this research was in progress, a Chinese team led by subgenus Chun Hongkui Deng and Hu revealed their first case in a clinical trial using CRISPR-based gene editing. During their research, they manipulated Hematopoietic stem and root cells from AN HLA-matched donors with spCas9 and transplanted CRSPPR-edited HSPCs into patients with HIV-1 infection and acute lymphoblastic leukemia. This study systematically examined the possibility and relative safety of the CD34 + HSPC machining approach [5].

Additionally, enhanced lymphocyte treatment with CRISPR is in active development. Portion of the first clinical study with semiconductor diodes by researchers from the China PLA General Hospital examined the defence and efficiency of CAR-T cells with PDCD1, which was destroyed by CRISPR. In another study, Cluster Carl June published their study of TCR T cells with multiple cistron editing of PDCD1, TRAC and TRBC in 3 patients with refractory cancer. Semi-permanent transplantation and removal of adverse outcomes are unquestionable for the survival of this technique [5].

Application of CRISPR/Cas9-based genome editing in the production of gibberellic acid by Fusarium fujikuroi

Gibberellic acids (GAs) are a category of natural plant growth hormones that are notably created in *F. fujikuroi* and are widely used to promote the growth of various plant species. Among all Gibberellic Acids, the most promising biologically active ones are GA1, GA3, GA4, and GA7. Of these, the production and application of GA_3 has touched a particularly mature stage, whereas that of GA4 and GA7 are stalled by the low strength of the present synthesis techniques (Fig 6). To overpower this CRISPR can be utilized to manipulate the GA 4 and GA7 genes [6].

Nuclease-mediated genome editing in higher eukaryotes: Chinese hamster ovary (CHO) cells are usually used mammalian cells for synthesis of biotherapeutic proteins. Genetic engineering of CHO cells has been pursued to expand product diversity moreover on improve product quality and yields. In higher eukaryotes, precise knock-in is extremely fascinating for the simplest and foreseeable production of recombinant proteins, since gene expression level is impressively plagued by chromosomal organization. HDR-mediated knock-in using short homology arms of 250bp and CRISPR-Cas9 was able to incorporate a single-copy gene cassette into the targeted site, showing uniform transgene expression [7]. As protein modifications like glycosylation might change its perform and immunogenicity, CRISPR-Cas9-mediated gene knockout of MGAT1, that limits N-linked glycosylation to early oligomannose glycans permitted synthesis of HIV immunizing agent protein with less glycosylation nonuniformity that exhibited improved binding to prototypical glycan-dependent protein. The cells were any designed by deleting a serine protease gene to stop proteolytic cleavage of the sensitive recombinant protein [7]. To stimulate protein yields through optimizing cellular metabolism, metabolic network reconstruction of amino acid catabolism was implemented for antibody production. The authors incontestable that disruption of amino acid catabolic genes such as Hpd (L-tyrosine and L-phenylalanine) and Gad2 (L-glutamate) greatly reduced specific suck and ammonium discharge whereas rising precise rate and integral of live cell density [7].

However, full understanding of the potential of CRISPR/Cas9 approaches would need addressing many challenges [8]. This is often a significant space for continuing study if CRISPR/Cas9 is to study its potential. Gene cargo delivery system is main obstacle for CRISPR/Cas9 utilization, in addition to it delivery systems are also limited. Each methodology has each benefit and drawbacks, and a few is quite specific or ill-suited to certain forms of delivery (e.g., delivery to cells in a flask vs. Further, the most effective gene editing results with lowest off-target effects are typically obtained from delivery of the ribonucleoprotein, as opposition plasmid DNA or RNA. Emergence of recent delivery approaches that alter effective RNP delivery can build a purposeful impact to the field. Still another barrier for delivery systems is guaranteeing that the chosen system is each safe and specific. Safety in living organisms can invariably be a priority, and a delivery vehicle which will target the desired cells with high-specificity will limit off-target effects and improve safety. There's presently restricted info out there on wherever varied parts of nanoparticle delivery systems end up in the body, however, and whether there's any long-term toxicity related to any constituent. As evident through the numerous examples bestowed within the 'Delivery Methods' portion of this, the therapeutic potential of CRISPR/Cas9 is great. Work has even moved into animal models, and therapeutic effects square measure broad-ranging, as well as inhibition of infection, reversal of enfeebling conditions like genetic

disorder, and elimination of tumors in cancer models. Because the technology evolves and CRISPR becomes even a lot of mechanistically precise and may be delivered with ever-increasing preciseness, its therapeutic potential can still rise. Prominently, the CRISPR field is emerging at an unlikely pace, with the quantity of peer-reviewed scientific papers with the term CRISPR increasing by one, 453% since 2011. Indeed, CRISPR is even getting down constructing its methodology into contemporary common culture, with informal references in multiple media set ups [8].

