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CRISPR-based Gene Editing in *Bt* for Improved Insecticidal Properties

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Abstract CRISPR-based gene editing has emerged as a powerful tool for enhancing the insecticidal properties of *Bacillus thuringiensis* (*Bt*) by targeting specific genetic components associated with resistance in insect pests. This study explores the application of CRISPR/Cas9 technology in *Bt* to improve its efficacy against various insect species. Key studies demonstrate the successful knockout of ATP-binding cassette (ABC) transporter genes, such as *ABCC2* and *ABCC3*, which are crucial for mediating resistance to *Bt* toxins in pests like the diamondback moth and cotton bollworm. These genetic modifications have resulted in significantly increased resistance levels, providing insights into the molecular mechanisms underlying *Bt* toxin resistance. Additionally, this study highlights the potential of CRISPR-mediated gene deletions in *Bt* strains to enhance their pesticidal protein profiles, thereby broadening their spectrum of activity against multiple insect pests. The findings underscore the importance of CRISPR technology in developing next-generation biopesticides with improved insecticidal properties and reduced likelihood of resistance development.

Keywords CRISPR/Cas9; *Bacillus thuringiensis*; Insecticidal proteins; ABC transporter genes; Pest resistance

1 Introduction

Bacillus thuringiensis (*Bt*) is a Gram-positive, spore-forming bacterium widely recognized for its insecticidal properties. It produces crystal (Cry) proteins during sporulation, which are toxic to a variety of insect pests upon ingestion. These Cry proteins have been effectively utilized in agriculture for pest control, reducing the reliance on chemical pesticides and thereby mitigating environmental pollution and health risks (Nair et al., 2020; Arsov et al., 2023; Sauka et al., 2023). *Bt*'s specificity towards target pests, such as moths, beetles, and mosquitoes, makes it a valuable tool in integrated pest management programs (Nair et al., 2020; Arsov et al., 2023).

Despite the success of *Bt*-based insecticides, several limitations hinder their long-term efficacy. One significant challenge is the rapid evolution of resistance in target insect populations. For instance, resistance to *Bt* Cry1 toxins has been observed in various lepidopteran pests, which compromises the effectiveness of *Bt* crops and biopesticides (Guo et al., 2019). Additionally, the narrow spectrum of activity of certain *Bt* strains limits their utility against a broader range of pests (Reyaz et al., 2019; Sauka et al., 2023). Environmental factors, such as soil enzymatic activities, can also influence the persistence and efficacy of *Bt* proteins, necessitating careful evaluation of their environmental impact (Li et al., 2019; Li et al., 2022).

Genetic modification offers a promising approach to overcome the limitations of traditional *Bt* insecticides. Traditional methods of genetic modification in *Bt* have been labor-intensive and time-consuming (Liu and Zhang, 2024). By employing techniques such as CRISPR/Cas9, researchers can enhance the insecticidal properties of *Bt* strains, broaden their spectrum of activity, and mitigate resistance development in target pests (Guo et al., 2019). For example, the construction of chimeric *Bt* proteins with novel domain combinations has shown enhanced activity against multiple soybean pests, demonstrating the potential of genetic engineering in developing more effective biopesticides (Chen et al., 2021). Additionally, the identification and characterization of novel Cry proteins, such as Cry78Ba1, provide new avenues for specific and safe pest control (Cao et al., 2020).

This study provides a comprehensive overview of the advancements in CRISPR-based gene editing of *Bt* for improved insecticidal properties, including summarizing the current state of *Bt* as a biopesticide and its role in pest control, identifying the limitations of traditional *Bt* insecticides, exploring the potential of genetic

modification techniques, particularly CRISPR/Cas9, in enhancing *Bt*'s insecticidal properties, and evaluating the environmental and ecological implications of genetically modified *Bt* strains. By addressing these objectives, this study aims to highlight the potential of CRISPR-based gene editing in developing next-generation *Bt* biopesticides with improved efficacy and sustainability.

