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CRISPR-Cas9 Technology in Bt Genome Editing and Functional Studies

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Abstract CRISPR-Cas9 technology, with its unparalleled precision and efficiency, has revolutionized the field of genome editing. In *Bacillus thuringiensis* (Bt), widely used as a biopesticide, genome editing holds great promise for significantly enhancing its efficacy and functional understanding. This study provides an overview of the mechanisms and advancements of CRISPR-Cas9, comparing it with other genome editing technologies. It delves into its applications in Bt, including gene knockout, knock-in strategies, multiplex genome editing, and targeted mutagenesis. Additionally, it explores the functional studies of Bt genes, covering gene function identification, overexpression, gene silencing, and toxin gene analysis. This study discusses methodological advancements and challenges such as delivery methods, off-target effects, and optimization. Case studies showcase successful gene editing examples, insights gained, and best practices. It also examines ethical and regulatory issues as well as public perception. Finally, it discusses future directions, emerging trends, novel applications, and the potential impact on industrial and agricultural biotechnology. Continuous research and development in CRISPR-Cas9 technology are crucial to fully realizing its potential in Bt genome editing.

Keywords CRISPR-Cas9; *Bacillus thuringiensis*; Genome editing; Gene knockout; Biopesticides

1 Introduction

CRISPR-Cas9 technology has revolutionized the field of genome editing since its discovery as a bacterial adaptive immune system (Wang et al., 2016). The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system, coupled with the Cas9 (CRISPR-associated protein 9) endonuclease, allows for precise and targeted modifications of DNA sequences in a wide range of organisms (Hsu et al., 2014). This system operates by utilizing a guide RNA (gRNA) to direct the Cas9 protein to specific genomic loci, where it introduces double-strand breaks. These breaks can then be repaired through non-homologous end joining (NHEJ) or homology-directed repair (HDR), enabling the insertion, deletion, or modification of genetic material (Ran et al., 2013). The simplicity, efficiency, and versatility of CRISPR-Cas9 have made it an indispensable tool in genetic research, with applications spanning from basic biology to therapeutic interventions (Hossain, 2021).

Bacillus thuringiensis (Bt) is a gram-positive bacterium renowned for its ability to produce insecticidal toxins, which are widely used in agriculture to control pest populations (Li et al., 2021). The genetic manipulation of Bt is crucial for enhancing its insecticidal properties, understanding its pathogenic mechanisms, and developing new biotechnological applications (Chen et al., 2017). Traditional methods of genetic modification in Bt have been labor-intensive and time-consuming. However, the advent of CRISPR-Cas9 technology has provided a powerful and efficient means to edit the Bt genome with high precision (Kirchner and Schneider, 2015). This has opened new avenues for the functional study of Bt genes, the development of novel Bt strains with improved insecticidal activity, and the exploration of Bt's potential in other biotechnological fields (Demirci et al., 2018).

This study is to provide a comprehensive overview of the current state of CRISPR-Cas9 technology as it pertains to genome editing and functional studies in *Bacillus thuringiensis*. This review aims to summarize the advancements in CRISPR-Cas9 technology and its applications in genome editing and highlight the specific challenges and solutions associated with using CRISPR-Cas9 in Bt, discuss the potential biotechnological applications of CRISPR-Cas9-mediated genome editing in Bt and Identify future research directions and technological improvements needed to enhance the efficiency and precision of CRISPR-Cas9 in Bt. By

synthesizing the latest research findings, this study seeks to underscore the transformative impact of CRISPR-Cas9 on Bt genome editing and its broader implications for biotechnology and agriculture.

2 Overview of CRISPR-Cas9 Technology

2.1 Mechanism of CRISPR-Cas9

The CRISPR-Cas9 system, derived from the adaptive immune system of bacteria, has revolutionized genome editing by providing a precise and efficient method for modifying DNA sequences. The core components of this system include the Cas9 protein, an RNA-guided DNA endonuclease, and a guide RNA (gRNA) that directs Cas9 to specific genomic loci. The gRNA contains a 20-nucleotide sequence complementary to the target DNA, enabling Cas9 to introduce double-strand breaks (DSBs) at the desired location (Ran et al., 2013). Upon binding to the target DNA, the Cas9 protein induces DSBs, which can be repaired by the cell's endogenous repair mechanisms, primarily non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ often results in insertions or deletions (indels) that can disrupt gene function, while HDR can be used to introduce precise genetic modifications using a donor DNA template (Zhang et al., 2014). To enhance specificity and reduce off-target effects, a double-nicking strategy using a Cas9 nickase mutant with paired gRNAs has been developed (Gupta et al., 2019).

