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Recent Developments in Gene Editing Technologies: A Comprehensive Review of CRISPR applications in Biotechnology

Bairu Himalayeshwari, *Osmania University.*

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Abstract: This review article offers a thorough summary of recent advancements related to CRISPR technology that investigates its mechanisms, principles, and potential applications in biotechnology. The paper critically discusses the transformative potential of CRISPR in relevant domains such as biomedical studies, agriculture, industry, and environmental science. Even with transformative potential, CRISPR is limited by off-target effects, requirements for PAMs and efficient delivery mechanisms. The literature reviewed indicates that although the potential for reliable therapy for treating genetic conditions and enhancing stress resilience in plants is promising, there is a lack of standardized practices for assessing off-target activity and gene editing safety. Compounding surveillance, there is relatively little knowledge of the long-term implications of CRISPR usages on different ecosystems and human health. The review calls for further research into new delivery systems to improve both the efficiency and specificity of biotechnological research and practice associated with CRISPR technologies. So, the study suggests subsequent research efforts go towards creating robust protocols for the assessment of off-target effects and enhancing delivery methods. Analyses of new CRISPR technologies like base editing and prime editing should be done to establish their strengths. Ethical implications of germline editing should also be addressed to allow for the use of CRISPR technology in a responsible manner. By integrating existing literature and laying out key areas for future study this research hopes to contribute to the ongoing debate concerning CRISPR technology and its relevance to biotechnology.

Keywords: CRISPR, Gene editing, CRISPR-Cas systems, Biotechnology, CRISPR/Cas9, DNA

1. Introduction

Genome editing (GE) technology is an engineering technique that employs the method using which Intracellular DNA undergoes sequence-specific modifications. The modifications comprise of functions such as insertions, Deletions, integrations, and sequence substitutions. Research on DNA damage repair mechanism along with the resulting structural modifications of DNA have been produced as the basis of targeted GE (Guha, Wai, & Hausner, 2017).Programmable sequence-specific nucleases (SSNs) were the initial means of gene editing. Non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ) and homology-directed repair (HDR) are all types of subsequent interconnections. However, there are some that are more complex (Gao, 2021). Also, site-specific genetic or epigenetic regulations were made possible by integrating regulatory proteins and sequence identifiable programmable nucleases. Targeted GE concept was examined through the meganuclease development which was formed with fusion of engineered I-Scel and catalytically active nuclease (folk domain) with a sequence targeting capability for 18 base pairs (Lippow et al., 2009). Zinc finger nuclease (ZFN) utilizes zinc finger modules, which are 3-nt DNA sequence-recognition units each. Transcription activator-like effector nucleases (TALENs) utilize essentially the same platform as ZFN but substitute ZF proteins with 14-24 TALENs, each of which uniquely binds a 1-bp oligonucleotide by the various base of the repeat variable diresidue (RVD) (T. Li & Yang, 2013; Marraffini, 2015).

GE has become the most effective technology for gene function investigation (Z. Zhang et al., 2014). ZFN and TALEN are first- and second-generation GE tools and are popular methods. Nevertheless, owing to the complexity of the construct design and the high rate of off-targeting (Gaj, Gersbach, & Barbas, 2013), their application is restricted. CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated Protein 9), a groundbreaking third generation GE technology that has facilitated effective targeting of genes in a diverse model organism (Gagnon et al., 2014) .It has a high rate of success (80%) with an easier construction design (Hwang et al., 2013).The origin of CRISPR was traced back to the identification of a 29 bp repeat cluster downstream of the *iap* gene locus in *E. coli* by Nakata and coworkers in 1987. These DNA sequences were a specific type of clustered repeats present in >40% of bacterial species and subsequently were all grouped under Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Jansen, Embden, Gaastra, & Schouls, 2002). With these repeats were also several CRISPR72 associated genes (Cas) such as Cas9, which was found by Moineau and colleagues to be an RNA73 guided endonuclease in 2010 (Garneau et al., 2010). The Cas9 protein

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of the Type II CRISPR system of S. pyogene, the most extensively utilized for genome engineering, specifically binds two RNAs encoded by the CRISPR repeats, the crRNA (CASCADE complex in type I, Cmr or Csm RAMP complexes in type III) and the tracrRNA (transactivating CRISPR RNA) (Deltcheva et al., 2011). The hybrid crRNA/tracrRNA hybridizes to stimulate host RNASe III cleavage to a double-stranded hybrid crRNA/tracrRNA which when paired with the Cas9 protein acts to guide the scission of the target DNA 3 nucleotides 5′ from the protospacer-adjacent motif (PAM) a 3-nucleotide element adjacent to the gRNA-binding site in the target DNA. The crRNA/TracrRNA can be combined into a single chimeric guide RNA (gRNA) and Charpentier and Doudna demonstrated that a single gRNA can mediate site specific cleavage of DNA (Jinek et al., 2012). CRISPR-Cas9 was then applied for GE in mammalian cells by the groups of George Church and Feng Zhang in papers in the same issue of Science in (Cong et al., 2013; Mali et al., 2013).