2 CRISPR-Cas9 Technology: A Tool for Genetic Enhancement

2.1 Introduction to CRISPR-Cas9 gene editing

CRISPR-Cas9, derived from a bacterial immune defense mechanism, has revolutionized genetic engineering by providing a precise, efficient, and versatile tool for genome editing. Initially recognized for its role in bacterial immunity against viruses, CRISPR-Cas9 has been adapted for use in a wide range of organisms, including plants and animals, to facilitate targeted genetic modifications (Demirci et al., 2018; Li et al., 2021). This technology employs a guide RNA (gRNA) to direct the Cas9 nuclease to a specific DNA sequence, where it introduces double-strand breaks (DSBs). These breaks are then repaired by the cell's natural repair mechanisms, leading to targeted mutations or insertions (Bao et al., 2019).

2.2 Mechanism of CRISPR-Cas9 in gene editing

The CRISPR-Cas9 system operates through a simple yet highly effective mechanism. The Cas9 protein, guided by a single-guide RNA (sgRNA), binds to a complementary DNA sequence and introduces a DSB at the target site. The cell's repair machinery then attempts to fix the break, typically through non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ often results in small insertions or deletions (indels) that can disrupt gene function, while HDR can be used to introduce specific genetic changes using a donor template (Bao et al., 2019). This precise targeting capability allows for the modification of specific genes, enabling the study of gene function and the development of organisms with desirable traits (Eş et al., 2019; Erdoğan et al., 2023).

2.3 Advantages of CRISPR in agricultural biotechnology

CRISPR-Cas9 offers several advantages over traditional breeding and earlier genome editing technologies such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). These advantages include higher efficiency, ease of design, lower cost, and the ability to target multiple genes simultaneously (multiplexing) (Demirci et al., 2018; Eş et al., 2019). In agriculture, CRISPR-Cas9 has been used to enhance crop yield, quality, and resistance to diseases and environmental stresses. For instance, it has been employed to develop crops with improved resistance to pests and pathogens, increased tolerance to abiotic stresses like drought and salinity, and enhanced nutritional profiles (Bisht et al., 2019; Chen et al., 2019; Zhu et al., 2020a). The technology's ability to create transgene-free plants also addresses regulatory and public acceptance issues associated with genetically modified organisms (GMOs) (Erdoğan et al., 2023).

2.4 Specific considerations for applying CRISPR to *Bt*

When applying CRISPR-Cas9 to *Bacillus thuringiensis* (*Bt*), several specific considerations must be taken into account. *Bt* is widely known for its insecticidal properties, which are primarily due to the production of crystal (Cry) proteins that target specific insect pests. Enhancing *Bt*'s insecticidal properties through CRISPR-Cas9 involves precise modifications to the genes encoding these Cry proteins to increase their efficacy or broaden their spectrum of activity (Figure 1) (Komal et al., 2023). Additionally, CRISPR can be used to engineer *Bt* strains with improved stability and environmental persistence, ensuring sustained pest control (Bisht et al., 2019; Komal et al., 2023). However, challenges such as off-target effects, delivery methods, and regulatory hurdles must be carefully managed to ensure the successful application of CRISPR technology in *Bt* (Rao and Wang, 2021; Erdoğan et al., 2023).

Komal et al. (2023) highlights the use of genome-editing technologies such as ZFNs, TALENs, and CRISPR/Cas9 to develop insect-resistant crops, offering a novel approach to pest management. By targeting specific genes in either plants or insects, these technologies can increase plant resistance and reduce insecticide resistance in pests. For plants, gene editing can enhance their natural defense mechanisms, such as increasing salicylic acid levels or altering volatile compounds to repel pests. In insects, modifying genes responsible for detoxifying insecticides can make them more susceptible, reducing pest populations. These strategies present a promising path for sustainable agriculture by minimizing the reliance on chemical pesticides and improving crop productivity.