2.2 Advances in CRISPR-Cas9 applications

CRISPR-Cas9 technology has seen rapid advancements and diverse applications across various fields. In biomedical research, it has been employed for gene knockouts, regulation of gene expression, and creation of disease models (Wang et al., 2016). The versatility of CRISPR-Cas9 extends to its use in live-cell imaging and epigenetic modifications, where a nuclease-deactivated form of Cas9 (dCas9) is used to target specific genomic regions without inducing DSBs (Sander and Joung, 2014). In agriculture, CRISPR-Cas9 has been instrumental in crop improvement by enabling precise genetic modifications to enhance traits such as disease resistance, drought tolerance, and nutritional content (Bortesi et al. 2015). The development of CRISPR ribonucleoproteins (RNPs) has further improved the efficiency and specificity of genome editing in plants, addressing some limitations associated with plasmid-based systems8.

2.3 Comparison with other genome editing techniques

CRISPR-Cas9 stands out among genome editing technologies due to its simplicity, efficiency, and versatility. Compared to earlier methods such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR-Cas9 is easier to design and implement, as it requires only the synthesis of a gRNA complementary to the target sequence (Arora and Narula, 2017). This ease of use has democratized genome editing, making it accessible to a broader range of researchers. While ZFNs and TALENs also induce DSBs at specific genomic loci, they rely on protein-DNA interactions, which are more complex to engineer and less flexible than the RNA-DNA interactions used by CRISPR-Cas9. Additionally, CRISPR-Cas9 can be multiplexed to target multiple genes simultaneously, a feature that is more challenging to achieve with ZFNs and TALENs. Despite its advantages, CRISPR-Cas9 is not without challenges. Off-target effects, where unintended genomic sites are edited, remain a concern. However, ongoing improvements in gRNA design, delivery methods, and the development of high-fidelity Cas9 variants are addressing these issues, enhancing the precision and reliability of CRISPR-Cas9-mediated genome editing (Wang et al., 2017). In summary, CRISPR-Cas9 technology has transformed the landscape of genome editing, offering unprecedented precision and flexibility. Its continued development and application hold great promise for advancing our understanding of genetics and improving human health and agriculture.

3 Applications of CRISPR-Cas9 in Bt Genome Editing

3.1 Gene knockout and knock-in strategies

CRISPR-Cas9 technology has revolutionized the field of genome editing by enabling precise modifications such as gene knockouts and knock-ins. This system utilizes small guide RNAs (sgRNAs) to direct the Cas9 nuclease to specific genomic loci, where it creates double-strand breaks. These breaks can be repaired in an error-prone manner to generate knockouts or through homologous recombination with donor DNA to create knock-ins

(Tavakoli et al., 2021). For instance, in mice, CRISPR-Cas9 has been employed to rapidly generate both knockout and knock-in models by microinjecting Cas9, sgRNA, and donor DNA into zygotes (Hall et al., 2018). Additionally, the system has been optimized to enhance targeting efficiency and specificity, which is crucial for successful genome editing experiments (Campenhout et al., 2019).

3.2 Multiplex genome editing

One of the significant advancements of CRISPR-Cas9 technology is its ability to perform multiplex genome editing, where multiple genes can be targeted simultaneously. This is particularly useful for studying complex genetic interactions and pathways. For example, in *Dictyostelium*, the CRISPR-Cas9 system has been used to generate mutants in five different *PI3K* genes simultaneously, achieving high mutagenesis frequencies. The use of an all-in-one vector containing Cas9 and multiple sgRNAs facilitates this process (Figure 1), allowing for efficient and transient expression without integrating drug resistance cassettes into the genome (Sekine et al., 2018). This multiplex capability has also been demonstrated in primary cell cultures, where CRISPR-Cas9 ribonucleoprotein complexes have been used to achieve high-efficiency gene knockouts and targeted deletions (Hoellerbauer et al., 2020).

Sekine et al. (2018) found that using an endogenous tRNA system to express sgRNAs can lead to efficient sgRNA expression, as demonstrated by RT-PCR analysis. The study also predicted the secondary structure of tRNA-sgRNA and identified the tRNA cleavage site necessary for releasing sgRNA. Sequence analysis of the tRNA-sgRNA junction through cRT-PCR revealed consistent sequences across multiple clones, with specific recognition sites for inserting oligonucleotides. Additionally, the sub-nuclear localization of dCas9-GFP was examined under different concentrations of hygromycin B, showing varying fluorescence distributions. This indicates the uniformity and efficiency of dCas9-GFP expression when maintained with specific antibiotic concentrations. The findings suggest that tRNA systems can be effectively utilized for sgRNA expression and dCas9 targeting within the nucleus.

3.3 Targeted mutagenesis

CRISPR-Cas9-mediated targeted mutagenesis offers a powerful tool for functional genomics and crop improvement. This technology allows for precise modifications at specific genomic sites, enabling the study of gene function and the development of crops with enhanced traits. For instance, targeted mutagenesis has been employed to improve crop yields under biotic and abiotic stresses by creating gene knockouts and modifications. The high efficiency and accuracy of CRISPR-Cas9 make it a valuable tool for generating targeted mutations, which can lead to the development of crops with greater resilience to environmental stressors (Abdelrahman et al., 2018). Additionally, the system's ability to perform high-throughput gene screening and live-cell labeling further expands its applications in functional genomics (Gupta et al., 2019).