With the landmark contributions of the Charpentier/Doudna and Church/Zhang labs mentioned earlier, the count of articles that refer to CRISPR utilization is doubling, about annually with 1400 papers in 2015. The CRISPR-Cas9 system is highly versatile and is currently being used not just for gene knock-out research, but for gene therapies like muscular dystrophy (Long et al., 2014). Also enzymatically lifeless dCas9 can be utilized for gene control when it is combined with transcriptional repressors and activators (reviewed in (Dominguez, Lim, & Qi, 2016) or epigenetic modification by dCas9 targeting histone modifying enzymes (Hilton et al., 2015), and chromosome tagging studies (B. Chen et al., 2013).

Investigators have employed CRISPR-Cas systems in single cell microbes to preserve genomic integrity, by counteracting the mobile/ foreign genomic elements effects (Barrangou et al., 2007), to edit and modify genomic DNA by generating double-stranded breaks (DSBs) that can subsequently lead to sequence alterations due to endogenous repair pathways (Mans et al., 2015; Ronda et al., 2015) and to modulate gene expression (Didovyk, Borek, Hasty, & Tsimring, 2016). As well, CRISPR-Cas systems offer the capacity for multiplex targeting, or targeting across multiple loci, and the possibility of creating scalable platforms to achieve genome-wide modifications. CRISPR-Cas systems can be used to complement existing methods of genetic manipulation, or add new methods of genetic manipulation for organisms that presently lack available tools for genetic manipulation (Donohoue, Barrangou, & May, 2018).

The review scope is comprehensive, detailing the study of CRISPR-Cas systems with respect to their principles, mechanisms, innovations, and issues. The review also analyzing the revolutionary reach of CRISPR in a variety of sectors such as medicine, agriculture, industry, and environmental science. The review spotlights biomedical examples such as gene therapy and disease modeling, agricultural breakthroughs such as pest-resistance crops, industrial applications in bio-manufacturing, and environmental contributions in conservation and bioremediation. Furthermore, the review discusses ethical concerns, limitations like off-target effects, and gaps in research that require further exploration. Through an examination of recent advances and suggesting future recommendations, the review seeks to present a thorough understanding of CRISPR's potential and limitations in biotechnology.

The main objectives of the review are as follows:

- To present an overview of CRISPR technology, its mechanisms, and principles, as well as recent developments.
- To critically evaluate the existing applications of CRISPR in different biotechnology industries, emphasizing its revolutionary potential.
- To discuss limitations and challenges of CRISPR technology that could hinder its extensive use.
- To address existing knowledge gaps to improve the efficiency and safety of CRISPR applications.
- To suggest future recommendations for researchers and practitioners in using CRISPR technology effectively in biotechnology.

In Section 2, the paper describes the methodology. In Section 3 outlines the mechanisms and principles bordering CRISPR technology and evaluates the different advances and methods that have occurred in the gene editing space Section 4, the study will address the application and section 5 limitations and challenges of CRISPR technology,

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Finally, the study will conclude with an assessment of the current applications of CRISPR in biotechnology and potential applications moving forward.

2. Methodology

The survey has gone through various databases to fetch the papers that are relevant to the topic. Here, the steps indulged in order to select the papers are given as follows as three predominant are exploration of databases, selection of keywords and inclusion and exclusion criteria.

Exploration of Databases

Numerous databases are explored with the aim of obtaining papers pertinent to the topic. Some of the databases that are investigated are,

- Google Scholar
- Wiley
- Elsevier
- Springer
- Pubmed

Hence, some of the databases considered are listed above. Using these databases, papers are fetched.

Selection of Keywords

Different keywords like' CRISPR', Gene Editing', 'CRISPR-Cas9, 'GE', 'Biotechnology', 'Genetic Engineering', 'Biomedical application' are some of the frequently used keywords in the databases to extract the desired papers.

Inclusion and Exclusion Criteria

Inclusion Criteria

- Only papers in English language are considered for reviewing the works.
- Papers with appropriate abstract, aim and methodology are opted by the present survey.

Exclusion Criteria

- Studies not published in English will be excluded.
- Papers that do not specifically address CRISPR or those that focus on unrelated content will be excluded.

Implementing these criteria's can effectively assist in mining the papers.

3. CRISPR Gene Editing: Principles and Mechanisms

3.1 Principles and Mechanism of CRISPR-Cas Systems

The principle behind CRISPR-Cas systems is their nature to function as a programmable, sequence-specific tool for target and nucleic acid modification. Based on prokaryote adaptive immunity, CRISPR-Cas systems are dependent upon RNA-guided targeting of distinctive DNA sequences with the ability of precise genetic alteration. The two major components making up the system are a guide RNA (gRNA) and a CRISPR-associated (Cas) protein, such as Cas9. The gRNA matches a particular DNA sequence guiding the Cas protein to the exact spot for editing. A protospacer adjacent motif (PAM) helps the gRNA stick to the target DNA, which Cas9 needs to work. Once it attaches, Cas9 cuts both DNA strands at the target site. This either stops the gene from working or allows for specific changes through the cell's repair processes. Scientists can program CRISPR-Cas systems making them valuable in biotech. They have an impact on gene editing studying gene functions and creating new treatments in many areas.

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The CRISPR-Cas machinery operates in a sequence-specific way by recognizing and cutting invasive DNA or RNA. The mechanism of defence may be separated into three phases: (i) adaptation or spacer acquisition, (ii) crRNA biogenesis, and (iii) target interference.