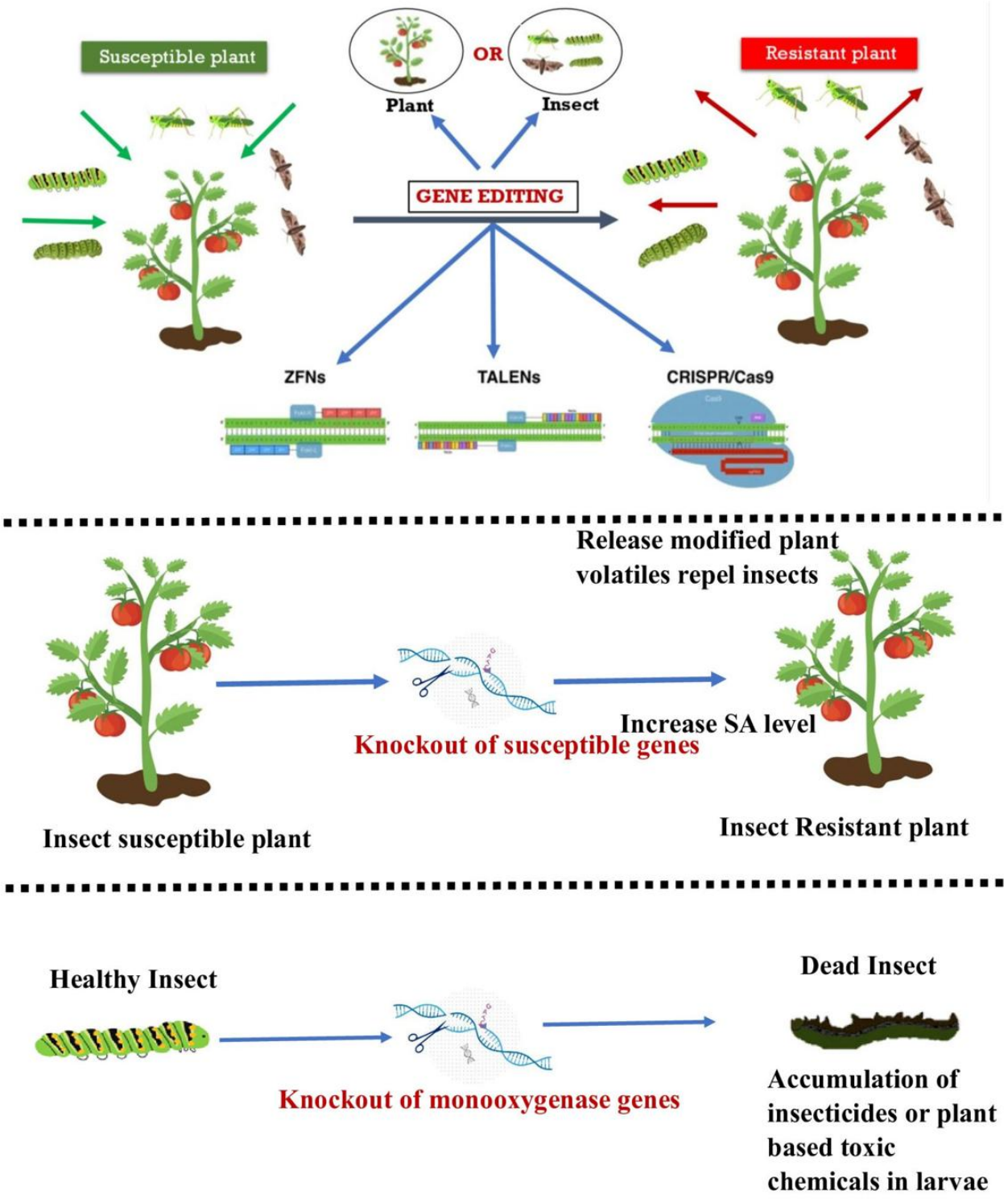

Figure 1 Depicting emerging tools of genome editing for resistance to insect pests. Genome editing of either plant or insect can make the insect susceptible plant to a resistant plant. The potential tools can be ZFNs, TALENs, or CRISPR/Cas9 (Adopted from Komal et al., 2023)