4 Functional Studies of Bt Genes

4.1 Identifying gene function through knockouts

CRISPR-Cas9 technology has revolutionized the ability to perform gene knockouts, allowing researchers to precisely disrupt specific genes to study their functions. This method has been widely used in various organisms, including Bt (*Bacillus thuringiensis*), to understand the roles of individual genes. For instance, the CRISPR-Cas9 system has been employed to generate gene knockouts in primary T cells, achieving near-complete loss of target gene expression without the need for selection, thus simplifying the process of gene function discovery (Figure 2) (Seki and Rutz, 2018). Additionally, the use of CRISPR-Cas9 in patient-derived xenografts (PDXs) has enabled the analysis of genetic dependencies by targeted gene disruption, demonstrating the system's utility in functional genomics (Hulton et al., 2020).

Seki and Rutz (2018) found that using an endogenous tRNA system for expressing sgRNAs significantly enhances sgRNA production, as shown by RT-PCR results. They also predicted the secondary structure of the tRNA-sgRNA complex and identified a crucial tRNA cleavage site required for sgRNA release. Sequence analysis confirmed the accuracy of the tRNA-sgRNA junction, indicating consistent integration across multiple

clones. Furthermore, varying concentrations of hygromycin B affected the sub-nuclear localization of dCas9-GFP, with higher concentrations leading to more uniform fluorescence distribution. These findings demonstrate that tRNA systems can effectively be employed to regulate sgRNA expression and ensure precise dCas9 targeting within the nucleus, paving the way for more efficient CRISPR/Cas9 applications in genetic engineering and research.