Adaptation

In an initial phase, a unique sequence of the invading MGE known as a protospacer is integrated into the CRISCR array resulting in a new spacer. This process allows the host organism to remember the intruder's genetic material and illustrates the adaptive character of this immune system (Barrangou et al., 2007). Two proteins, Cas1 and Cas2, appear to be universally implicated in the spacer acquisition process since they are present in nearly all CRISPR-Cas types. Exceptions are type III-C, III-D and IV CRISPR-Cas systems, with no homologous proteins. Furthermore, type V-C exhibits a minimal structure as it has only a putative effector protein known as C2C3 and a Cas1 homologue (Makarova et al., 2015; Shmakov et al., 2015). Over the past years, great progress has been made in disclosing the biochemical and genetic mechanisms of CRISPR-Cas immunity. Yet, spacer acquisition remains poorly understood (Rath, Amlinger, Rath, & Lundgren, 2015). The choice of protospacers and their processing prior to integration are still largely unknown in most CRISPR-Cas systems. More recent discoveries, however, have unraveled the biochemistry behind spacer integration. It has been shown that Escherichia coli type I-E system's Cas1 and Cas2 assemble a complex that facilitates the integration of new spacers in a process that is reminiscent of viral integrases and transposases (Nuñez, Lee, Engelman, & Doudna, 2015; Rollie, Schneider, Brinkmann, Bolt, & White, 2015). While both Cas1 and Cas2 are nucleases (Babu et al., 2011), the spacer acquisition is dispensable for catalytically active site of Cas2 (Nuñez et al., 2014). A new spacer is typically inserted at the boundary of leader-repeat of the CRISPR array whereas the first repeat of the array is replicated (Wei, Chesne, Terns, & Terns, 2015).

The mechanisms of the various CRISPR-Cas types could be conserved only partially as various studies have revealed differences with respect to adaptation machinery requirements and targets. Cas1 and Cas2 are enough to facilitate spacer acquisition in the majority of examined type I CRISPR-Cas systems, but type I-B also necessitates Cas4 for adaptation (M. Li, Wang, Zhao, & Xiang, 2014). Pseudomonas aeruginosa's type I-F CRISPR-Cas system also necessitates the interference machinery to encourage the acquisition of new spacers (Vorontsova et al., 2015). In the same way, type II-A systems need Csn2, Cas9 and tracrRNA (trans activating CRISPR RNA) for acquisition (Wei, Terns, & Terns, 2015). Yet another, to date exclusive, mode of adaptation was disclosed for a type III-B Cas1 protein that carries a reverse transcriptase. There, acquisition from DNA and RNA was described (Silas et al., 2016).

Biogenesis

To facilitate immunity, the CRISPR array is transcribed as a long precursor crRNA that is processed to mature guide crRNAs harboring the remembered sequences of invaders (Haurwitz, Jinek, Wiedenheft, Zhou, & Doudna, 2010). In type I and III systems, Cas6 family members catalyze the processing step giving rise to intermediate species of crRNAs flanked by a short 50 tag. Exception is provided by the type I-C systems, which do not encode Cas6 proteins. In this case, the protein Cas5d cuts pre-crRNA to yield intermediate crRNAs with an 11 nt 50 tag (Sashital, Jinek, & Doudna, 2011). Additional trimming of the 30 end of the intermediate crRNA by a putative nuclease can be done and yields mature crRNA species consisting of a complete spacer region (50 end) and repeat-part (30 end), which generally shows a hairpin conformation in the majority of type I systems (Charpentier, Richter, van der Oost, & White, 2015). CrRNA maturation in class 2 CRISPR-Cas systems varies considerably. In type II systems, tracrRNA is necessary for processing of the pre-crRNA. The anti-repeat sequence of tracrRNA allows for the formation of an RNA duplex with each of the repeats of the pre-crRNA, stabilized by Cas9.

The duplex is then identified and processed by the host RNase III to produce an intermediate form of crRNA that further matures by an as yet unidentified mechanism to result in the mature small guide RNA (Deltcheva et al., 2011). An RNase III-independent process was found in the type II-C CRISPR-Cas system of Neisseria meningitidis. In this, promoter sequences were found to be located within each repeat, and a few were capable of initiating transcription resulting in intermediate crRNA species. Although RNase III-mediated 30 processing of the crRNA, tracrRNA duplex was detected, it was not required for interference (Y. Zhang et al., 2013). In the type V-A CRISPR-Cas system, Cpf1 has been revealed to have a dual role during CRISPR-Cas immunity. Cpf1

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processes premature crRNAs and after another maturation event of unknown cause, employs processed crRNAs that it has produced to cut target DNA.

Interference

In the final stage of immunity, mature crRNAs are utilized as guides to specifically disrupt the invading nucleic acids. Class 1 systems utilize Cascade (CRISPR-associated complex for antiviral defence)-like complexes to bring about target degradation, whereas in class 2 systems, a single effector protein is enough for target interference. To prevent self-targeting, type I, II and V systems recognize the PAM specifically sequence that lies upstream (types I and V) or downstream (type II) of the protospacer. In type III systems, self-non-self-discrimination is facilitated through the 50 tag of the mature crRNA, which should not base pair with the target in order to facilitate degradation by the complex (Marraffini & Sontheimer, 2010).