3 Genetic Modifications in *Bt* for Improved Insecticidal Properties

3.1 Target genes in *Bt* for enhanced toxin production

Genetic modifications in *Bacillus thuringiensis* (*Bt*) have primarily focused on enhancing the production and efficacy of its insecticidal toxins. Key target genes include those encoding ATP-binding cassette (ABC) transporters, which play a crucial role in the susceptibility of insects to *Bt* toxins. For instance, mutations in the *ABCC2* and *ABCC3* genes have been linked to high levels of resistance to Cry1Ac toxin in the diamondback moth,

Plutella xylostella (Guo et al., 2019). Similarly, the *ABCA2* gene in the pink bollworm, *Pectinophora gossypiella*, has been associated with resistance to Cry2Ab toxin (Fabrick et al., 2021). These findings underscore the importance of targeting ABC transporter genes to enhance *Bt* toxin production and efficacy.

3.2 Engineering *Bt* toxins: Cry and Vip proteins

Bt produces a variety of insecticidal proteins, including Cry and Vip proteins, which have been engineered to improve their insecticidal properties. Cry proteins, such as Cry1Ac and Cry1Fa, have been extensively studied and modified to enhance their toxicity. For example, CRISPR-mediated knockouts of the *ABCC2* gene in *Ostrinia furnacalis* have conferred high-level resistance to Cry1Fa toxin, highlighting the potential for genetic modifications to improve toxin efficacy (Wang et al., 2020a). Additionally, Vip3Aa proteins have been engineered to increase their insecticidal activity. Mutations in domains IV and V of Vip3Aa have resulted in significantly higher toxicity against pests like *Spodoptera frugiperda* and *Helicoverpa armigera* (Yang et al., 2022). The coexistence of Cry9 and Vip3A genes in the same plasmid has also been shown to provide synergistic insecticidal toxicity, further enhancing the effectiveness of *Bt* strains (Wang et al., 2020b).

3.3 Strategies for enhancing *Bt* strains using CRISPR

CRISPR/Cas9 technology has emerged as a powerful tool for enhancing *Bt* strains by enabling precise genetic modifications. This technology has been used to knockout specific genes associated with resistance to *Bt* toxins, thereby increasing the susceptibility of pests. For example, CRISPR/Cas9-mediated knockouts of the *ABCC2* and *ABCC3* genes in Plutella xylostella have demonstrated the critical role of these genes in mediating resistance to Cry1Ac toxin (Guo et al., 2019). Similarly, CRISPR-mediated mutations in the *ABCA2* gene in Pectinophora gossypiella have confirmed its role in resistance to Cry2Ab toxin (Fabrick et al., 2021). These studies highlight the potential of CRISPR technology to enhance *Bt* strains by targeting genes that confer resistance to *Bt* toxins.

3.4 Potential for multi-toxin gene editing

The potential for multi-toxin gene editing in *Bt* offers a promising strategy for improving insecticidal properties and delaying resistance development. By combining multiple toxin genes, such as Cry and Vip proteins, in a single *Bt* strain, it is possible to achieve synergistic effects and enhance overall toxicity. For instance, the coexistence of Cry9 and Vip3A genes in the same plasmid has been shown to provide synergistic insecticidal activity against pests like *Chilo suppressalis* (Wang et al., 2020b). Additionally, the use of chimeric toxins, such as Cry1AcF, which combine domains from different Cry proteins, has been explored to overcome resistance and enhance toxicity (Dutta et al., 2023). These multi-toxin strategies, facilitated by advanced gene editing techniques like CRISPR, hold great potential for developing more effective and sustainable *Bt*-based biopesticides.

4 Case Study: CRISPR-Based Enhancement of *Bt* Strains

4.1 Overview of the case study and selection criteria

This case study focuses on the application of CRISPR technology to enhance *Bacillus thuringiensis* (*Bt*) strains for improved insecticidal properties. The selection criteria for this study included the identification of key genes in insect pests that mediate resistance to *Bt* toxins and the subsequent use of CRISPR/Cas9 to modify these genes. The primary goal was to understand the genetic basis of resistance and to develop *Bt* strains with enhanced efficacy against resistant pest populations.