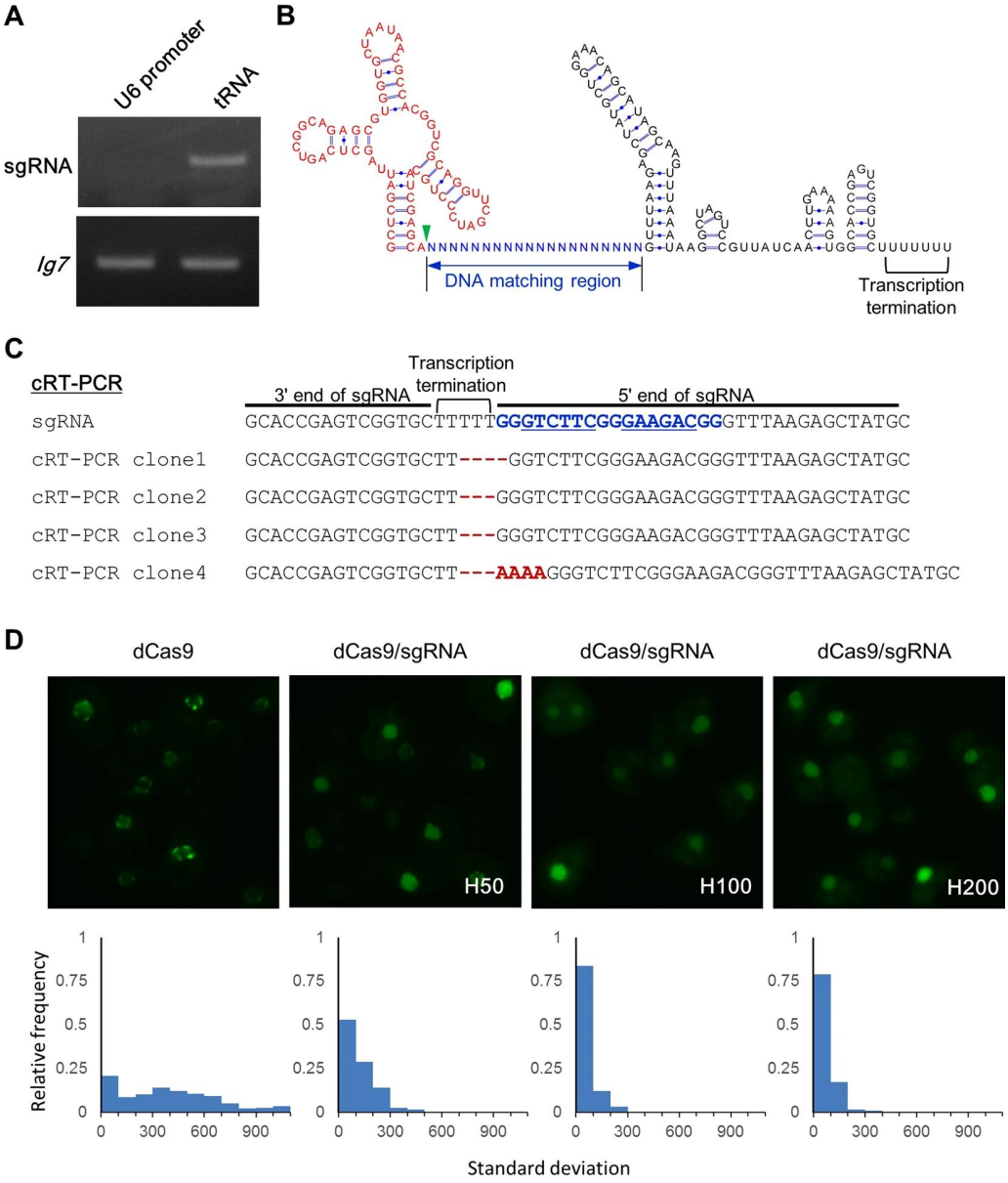

Figure 1 Endogenous tRNA system for expressing sgRNAs (Adopted from Sekine et al., 2018)

Image capton: (A) Comparison of sgRNA expression using different promoters. RT-PCR of sgRNA is presented. The lower panel reveals Ig7 as an internal control. Gel images were cropped, but no other bands were present. (B) Predicted secondary structure of tRNA-sgRNA. The nucleotides of isoleucine tRNA are indicated in red, and the DNA matching region is presented in blue. The green arrowhead indicates the tRNA cleavage site to release sgRNA. (C) Sequence analysis of the tRNA-sgRNA junction by cRT-PCR. Sequences of four independent clones are presented. As empty sgRNA vector was sequenced, the DNA matching region shown in blue contains two BpiI sites (underlined) to insert a pair of annealed oligonucleotides. The extra nucleotide at 5′ region is given in red. (D) The sub-nuclear localisation of dCas9-GFP. All the dCas9-expressing transformants were maintained with 60 μg/ml G418 for a few days before imaging. Cells expressing sgRNA were cultured with hygromycin B at 50, 100 and 200 μg/ml, respectively. Graphs reveal a histogram of standard deviation (SD) of fluorescence distribution within the nucleus. Lower SD indicates a uniform distribution of dCas9-GFP in the nucleus (Adopted from Sekine et al., 2018)