In type I systems, Cascade recognizes invading DNA in a crRNA-dependent process and then additionally recruits the nuclease Cas3 for target cleavage. Cas3 causes a nick on the foreign DNA and then degrades the target DNA (Westra et al., 2012). In type II CRISPR-Cas systems, the tracrRNA, crRNA duplex directs the effector protein Cas9 to create a double-strand break in the target DNA [45]. The interference apparatus of type III systems include Cas10-Csm (types III-A and III-D) and Cas10-Cmr (types III-B and III-C) complexes, which can target both DNA and RNA (Garneau et al., 2010). Interestingly, it has been revealed that interference of type III-A and type III-B systems is dependent on the transcription of the target DNA (Tamulaitis et al., 2014). More specifically, the subunit Cas10 cuts the DNA whereas Csm3(Samai et al., 2015) and Cmr4 cut the transcribed mRNA in type III-A and type III-B CRISPR-Cas systems, respectively. Interference in type V CRISPR-Cas systems bears resemblance to interference in type II. An RNA duplex, which consists of tracrRNA and crRNA, are specifically necessary for target cleavage in type V-B systems Type V-A, but only utilize crRNA for localization and degradation of the target (Fonfara, Richter, Bratovič, Le Rhun, & Charpentier, 2016).

3.2 Advancements in CRISPR Technology

Since the inaugural demonstration of CRISPR-mediated gene editing, the field has progressed at record speed. First-generation DSB-dependent genome editors based on Cas9 and Cas12a nucleases' capabilities have been augmented by ongoing advancements that not only made these instruments more versatile but also precision-tuned their performance and lessened undesirable editing effects. However, fears of their safety persist, both as a result of off-target editing activity and possible genotoxic effects of on-target DSBs, (Kosicki, Tomberg, & Bradley, 2018) such as the induction of p53 (Haapaniemi, Botla, Persson, Schmierer, & Taipale, 2018).To minimize the frequency of unwanted edits, several strategies have been investigated for precise spatiotemporal control of CRISPR genome editors.

Base Editing

CRISPR-based base editors (BEs) have been created as powerful technology to introduce targeted point mutations without having to generate DSBs and offer homology. repair templates, thus facilitating editing in HDR-deficient cells (Gaudelli et al., 2017). BEs are modular chimeras of a RuvC-inactivated nickase variant of Cas9 and a nucleotide deaminase enzyme (Anzalone, Koblan, & Liu, 2020). Two classes of BEs were initially developed. Cytosine BEs (CBEs), which have catalytic domains from cytidine deaminases like APOBEC1, and an uracil glycosylase inhibitor (UGI) domain to catalyze C-to-T conversion. Adenine BEs (ABEs) produce A-to-G conversions utilizing an adenosine deaminase domain from the tRNA-specific deaminase TadA that has been evolved by direct evolution to act on ssDNA.158 When the Cas9 module is bound, BEs deaminate a cytosine or adenine within an ‘editing window’ of the displaced non-target DNA strand to uracil or inosine, respectively.

These are read out during DNA replication as thymine and guanine, respectively, causing transition point mutations. Since their discovery, the original CBE and ABE editors have undergone multiple design cycles to enhance activity and minimize the level of deaminase-induced off-target edits (Doman, Raguram, Newby, & Liu, 2020; Lam et al., 2023) and Cas12a BEs have also been engineered.165 The base editing repertoire has also been broadened to also include A-to-C (L. Chen et al., 2024) A-to-Y (Tong et al., 2023) and C-to-G (Kurt et al., 2021) transversions. Because of their predominantly predictable editing results, BEs have been used for genome-wide knockout and mutational screens (Hanna et al., 2021). The accuracy of BEs renders them ideal for therapeutic correction of single-point mutation-based diseases.

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Prime Editing

Prime editing is an HDR-independent method that was developed to make targeted point mutations, in addition to insertions or deletions. It involves a prime editing guide RNA (pegRNA) and a fusion protein construct consisting of the Cas9 nickase with an inactive HNH domain and an engineered reverse transcriptase (RT) domain. The pegRNA has a 30 -terminal extension that is complementary to the NTS of the target genomic site and bears the desired mutation. Cas9 creates a nick in the NTS, which base pairs with the complementary pegRNA extension. The mutation is introduced by RT-catalyzed extension of the 30 ends of the NTS from the pegRNA as a template. This is followed by reannealing the DNA strands to generate a 50-flap intermediate which is exercised and ligated, correcting the edit in genomic DNA. Such targeted strand synthesis enables the introduction of insertions of up to 40 bp or deletions of up to 80 bp in size, as well as point mutations as distant as 30 bp from the Cas9 nicking position. Based on the initial first-generation prime editor design, where a nickase Cas was combined with a wild-type RT from Moloney murine leukemia virus (MMLV), later PE design iterations made advancements in prime editing efficiency through the incorporation of engineered MMLV RT domains with higher thermostability and the addition of a second sgRNA that to create a nick on the non-edited strand to ensure retention of the edit in the genomic DNA (Anzalone et al., 2019).