4.2 Applying CRISPR in the genetic modification of *Bt*

The methodology involved using CRISPR/Cas9 gene editing to target specific genes in insect pests that are known to confer resistance to *Bt* toxins. For instance, in the beet armyworm (*Spodoptera exigua*), CRISPR-mediated knockouts of five candidate *Bt* toxin receptor genes were performed to evaluate their roles in mediating toxicity of Cry1Ac, Cry1Fa, and Cry1Ca toxins. The genes targeted included *SeAPN1*, *SeCad1*, *SeABCC1*, *SeABCC2*, and *SeABCC3* (Huang et al., 2020). Similarly, in the pink bollworm (*Pectinophora gossypiella*), CRISPR/Cas9 was used to introduce disruptive mutations in the *ABCA2* gene, which was hypothesized to confer resistance to Cry2Ab toxin (Figure 2) (Fabrick et al., 2021). Additionally, in the diamondback moth (*Plutella xylostella*), CRISPR/Cas9 was employed to create knockout strains for the *PxABCC2* and *PxABCC3* genes to study their roles in resistance to Cry1Ac toxin (Guo et al., 2019).

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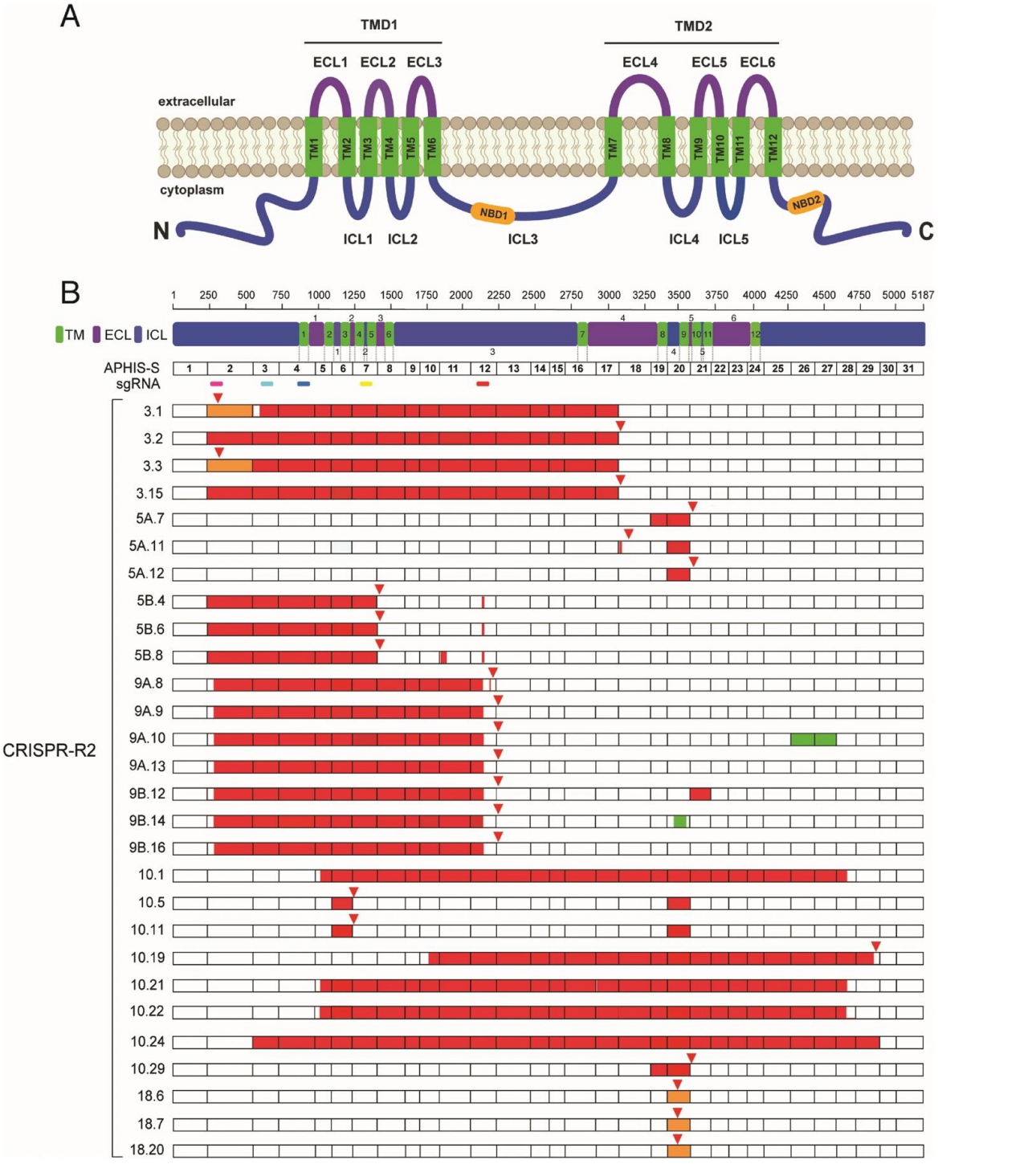