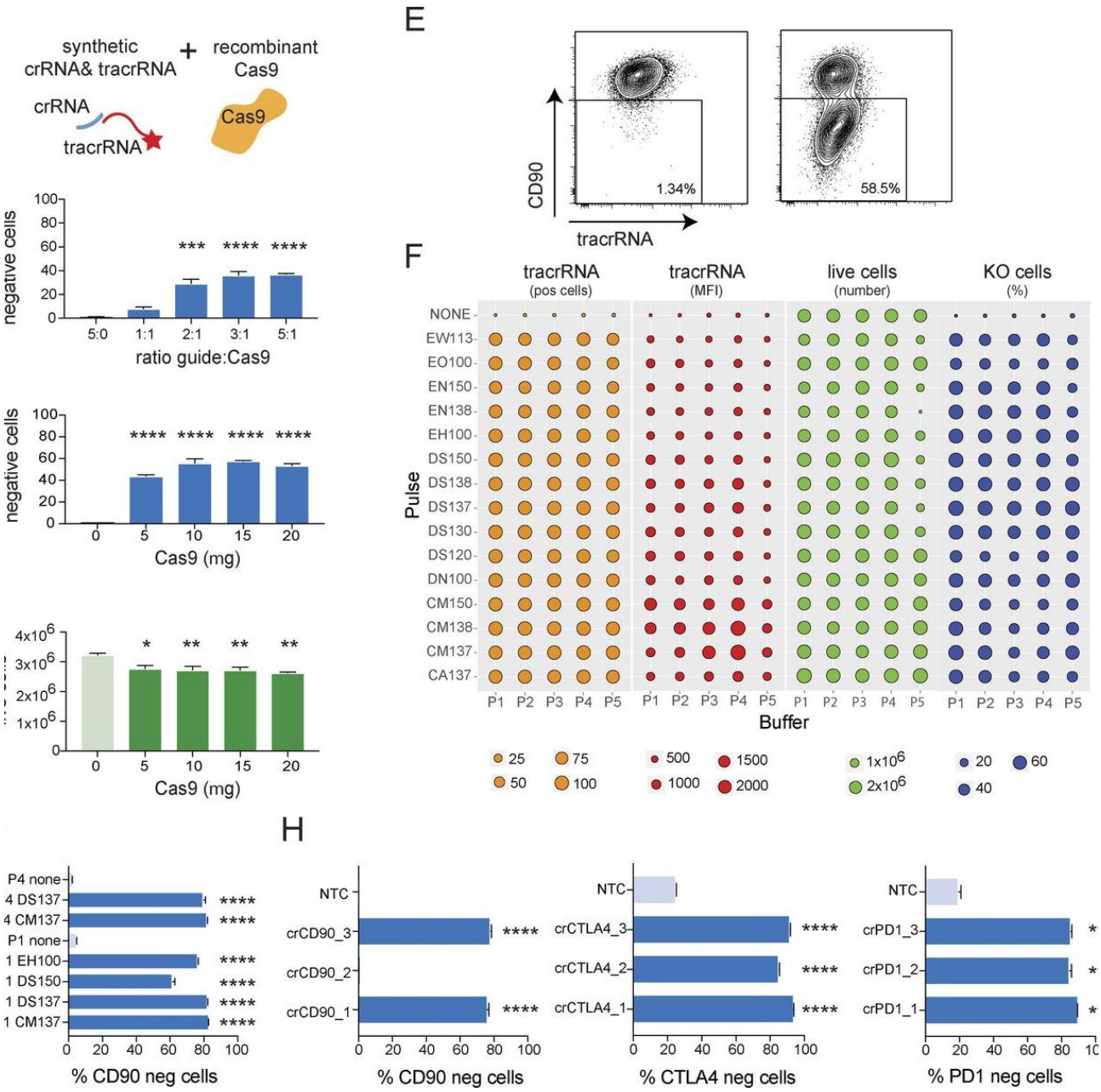

Figure 2 Nucleofection of RNPs leads to highly efficient target gene KO in activated mouse T cells (Adopted from Seki and Rutz, 2018)

Image capton: (A) Schematic depiction of RNP components, chemically stabilized crRNA, fluorescently labeled tracrRNA, and recombinant Cas9 protein. (B) KO efficiency as measured by CD90-negative CD8+ T cells 72 h after nucleofection of RNPs (DN100/P3) targeting CD90, and titration of gRNA to Cas9 ratio. Data are presented as mean ± SD (n = 2) and representative of two independent experiments. (C and D) KO efficiency as measured by CD90-negative CD8+ T cells (C) and cell viability 72 h after nucleofection of RNPs (DN100/P3) targeting CD90 (D), and titration Cas9 amount. Data are presented as mean ± SD (n = 2) and representative of two independent experiments. (E) Example of KO efficiency of RNP transfection targeting CD90 with 3:1 RNA/Cas9 ratio and 10 µg Cas9 3 d after transfection. (F) Systematic optimization of nucleofection parameters for RNP transfection of activated mouse CD8+ T cells. Analysis of transfection efficiency (ATTO550 expression and MFI), cell viability, and CD90 KO frequency 48 h after transfection. Data are from one experiment. (G) Comparison of KO efficiency by flow cytometry after RNP transfection using selected nucleofection pulses and buffers for targeting CD90 in CD8+ activated mouse T cells. Data are presented as mean ± SD (n = 2) and representative of two independent experiments. (H) KO efficiency as measured by flow cytometry using optimized RNP transfection in activated mouse CD8 T cells targeting CD90, CTLA4, or PD1 compared with target expression in cells transfected with NTC. Data are presented as mean ± SD (n = 2) and representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by one-way ANOVA (Adopted from Seki and Rutz, 2018)

4.2 Overexpression and gene silencing

Beyond gene knockouts, CRISPR-Cas9 can also be used for gene overexpression and silencing, providing a comprehensive toolkit for functional studies (Zhou et al., 2024). The CHOPCHOP web tool, for example, supports CRISPR activation and repression, allowing researchers to modulate gene expression levels in Bt and other organisms. This capability is crucial for studying gene function in a more dynamic context, as it enables the investigation of both loss-of-function and gain-of-function phenotypes. Moreover, the CRISPR-Cas9 system has been adapted to target RNA, expanding its applications to include the regulation of endogenous gene expression and the study of alternative transcript isoforms (Labun et al., 2019).

4.3 Functional analysis of toxin genes

Bt is well-known for its production of insecticidal toxins, and CRISPR-Cas9 technology has been instrumental in dissecting the functions of these toxin genes. By introducing targeted mutations, researchers can study the effects of specific gene alterations on toxin production and activity. For example, CRISPR-Cas9 has been used to investigate the functional impact of gene modifications in immune cells, which can be analogous to studying toxin gene function in Bt. Additionally, the system's ability to induce large gene modifications, such as deletions and insertions, allows for a detailed analysis of the genetic elements involved in toxin gene regulation and expression (Hulton et al., 2020). In summary, CRISPR-Cas9 technology provides a versatile and powerful approach for functional studies of Bt genes, enabling precise gene knockouts, overexpression, and silencing, as well as detailed analyses of toxin gene functions. This technology continues to advance our understanding of Bt biology and its applications in biotechnology and agriculture.

5 Methodological Advances and Challenges

5.1 Delivery methods for CRISPR-Cas9 in Bt

The delivery of CRISPR-Cas9 components into *Bacillus thuringiensis* (Bt) cells is a critical step for successful genome editing. Various strategies have been developed to enhance the efficiency and specificity of CRISPR-Cas9 delivery. One promising approach is the use of ribonucleoprotein (RNP) complexes, which consist of Cas9 protein and single guide RNA (sgRNA). This method offers the advantage of transient genome editing and reduced off-target effects (Zhang et al., 2021). Additionally, both viral and non-viral delivery systems have been explored. Viral vectors, such as adenoviruses and lentiviruses, are effective but come with limitations like immunogenicity and limited packaging capacity. Non-viral methods, including lipid- or polymer-based nanocarriers, have shown potential due to their lower risk of carcinogenesis and immune response. Despite these advancements, challenges remain in achieving efficient and targeted delivery in vivo, necessitating further research and development.