Starting from the first-generation prime editor design, in which a nickase Cas9 was fused with a wild-type RT from the Moloney murine leukemia virus (MMLV), subsequent PE design generations brought improvements in prime editing efficiency by including engineered MMLV RT domains with enhanced thermosability and introducing a second sgRNA that to generate a nick on the non-edited strand to promote retention of the edit in the genomic DNA. Additional improvements have been achieved by inhibiting the DNA mismatch repair pathway, as well as by improving nuclear localization, expression, and DNA nicking (P. J. Chen et al., 2021; P. J. Chen & Liu, 2023).

High fidelity variants

To solve the problem of off-target activity, engineered SpCas9 variants with higher specificity have been created through two complementary strategies. An overview of currently utilized genome editor technologies based on CRISPR-associated nucleases and their derivatives. DSB-based GE: Cas9 and Cas12a nucleases allow effective gene knockouts and possess partial ability to make HDR-mediated knockin edits. Base editing: this technique involves a combination of a Cas9 nickase (nCas9) with nucleobase modification enzymes. Base editors allow one-nucleotide base direct conversion into another without inducing double-strand breaks.

This method is especially useful for the introduction of point mutations (A-to-G or C-to-T, and A-to-C or C-to-G), allowing for accurate gene correction or the introduction of stop codons for accurate gene knockouts. Prime editing: this method involves a combination of a Cas9 nickase with a reverse transcriptase (RT) and employs a prime editing guide RNA (pegRNA) that is a Cas9 sgRNA fused to an RT template (RTT) and a primer-binding site (PBS). Nicking of the non-target DNA strand facilitates its extension by RT upon the hybridization of the PBS within the pegRNA, thus reproducing the RTT sequence at the target location. Prime editing allows for inserting, deleting, and replacing short DNA sequences spanning several tens of nucleotides. Transcriptional modulators: these utilities allow RNA-guided gene transcription control by directing a deactivated Cas9 (dCas9) fused with transcriptional modulation domains (e.g., VP64 or KRAB) to gene promoters. RNA editors: departing from GE, RNA editors use RNA-targeting Cas13 nucleases, either for targeted transcript degradation (when catalytically active) or for transcript editing (when made catalytically dead and fused to adenosine deaminases). rational structure-based design of fidelity-promoting mutations, on the premise that removing particular contacts between bound DNA target and the Cas9 protein renders the Cas9-guide RNA complex more sensitive to mismatches in the substrate DNA and hence decreases the likelihood of off-target binding and cleavage (J. S. Chen et al., 2018).

Biochemical and biophysical analyses of the variants have demonstrated that the mutations significantly reduce the rate of DNA cleavage, thus enhancing off-target release (Liu et al., 2020). The second method employs directed evolution techniques to isolate mutations that lower off-target editing (Lee et al., 2018). Comparable work has been performed to design high-fidelity variants of other Cas9 and Cas12a enzymes. While the High-fidelity derivatives thus far developed provide significantly elevated specificities relative to wild-type enzymes, their efficiencies between them may differ with respect to DNA targets and use (Schmid-Burgk et al., 2020). In addition,

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since every target comes with its set of off targets with unpredictable editing frequencies (Schmid-Burgk et al., 2020) none of the nuclease variants currently available are likely to be applicable everywhere.

3.3 Limitation and Challenges

The redirection of CRISPR-Cas systems as straightforward and efficient programmable gene editing devices has significantly contributed to numerous fields of basic and applied research, paving the way for the creation of targeted gene therapies and other biotechnological applications. Nevertheless, the functional characteristics of a highly developed biological defense system are not the same as the functionalities anticipated from an accurate GE device. Therefore, the practical application potential of first-generation CRISPR-based gene editing machinery is constrained by a number of important factors, the main of which are specificity, scope for targeting, and the necessity of depending on endogenous DSB repair processes in order to realize genomic edits. Lastly, delivery of the CRISPR components is constrained by certain limitations of the delivery vectors and target cells or organisms.

Off-target activity

Natural CRISPR-Cas systems are tolerant, to some extent, of mismatches between the guide RNA and target, a probable evolutionary adaptation to the necessity of countering the high mutational rate of phages. This feature is not wanted, however, for genome engineering purposes since it can lead to the targeting and editing of partially complementary off-target sites throughout the genome alongside the aimed, on-target locus. The off-target activity of Cas9 has been reported by many studies to indicate that the enzyme is very tolerant of a significant number and diversity of nucleotide mismatches in the guide-target heteroduplex in a guide-dependent fashion. Off-targets can vary from sites with a single base mismatch to targets with several consecutive mismatches, or even nucleotide insertions or deletions (Boyle et al., 2021; Jones et al., 2021; Pacesa, Lin, et al., 2022).

Even with the tolerance of Cas9 for mismatch, most of the potential off-target locations are only bound and do not lead to dsDNA cleavage and editing as a result of intrinsic checkpoints in the Cas9 DNA binding and cleavage mechanism (Boyle et al., 2021; Jones et al., 2021; Pacesa, Loeff, et al., 2022). In addition, off-target profiling experiments have indicated that the frequency of off-target cleavage reactions is invariably lower in vivo compared with purified genomic DNA, which indicates that other factors, such as genome structure, may regulate the editing process of Cas9 within cells. However, off-target cleavage at multiple sites in the genome simultaneously can eventually lead to genomic rearrangements like deletions, inversions, or chromosomal translocations and initiate DNA damage and stress response pathways (Tsuchida et al., 2023). Off-target editing is still a primary concern for therapeutic use and has led to significant efforts to create strong and sensitive techniques for off-target edit prediction and detection and enhancing the specificity of CRISPR genome editors by molecular engineering (Lin, Zhang, Zhang, Chen, & Wong, 2020).