Figure 2 Mutations in 28 *PgABCA2* cDNA sequences from five Cry2Ab-resistant pink bollworm larvae from the CRISPR-R2 strain that lacked gDNA mutations in sgRNA target sites (Adopted from Fabrick et al., 2021)

Image caption: (A) The predicted PgABCA2 protein includes amino (N) and carboxyl (C) termini and transmembrane domains (TMD1 and TMD2). Each TMD contains six transmembrane-spanning regions (TM, green), three extracellular loops (ECL, purple), and two intracellular loops (ICL, blue). The two TMDs are connected by a single intracellular loop (ICL3). ICL3 and the C-terminal domain each contain a nucleotide-binding domain (NBD, orange). (B) Mutations in *PgABCA2* cDNAs from CRISPR-R2 (3–8 clones from each of five individuals: 3, 5, 9, 10 and 18) relative to the susceptible strain APHIS-S (MG637361). Individuals with two distinct PCR products cloned are indicated as A or B (e.g., 5A and 5B, etc.). Numbers to the right of the decimal point for each individual indicate the clone sequenced. Exon numbers are shown for APHIS-S. Location of sgRNAs 1–5 are shown as colored bars (sgRNA1, magenta; sgRNA2, cyan; sgRNA3, blue; sgRNA4, yellow; sgRNA5, red). Red triangles above bars indicate premature stop codons, which occur in all sequences except 10.1, 10.21, 10.22, and 10.24. Colors within bars show mutations: orange for insertions and deletions (indels), red for deletions, and green for insertions (Adopted from Fabrick et al., 2021)

Fabrick et al. (2021) focuses on the mutations in the *PgABCA2* cDNA of Cry2Ab-resistant pink bollworm larvae that do not have mutations in the sgRNA target sites of their genomic DNA. The findings indicate that even without gDNA mutations, larvae exhibited significant cDNA changes, such as premature stop codons, deletions, and insertions affecting multiple exons. This suggests that resistance mechanisms can occur at the cDNA level, contributing to insecticide resistance. The study highlights the complexity of developing genome-editing strategies for pest management, as targeting only the gDNA might not be sufficient to counteract resistance. Understanding these cDNA-level mutations can help design more effective interventions to combat resistance in pest populations.

4.3 Improvement in insecticidal efficacy post-gene editing

The results demonstrated significant improvements in insecticidal efficacy post-gene editing. In the beet armyworm, the knockout of *SeABCC2* resulted in a major increase in susceptibility to Cry1Ac and Cry1Fa toxins, indicating its crucial role in mediating toxicity (Huang et al., 2020). In the pink bollworm, the introduction of disruptive mutations in the *ABCA2* gene led to the creation of a Cry2Ab-resistant strain, confirming the gene's role in resistance (Fabrick et al., 2021). For the diamondback moth, knockout strains for *PxABCC2* and *PxABCC3* exhibited high levels of resistance to Cry1Ac, with the double knockout strain showing over 10,320-fold resistance to Cry1Ac and 380-fold resistance to Cry1Fa, highlighting the synergistic effects of these genes in mediating resistance (Zhao et al., 2020).