5.2 Off-target effects and specificity

Off-target effects are a significant concern in CRISPR-Cas9 genome editing, as unintended modifications can lead to adverse outcomes. Various strategies have been developed to minimize these effects. High-fidelity Cas9 variants and paired nickases have been engineered to enhance specificity (Manghwar et al., 2020). Computational tools and experimental methods have been employed to predict and validate off-target sites, thereby improving the precision of genome editing (Guo et al., 2023). Additionally, the use of truncated sgRNAs and partial DNA replacement in guide RNAs has been shown to reduce off-target activity while maintaining on-target efficiency8 10. Despite these advancements, off-target effects remain a critical hurdle, and ongoing research aims to develop more robust methods to mitigate these risks (Gupta et al., 2019).

5.3 Efficiency and optimization

The efficiency of CRISPR-Cas9 genome editing in Bt can be influenced by several factors, including the design of sgRNAs, the delivery method, and the cellular environment. Optimizing sgRNA design is crucial for enhancing target specificity and reducing off-target effects. Tools for sgRNA design and evaluation have been developed to assist researchers in selecting the most effective guides1. Additionally, the delivery method plays a significant role in the overall efficiency of genome editing. RNP delivery has been highlighted for its high efficiency and

reduced off-target effects compared to plasmid-based methods. Furthermore, optimizing the cellular environment, such as by enhancing the expression of repair proteins, can improve the efficiency of CRISPR-Cas9-mediated genome editing (Wei et al., 2020). Continuous advancements in these areas are essential for achieving high-efficiency and precise genome editing in Bt.

6 Case Studies of CRISPR-Cas9 in Bt Research

6.1 Successful gene editing examples

CRISPR-Cas9 technology has been successfully applied in *Bacillus thuringiensis* (Bt) for gene editing, demonstrating its potential in both chromosomal and plasmid gene deletions. One notable example is the deletion of the protease genes nprA (neutral protease A) and aprA (alkaline protease A) in Bt. This was achieved by using a *Lactobacillus plantarum*-derived plasmid, which reduced Cas9 toxicity due to its lower copy number, thereby facilitating efficient gene editing (Soonsanga et al, 2020). Additionally, the successful editing of the plasmid vip3A gene required the use of a Bacillus-derived plasmid with longer homology sequences for the repair template, showcasing the versatility of CRISPR-Cas9 in different genetic contexts within Bt (Figure 3) (Mollashahi et al., 2023).

Mollashahi et al. (2023) found that advancements in CRISPR technology have enabled precise genetic modifications through a series of innovations. Initially, CRISPR-Cas9 was utilized to create double-strand breaks in DNA, guided by synthetic single guide RNA (sgRNA) designed for specific targets. Subsequent modifications led to the development of nickase Cas9 (nCas9), which introduced single-strand cuts, allowing for targeted base editing by coupling base editor domains to the Cas9 protein. The latest iteration, prime editing, employs nCas9 to make precise cuts, with sgRNA designed to guide the reverse transcriptase enzyme to convert the sgRNA into cDNA, facilitating highly accurate genetic modifications. These advancements highlight the progressive refinement of CRISPR tools, enhancing their applicability and precision in genetic engineering.

6.2 Insights gained from functional studies

Functional studies using CRISPR-Cas9 in Bt have provided significant insights into gene function and regulation. For instance, the modulation of Cas9 levels was found to be crucial for efficient gene editing. By replacing the Cas9 promoter with a sporulation-specific promoter, researchers were able to obtain a Bt ΔnprA clone, although reproducibility was an issue with this construct. These studies highlight the importance of promoter selection and plasmid copy number in optimizing CRISPR-Cas9-mediated gene editing in Bt. Furthermore, the differential impact of plasmid copy number and homology arm length on gene editing efficiency underscores the need for tailored approaches depending on the target gene and desired outcome (Li et al., 2018).

6.3 Lessons learned and best practices

Several lessons have been learned from the application of CRISPR-Cas9 in Bt research, leading to the development of best practices for future studies. One key lesson is the importance of selecting appropriate plasmid vectors to minimize Cas9 toxicity and enhance editing efficiency. Using plasmids with lower copy numbers, such as those derived from *Lactobacillus plantarum*, can significantly reduce Cas9-induced lethality and improve the chances of obtaining desired mutants. Additionally, the length of homology arms in the repair template plays a critical role in the success of plasmid gene editing, with longer sequences being more effective. Best practices also include careful consideration of promoter selection to control Cas9 expression levels, as this can impact the efficiency and reproducibility of gene editing. Employing sporulation-specific promoters or other context-specific regulatory elements can help fine-tune Cas9 activity to achieve optimal results. Finally, the use of multiple screening steps to identify successful gene edits is recommended to ensure the accuracy and reliability of the edited strains. By adhering to these best practices and leveraging the insights gained from previous studies, researchers can continue to advance the application of CRISPR-Cas9 technology in Bt, paving the way for new discoveries and innovations in genetic research and biotechnology (Zhou et al., 2024).