Targeting scope: PAM requirements

The DNA-binding mechanism of CRISPR nucleases limits their targeting scope to genomic target sites flanked by a PAM sequence. Several naturally occurring Cas9 orthologs with different PAM specificities have been discovered and used for GE, yet most of these possess even higher restrictive PAM requirements (Kim et al., 2017). Although this provides a greater specificity of targeting, and reduces off-target activity (Edraki et al., 2019), it often results in suboptimal DNA cleavage and editing efficiencies. While these significantly extend the scope of targetable locations, loose PAM targeting is correlated with a serious decline in targeting selectivity, (Collias & Beisel, 2021) enhancing the occurrence of off-target effects and lesser on-target editing efficacy owing to sequestration at off-target locations.

Cas12a nucleases are T-rich PAM sequence-specific (Swarts & Jinek, 2019) usually TTTV, but their targeting range is also limited. To bypass the limitation of PAM demands, there have been numerous artificial variants of Cas9 with altered or relaxed PAM specificities produced in the past few years, as discussed in the following section. While these significantly increase the scope of targetable locations, lenient PAM targeting is linked with a significant reduction in targeting specificity (Collias & Beisel, 2021) enhancing the possibility of off-target effects and decreased on-target editing efficiency as a result of sequestration at off-target locations.

Controlling editing outcomes

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The induction of DSBs in the genome by using targeted nucleases greatly increases the frequency of HDR in mammalian cells. Even with this, HDR application is limited to dividing cells and frequently leads to heteroallelic editing products because of concurrent editing due to end joining mechanisms (Yang et al., 2020). In human primary T cells, HDR editing efficiencies of over 80%–90% may be attained using engineered ssDNA repair templates capable of being formed into dsDNA ends that are recognizable by Cas9-RNPs, combined with small-molecule DNA repair modulators (Shy et al., 2023). Lastly, recent research has revealed that the efficacy of HDR may be enhanced through retargeting of NHEJ editing byproducts by secondary guide RNAs (Bodai, Bishop, Gantz, & Komor, 2022; Möller et al., 2022). Even with these developments, the process of introduction of knockin mutations, specifically long insertions, by means of homology templates still remains difficult. Post-mitotic, terminally differentiated cells like neurons, in which HDR does not take place at appreciable levels, at least (Nambiar, Baudrier, Billon, & Ciccia, 2022) and thus remain somewhat recalcitrant to precise editing by canonical DSB-based methodologies. These limitations, along with the genomic risks posed by unintended DSB formation, have spurred the design of GE technologies that are independent of DSB and avoid the use of HDR, such as base editing and prime editing, and most recently CRISPR-based recombinases and transposases.

Delivery

The targeted delivery of considered gene editor is still the limiting factors for many in vivo and ex vivo gene editing applications. The immunogenicity of CRISPR components and their delivery vectors presents a concern for in vivo therapeutic applications (Wagner, Peter, & Schmueck-Henneresse, 2021) Pre-existing anti-Cas9 antibodies and reactive T cells have been identified in humans (Wagner et al., 2021) and Cas9 immunity has been associated with compromised therapeutic outcomes in canine and non-human primate disease models (Hakim et al., 2021; Rothgangl et al., 2021). A number of methods for overcoming pre-existing immunity have been suggested, for instance, engineering Cas9 to remove immunogenic epitopes, regulating the immune responses, and restricting the time of Cas9 expression (Doudna, 2020).

In vivo delivery of CRISPR-Cas9 into mammalian cells is normally made using viral vectors. Adenoviruses, lentiviruses, and adeno-associated viruses (AAVs) may all be designed to replace the viral genes in the vector with gene editing modules. AAVs are still the vectors of choice for in vivo delivery because they are low in immunogenicity, highly transducible, and have a wide cell tropism. AAVs are, however, relatively small viruses with limited (4.7 kb) cargo packaging capacity (D. Wang, Zhang, & Gao, 2020).

4. Applications of CRISPR in Biotechnology

4.1 Biomedical Applications

Conventional cancer therapies (e.g., surgery, radiotherapy, chemotherapy) can postpone recurrence and extend the survival of cancer patients, but tumor recurrence or drug resistance usually results in unfavorable prognosis. Moreover, non-specifcity of chemotherapy and radiotherapy may cause toxic side effects and even death in others. Tus, novel cancer therapies continue to be required, and CRISPR/Cas9 technology promise for revolutionary advancements in cancer therapy. Somatic gene therapy has historically been defined as the introduction of new genetic information into somatic cells for the treatment of disease by the expression of therapeutic gene products. Gene therapy trials started as early as the 1980s, but these methods have met with only limited success due to issues such as gene silencing, host immune reactions, and off-target effects (B. Zhang, 2021). Despite the majority of these problems still being open, there are a number of studies demonstrating that somatic gene therapy holds favorable application prospects (Hirsch et al., 2017; Mendell et al., 2017; Russell et al., 2017).