4.4 Implications for agricultural pest management

The findings from this case study have significant implications for agricultural pest management. By using CRISPR/Cas9 to modify key resistance genes in insect pests, it is possible to develop *Bt* strains with enhanced insecticidal properties, thereby improving the efficacy of *Bt* crops and reducing the reliance on chemical insecticides. This approach not only helps in managing resistant pest populations but also contributes to sustainable agricultural practices by minimizing the environmental impact of pest control measures. The insights gained from these studies can guide the development of next-generation *Bt* crops with improved resistance management strategies, ensuring long-term efficacy and sustainability (Guo et al., 2019; Huang et al., 2020; Zhao et al., 2020; Fabrick et al., 2021).

5 Safety and Regulatory Aspects of CRISPR-Modified *Bt*

5.1 Biosafety concerns and risk assessment

The application of CRISPR/Cas9 technology in modifying *Bacillus thuringiensis* (*Bt*) for enhanced insecticidal properties brings forth significant biosafety concerns. One of the primary issues is the potential for off-target effects, which can lead to unintended genetic modifications that may have unforeseen ecological consequences (El-Mounadi et al., 2020; Movahedi et al., 2023). Additionally, there is a risk of horizontal gene transfer, where the modified genes could be transferred to non-target organisms, potentially disrupting local ecosystems (Movahedi et al., 2023). Strategies to mitigate these risks include the development of more precise gene-editing tools and robust detection methods to monitor and manage any unintended genetic changes (El-Mounadi et al., 2020; Movahedi et al., 2023).

5.2 Regulatory frameworks for gene-edited organisms

The regulatory landscape for CRISPR-modified organisms, including *Bt*, varies significantly across different countries. Some nations have established comprehensive guidelines to oversee the development and deployment of gene-edited crops, focusing on ensuring safety and efficacy (Tyagi et al., 2020; Zhang et al., 2020). For instance, the European Union has stringent regulations that classify CRISPR-modified organisms similarly to traditional GMOs, requiring extensive risk assessments and approval processes (Zhang et al., 2020). In contrast, countries like the United States have a more lenient approach, where gene-edited crops that do not contain foreign DNA may not be subject to the same rigorous regulations as GMOs (Zhang et al., 2020; Movahedi et al., 2023). These regulatory frameworks aim to balance the potential benefits of CRISPR technology with the need to protect public health and the environment.

5.3 Addressing ethical concerns related to genetic modification

The ethical implications of using CRISPR technology in *Bt* and other organisms are a subject of ongoing debate. Key concerns include the potential for unintended consequences, such as the creation of new pests or the

disruption of existing ecosystems (Zhang et al., 2020; Erdoğan et al., 2023). There is also apprehension about the long-term impacts of releasing genetically modified organisms into the environment, particularly regarding their persistence and potential to revert to their original phenotypes (Ahmad et al., 2020). Addressing these ethical concerns requires transparent communication with the public, thorough ethical reviews, and the development of policies that ensure responsible use of CRISPR technology (Ahmad et al., 2020; Zhang et al., 2020; Erdoğan et al., 2023). Engaging with various stakeholders, including scientists, policymakers, and the public, is crucial to build trust and ensure that the benefits of CRISPR-modified *Bt* are realized in a socially and ethically responsible manner.

6 Challenges and Future Directions

6.1 Technical limitations of CRISPR in *Bt* Editing

CRISPR technology, while revolutionary, faces several technical limitations when applied to *Bacillus thuringiensis* (*Bt*) for enhancing its insecticidal properties. One significant challenge is the delivery of CRISPR components into *Bt* cells, which can be particularly difficult due to the bacterium's robust cell wall. Additionally, achieving high efficiency and specificity in gene editing remains a hurdle. For instance, the need for microinjection in preblastoderm embryos can be a limiting factor in certain insect species, as seen in the white-backed planthopper (Zhang et al., 2023). Moreover, the mosaicism often observed in gene-edited insects, where only a fraction of cells are edited as intended, complicates the establishment of homozygous lines (Zhu et al., 2020b).