7 Ethical and Regulatory Considerations

7.1 Ethical issues in genome editing

The advent of CRISPR-Cas9 technology has revolutionized genome editing, offering unprecedented precision and efficiency. However, it has also raised significant ethical concerns. One of the most contentious issues is the potential for human germline modifications, which could lead to unforeseen and undesirable effects, including the risk of eugenics and the ethical dilemma of informed consent. The possibility of creating permanent changes in the human genome that can be passed on to future generations necessitates a thorough ethical review and stringent oversight. Additionally, the use of CRISPR-Cas9 in agriculture and the environment also poses ethical questions, such as the potential impact on biodiversity and ecosystem balance (Barman et al., 2019).

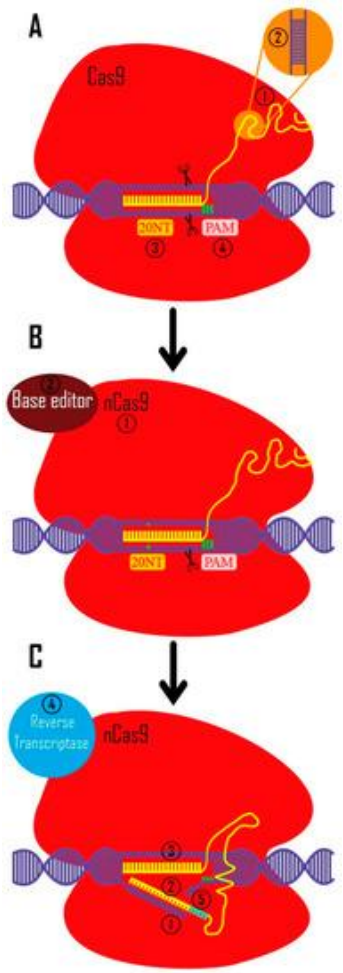

Figure 3 CRISPR toolkit (Adopted from Mollashahi et al., 2023)

Image capton: (A) CRISPR technology was originally used to create double-strand breaks in eukaryotic DNA (with a bacterial origin (*Streptococcus pyogenes*). 1. In bacteria, crRNA and tracrRNA guide Cas9 to target the intended region. These RNAs are artificially synthetized as a unique sgRNA to be more applicable in other creatures (yellow) 2. crRNA and tracrRNA are widely used in multiple experimental systems (e.g., mouse embryo microinjections, RNP electroporation into mammalian cell lines, etc.) [91,92] 3. Twenty nucleotides complementary to the target site are used to identify the target area (these nucleotides are designed in a targeted manner). 4. Before these 20 nucleotides, there are three PAM nucleotides (5′-NGG-3′ in *Streptococcus pyogenes* Cas9 system) which are necessary for CRISPR/Cas9 function. (B) 1. In order to modify the bases in a targeted way, the Cas9 protein was altered to cut only one strand of DNA by changing one amino acid in Cas9 protein (nickase Cas9 [nCas9]). 2. Additionally, they coupled the different base editor domains to the Cas9 protein. (C) 1. Prime editing, the subsequent iteration of this technique, cuts a DNA strand by creating a cut at the intended location. 2 and 3. The sgRNA is made in such a way that its 3′-end complements the two sides of cut site, and its 5′-end can recognize the target site. 4. The reverse transcriptase enzyme turns 3′ sgRNA into cDNA using this 3′ end as a primer. 5. In the cut region, bases are designed for knock-in to produce highly accurate results (Adopted from Mollashahi et al., 2023)

7.2 Regulatory frameworks

The rapid development and application of CRISPR-Cas9 technology have outpaced existing regulatory frameworks, necessitating the creation of new guidelines and regulations. Different countries have adopted varying approaches to regulate CRISPR-Cas9 applications. For instance, some nations have stringent regulations that limit the use of this technology to specific research contexts, while others have more permissive frameworks that allow broader applications under strict oversight (Shinwari et al., 2018). International cooperation and harmonization of regulatory standards are crucial to ensure the safe and ethical use of CRISPR-Cas9 technology globally. The establishment of comprehensive legislation that balances scientific freedom with ethical considerations is essential for the responsible advancement of genome editing technologies (Memi et al., 2018).