The first clinical trial to be reported in 2014 utilized ZFNs rather than CRISPR/Cas9. Patients were treated with chronic HIV viremia and received high-potency antiretroviral treatment with CD4+ T cells genetically engineered by ZFN, with the majority of patients manifesting decreased levels of HIV DNA in blood (Tebas et al., 2014). As much as therapy did not demonstrate a persistent effect and certain hazardous adverse effects have arisen, this trial established a precedent for gene therapy. ACT is a form of immunotherapy that employs immune cells, particularly T cells, to attack tumor cells. Tumor infiltrating lymphocyte therapy is one of the first ACTs. Yet, ACT is faced with numerous practical constraints, such as difficulty in isolating sufficient qualified T cells from patients with advanced cancer and infants. There are two primary ACT approaches under investigation today, chimeric antigen receptors (CAR)-T cell therapy and transgenic T cell receptor (TCR)-T cell therapy. In the majority of

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instances, autologous T cells of the patients' blood are separated as well stimulated in vitro, then subjected to modification (genetic). Thereafter, modifed T cells are grown in vitro and then administered back to the patients (S.-W. Wang et al., 2022).

4.2 Agriculture Biotechnology

CRISPR/Cas9-mediated mutagenesis has also been successfully executed on other high-impact plant species with high mutation efficiency. (Jacobs, LaFayette, Schmitz, & Parrott, 2015) carried out the first use of CRISPR/Cas9 gene editing in soybean where gene knockout was carried out on the green fluorescent protein (GFP) gene. This breakthrough work initiated countless attempts in using CRISPR/Cas9 gene editing in soybean. (Han et al., 2019) used CRISPR/Cas9 to introduce a targeted mutation in the E1 gene in soybean flowering regulation and determined that truncation of the E1 protein inhibited the repression of the GmFT2a/5a gene, enhanced its expression, and resulted in an accelerated flowering time under long-day conditions. This conversion resulted in the creation of a photo-insensitive soybean mutant, which is potentially ideal for the starter of soybean in higher latitudes. In a similar vein, (Cai et al., 2020) elucidated the function of the GmFT2a/5a gene in soybean for the regulation of flowering periods and yield under various photoperiods through a comparative study of double-knockouts and gene overexpression using CRISPR/Cas9 technology. All these results cumulatively proved the function of some genes in soybean that can possibly make it adaptable to various environments and situations.

Also, the GmFT2a/5a double-knockout mutants have been found to possess a far higher number of pods and seeds per plants than the wild-type plant, despite having a prolonged flowering time (Cai et al., 2020). Apart from that, GmPRR37 also triggered a delay in flowering time in LD conditions and was involved in downregulating the aforementioned GmFT2a/5a, an activator of flowering, and upregulating GmFT1a which is a repressor of flowering and hence aids in regional adaptability of soybean (Wang et al., 2020). Higher productivity variants of soybeans can be developed and adapted to a broader environment on the basis of these findings. GmF3H1, GmF3H2, and GmFNSII-1 were attained successfully using a multiplex CRISPR/Cas9 in soybean and resulted in increased is flavone content in the plant while at the same time causing increased resistance in the soybean against the soybean mosaic virus (SMV) (P. Zhang et al., 2020). A number of soybean genome edits were properly passed on to later generations, suggesting that selective breeding from CRISPR/Cas9-edited soybeans may be able to create useful new crop types. Still, the heredity of CRISPR/Cas9 mutations needs more research since the effectiveness of its incidence is still quite random.

4.3 Industrial Biotechnology

As the CRISPR array is a genetic history of immunization against invasive DNA upon exposure, like a genetic immunization card, arrays can indicate strain origin and divergence within a specific bacterial species depending on the set of shared or distinct spacer sequences within an array (Sheludchenko, Huygens, Stratton, & Hargreaves, 2015). Lactobacillus buchneri strains from rotten pickle fermentations were genotyped by spacer compositions and also by CRISPR repeat sequences, and grouped into 26 different L. buchneri strains(Briner & Barrangou, 2014). CRISPR-Cas systems may also be employed to vaccinate bacteria by fighting future phage attacks. Bacteria with endogenous systems can repeatedly be challenged with phage to gain spacer sequences that provide immunity. Conversely, a heterologous system may be produced in industrial strains to yield an artificial immune system. Customized CRISPR arrays can be pre-loaded with spacers against risky phage sequences in advance, significantly shortening the prolonged routine of repeated phage challenge and survivor recovery to generate holistic immunity(Barrangou et al., 2013).

These systems have also been employed as antimicrobial selection systems by introducing spacers complementary to endogenous DNA sequences. In bacteria, the absence of NHEJ pathways or reduced expression of NHEJ components result in the lethality of CRISPR-Cas-induced DNA cutting (Bikard et al., 2014). Various groups have tried self-targeting spacers and established sequence-specific bacterial selection (Caliando & Voigt, 2015). In addition, inducible control of CRISPR-Cas system expression enables timed self-elimination and removal of a strain population from culture following the completion of fermentation or other bioprocesses. Such bacterial selection methods are of potential value in industrial consortia in which selection of strains using antibiotics is not possible or is not desirable. Also, other CRISPR-Cas systems utilize a processive nuclease in their interference, as in the case of the Type I Escherichia coli Cascade system with the associated Cas3 exonuclease. These nucleases have proven useful in eliminating plasmids from a population of bacteria and thus preventing gene expression and

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also safeguarding against inadvertent release of a plasmid into the environment (Bikard, Hatoum-Aslan, Mucida, & Marraffini, 2012; Donohoue et al., 2018).