As CRISPR–Cas-based treatment enters clinical testing; they hold excellent potential and promising future for curing genetic diseases and increasing cell treatments. A possible risk of utilizing gene editing technique is that the introduction of off-target changes to genome sequence, and therefore enhancing ways for detection of rare mutations and quantifying their potential risks are important for future clinical advancement. Cas9-based genome engineering serves as a latest generation tool which are based on components from the microbial antiphage defence system. It is concluded that CRISPR technology is an excellent solution for efficient and precise gene modification and can be used to describe unexplored corners of the rich biological diversity of nature in future.

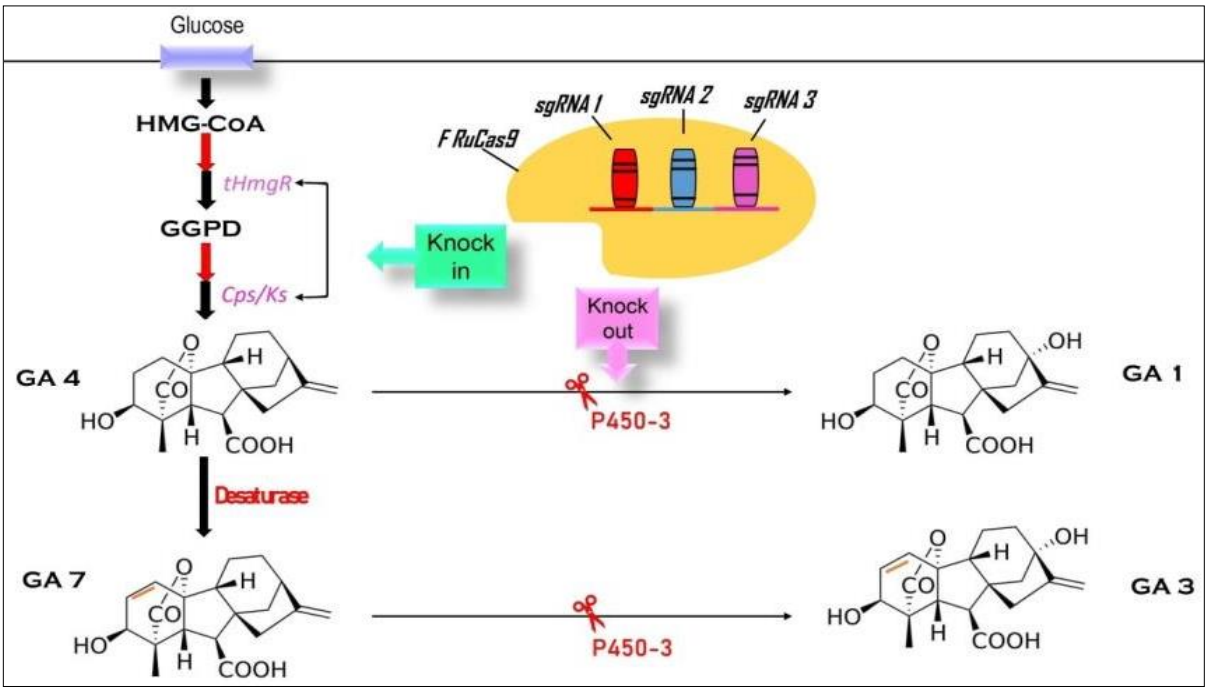

Fig 6 Application of the CRISPR/Cas9-based genome editing tool in Fusarium fujikuroi for improving the production of the gibberellic acids GA4 and GA7

CONCLUSION

As CRISPR–Cas-based treatment enters clinical testing; they hold excellent potential and promising future for curing genetic diseases and increasing cell treatments. A possible risk of utilizing gene editing technique is that the introduction of off-target changes to genome sequence, and therefore enhancing ways for detection of rare mutations and quantifying their potential risks are important for future clinical advancement. Cas9-based genome engineering serves as a latest generation tool which are based on components from the microbial antiphage defence system. It is concluded that CRISPR technology is an excellent solution for efficient and precise gene modification and can be used to describe unexplored corners of the rich biological diversity of nature in future.

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