7.3 Public perception and acceptance

Public perception and acceptance of CRISPR-Cas9 technology play a critical role in its development and application (Zhou et al., 2024). There is a need for transparent communication and public engagement to address the ethical, moral, and safety concerns associated with genome editing. Public awareness campaigns and educational initiatives can help demystify the technology and foster informed discussions about its potential benefits and risks. Engaging with various stakeholders, including ethicists, policymakers, religious scholars, and the general public, is essential to build trust and ensure that the societal implications of CRISPR-Cas9 are thoroughly considered. Public acceptance is likely to be influenced by the perceived benefits of the technology, such as its potential to treat genetic diseases and improve agricultural productivity, as well as the robustness of regulatory frameworks in place to mitigate risks (Ayanoğlu et al., 2020). In conclusion, while CRISPR-Cas9 technology holds immense promise, addressing the ethical and regulatory challenges is crucial for its responsible and sustainable development. A balanced approach that considers ethical issues, establishes robust regulatory frameworks, and engages the public will be key to harnessing the full potential of this revolutionary technology (Eş et al., 2019).

8 Future Directions and Potential Applications

8.1 Emerging trends in genome editing

The CRISPR-Cas9 technology has revolutionized genome editing, and recent advancements continue to expand its capabilities. Emerging trends include the development of more precise gene editing techniques such as base editing and prime editing, which allow for targeted nucleotide changes without inducing double-strand breaks (Zhu et al., 2020). These advancements are crucial for minimizing off-target effects and increasing the specificity of genome edits. Additionally, novel CRISPR variants like xCas9 and Cas12a (Cpf1) have been engineered to overcome PAM restrictions and improve editing efficiency. The integration of CRISPR with other biotechnologies, such as RNA interference and transcription activator-like effector nucleases (TALENs), further enhances its versatility and application scope (Eş et al., 2019)。

8.2 Novel applications in Bt research

In the context of *Bacillus thuringiensis* (Bt) research, CRISPR-Cas9 technology offers promising avenues for functional studies and genetic modifications. The ability to create targeted gene knockouts and insertions can help elucidate the roles of specific genes in Bt's pathogenicity and environmental interactions (Bishnoi, 2023). Moreover, CRISPR can be employed to engineer Bt strains with enhanced insecticidal properties or broader host ranges, thereby improving their efficacy as biopesticides. The development of multiplexed gene editing techniques allows for simultaneous modifications of multiple genes, facilitating comprehensive functional analyses and the creation of Bt strains with complex trait improvements (Huang and Puchta, 2021).

8.3 Potential for industrial and agricultural biotechnology

The potential applications of CRISPR-Cas9 in industrial and agricultural biotechnology are vast. In agriculture, CRISPR can be used to develop crop varieties with improved yield, quality, and resistance to diseases and environmental stresses. For instance, CRISPR-edited crops with enhanced nutritional profiles or reduced allergenicity are already being explored. In industrial biotechnology, CRISPR can be utilized to optimize

microbial strains for the production of biofuels, pharmaceuticals, and other valuable biochemicals (Schulze and Lammers, 2020). The ability to precisely edit metabolic pathways and regulatory networks in industrial microorganisms can lead to more efficient and sustainable bioprocesses. In summary, the future of CRISPR-Cas9 technology in Bt genome editing and functional studies is promising, with emerging trends focusing on increased precision and novel applications in both research and biotechnology. The continued development and refinement of CRISPR tools will undoubtedly drive further innovations and applications in these fields.

9 Concluding Remarks

The application of CRISPR-Cas9 technology in Bt genome editing and functional studies has demonstrated significant advancements and potential. The key findings from various studies highlight the efficiency, precision, and versatility of CRISPR-Cas9 in targeted genome editing. This technology has been successfully employed to achieve gene knockouts, regulate endogenous gene expression, and create disease models, thereby expanding the toolkit available for genetic research and therapeutic applications. Despite its transformative impact, challenges such as off-target effects and unintended large gene modifications remain critical concerns. Efforts to mitigate these issues through high-fidelity CRISPR variants and improved sgRNA design are ongoing and show promise in enhancing the specificity and safety of CRISPR-Cas9 applications.

The importance of continued research and development in this field cannot be overstated. As we delve deeper into the mechanisms and applications of CRISPR-Cas9, the potential for novel discoveries and technological innovations grows. This ongoing research is crucial for refining the technology, addressing existing limitations, and expanding its applicability to a broader range of organisms and cell types. The development of new Cas effectors and the exploration of microbial diversity are particularly promising areas that could lead to the discovery of new genome editing tools and techniques.

Future studies should focus on several key areas to further advance CRISPR-Cas9 technology. First, there is a need for more comprehensive methods to detect and quantify off-target effects and large gene modifications to ensure the safety and efficacy of genome editing. Second, the development of more efficient delivery systems for CRISPR components will be essential for clinical applications. Third, exploring the potential of CRISPR-Cas9 in creating more accurate and diverse disease models will enhance our understanding of genetic disorders and facilitate drug discovery. Finally, interdisciplinary collaborations will be vital in translating CRISPR-Cas9 research from the laboratory to clinical settings, ultimately improving human health and disease treatment.

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Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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