4.4 Environmental Applications

Fourth-generation biofuels can be grouped into three classes: solar biofuels, which use photosynthetic microbes that, via synthetic biology, are able to transform solar light into fuels by the use of CO2 and water as substrates to obtain lactic acid, ethanol, hydrogen, and butanol, electro fuels are produced from photovoltaic cells and bio electrochemical systems in which microorganisms utilize CO2 as a source of carbon and electrons from electrodes as a source of energy (Patil et al., 2015) lastly, synthetic biofuels, make up the biological systems, novel devices, and metabolic pathway development for cost-effective biofuel synthesis via CO_2 and excretion of sugars (Moravvej, Makarem, & Rahimpour, 2019). Among the most prospective organisms for fourth-generation biofuel production are algae, which naturally contain a significant lipid content, which, depending on every species, may range from 2 to 63% on dry weight basis (Jacob-Lopes, Zepka, Severo, & Maroneze, 2022), meaning that its oil yield is 15 to 300 times greater than conventional crops (Rizza, Smachetti, Do Nascimento, Salerno, & Curatti, 2017).

Genetic engineering has enabled the use of the CRISPR/Cas9 system for gene editing to enhance the capabilities of various algae species, notably in increasing triacylglycerol (TAG) production and rates of cell growth. For instance, in Chlamydomonas reinhardtii, silencing the phospholipase A2 gene resulted in an increase in the lipid fraction up to 64.25% (Shin et al., 2019) moreover, C. reinhardtii mutants were developed by nullifying fatty acid degrading genes, thereby enabling lipids of 28% on a dry weight basis to be produced (Nguyen et al., 2020) in the marine microalga Tetraselmis sp., alternately, the activity of ADP-glucose pyrophosphorylase (AGP), the enzyme that catalyzes carbohydrate synthesis, was reduced because it is a metabolic route with lipid biosynthesis and resulted in mutants with 2.7 - 3.1 fold increases in lipid content over wild type(Chang et al., 2020).

5. Research Gap

There are some inherent limitations to the study, even though this review offers a thorough overview of CRISPR technology, including its mechanisms, recent developments, and biotechnology applications. First, because it is a review, this study draws from the body of existing literature and excludes experimental data and original research. As a result, the conclusions are restricted to the extent and caliber of previously published research, which might not adequately account for new or unreported developments in CRISPR technology. Secondly, although this research critically assesses the uses of CRISPR in biomedical, agricultural, industrial, and environmental sectors, it fails to examine in-depth case studies or industry-specific issues of implementation. This general strategy is likely to miss incredibly detailed insights that might be gained by more in-depth examination within individual industries. The review also reviews limitations and challenges of CRISPR technologies with regard to off-target activity, PAM requirement, delivery modalities (factors that impact utility especially in preclinical and clinical testing), and ethical considerations. Nonetheless, because of the many topics discussed, some of the technical issues, such as comparative performance metrics for next-generation tools such as base editing and prime editing, are described more generally than with exhaustive detail.

Overall, while the study does a great job of pinpointing research gaps and suggesting future directions for enhancing CRISPR applications in biotechnology, it falls short of offering practical experimental frameworks or methods to tackle these issues. Consequently, the study acts more like a roadmap for researchers rather than a direct solution to these challenges.

6. Conclusion

This review concludes by highlighting the remarkable developments and revolutionary potential of CRISPR technology in the biotechnology industry. CRISPR's capacity to precisely modify genes has created new opportunities for innovation in several fields, including environmental management, industrial processes, agriculture, and medicine. From improving crop resilience and developing gene therapies for genetic disorders to developing sustainable bioprocesses and tackling ecological issues, the applications are numerous. But even with these encouraging advancements, there are still critical issues that need to be resolved if CRISPR is to reach its full potential. Major limitations including off-target effects that may cause unforeseen genetic alterations and the high demands of PAM that limit the scope of accessible genomic sites are key concerns requiring additional

investigation. The intricacies of delivery systems for CRISPR elements also present a limitation to successful application in vivo. Ethical implications regarding germline editing and the longer-term ecological consequences of CRISPR uses also require keen analysis. Thus, a combined effort must be made to surmount these issues while ensuring that CRISPR technology is responsibly and ethically developed.

7. Future Recommendation

Several important suggestions are made to close the gaps found and progress the field of CRISPR biotechnology. Standardized procedures must be established first to assess off-target effects and the effectiveness of high-fidelity variants. This standardization will improve clinical application safety and streamline regulatory approval procedures. Second, novel approaches to get around PAM restrictions should be sought, like creating new Cas proteins or using different GE programs that do not require PAM sequences. The range of targetable genomic regions may be expanded by this research. Finally, there is a need for substantial investment in optimizing delivery mechanisms for CRISPR components to maximize editing efficiency and reduce side effects. This may include investigating new delivery vehicles like nanoparticles or viral vectors designed for tissues or cell types. Through such consensus-building between research, industry players, and government regulators, however, the world can unlock CRISPR's full potential as a transformative scientific technology and drive innovative breakthroughs for society in accordance with what is ethically acceptable.

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