6.2 Addressing off-target effects in gene editing

Off-target effects are a major concern in CRISPR-based gene editing, as unintended modifications can lead to undesirable traits or reduced fitness. Strategies to mitigate these effects include the use of high-fidelity Cas9 variants and thorough validation of guide RNA (gRNA) specificity. For example, the use of multiple sgRNAs targeting a single exon has been shown to improve the precision of gene knockouts in the fall armyworm (Zhu et al., 2020b). Additionally, the development of novel CRISPR systems, such as CRISPR/Cas12a, which has demonstrated high editing efficiencies and reduced off-target effects in Bombyx mori, offers promising alternatives (Dong et al., 2020).

6.3 Potential for resistance development in target insects

The rapid evolution of resistance in target insects poses a significant threat to the long-term efficacy of *Bt* crops. Studies have shown that mutations in specific genes, such as *ABCC2* and *ABCC3*, can confer high levels of resistance to *Bt* toxins in insects like the diamondback moth and pink bollworm (Guo et al., 2019; Fabrick et al., 2021). This highlights the need for continuous monitoring and management strategies to counteract resistance. The use of CRISPR to create gene knockouts has provided valuable insights into the genetic basis of resistance, enabling the development of more effective pest management strategies (Douris et al., 2020; Huang et al., 2020).

6.4 Future prospects for *Bt* improvement using advanced gene editing

The future of *Bt* improvement lies in the integration of advanced gene editing technologies to create more robust and effective biopesticides. The potential of CRISPR/Cas9 and other genome editing tools to engineer durable resistance against insect pests has been demonstrated in various studies (Bisht et al., 2019; Tyagi et al., 2020). For instance, the use of CRISPR/Cas9 to knockout specific genes in insects has shown promise in enhancing the insecticidal properties of *Bt* (Guo et al., 2019; Fabrick et al., 2021). Additionally, the development of novel CRISPR systems, such as CRISPR/Cas12a, offers new opportunities for targeted genome engineering and improved pest resistance (Dong et al., 2020). As these technologies continue to evolve, they hold the potential to revolutionize agricultural pest management and ensure sustainable crop protection.

7 Concluding Remarks

The application of CRISPR-based gene editing in *Bacillus thuringiensis* (*Bt*) has shown significant promise in enhancing the insecticidal properties of *Bt* proteins. The development of chimeric *Bt* proteins such as Cry1A.2 and Cry1B.2 has expanded the spectrum of activity against key lepidopteran pests, demonstrating distinct receptor utilization and minimizing cross-resistance issues. Additionally, CRISPR/Cas9-mediated knockout studies have

provided in vivo evidence of the roles of *ABCC2* and *ABCC3* proteins as functional receptors for *Bt* Cry1 toxins, offering insights into the molecular mechanisms of insect resistance. The integration of *Bt* genes into other bacterial strains, such as Bacillus su*Bt*ilis, has also been explored, resulting in enhanced biocontrol efficacy against both insect pests and plant pathogens. Furthermore, whole genome sequencing of various *Bt* strains has revealed a plethora of pesticidal protein genes, underscoring the genetic diversity and potential for biotechnological applications.

CRISPR technology has revolutionized the field of *Bt* insecticides by enabling precise genetic modifications that enhance the efficacy and specificity of *Bt* proteins. The ability to knockout specific genes, such as *PxABCC2* and *PxABCC3*, has provided a deeper understanding of the genetic basis of resistance in target pests, facilitating the development of more effective *Bt* strains. This technology has also allowed for the creation of novel chimeric proteins with improved insecticidal properties, as seen with Cry1A.2 and Cry1B.2, which offer new tools for pest management. The integration of CRISPR with *Bt* technology not only enhances the insecticidal spectrum but also addresses the issue of resistance, thereby ensuring the long-term sustainability of *Bt*-based biopesticides.

The advancements in CRISPR-based gene editing of *Bt* have the potential to significantly impact global agricultural practices. By developing *Bt* strains with enhanced insecticidal properties and reduced resistance, farmers can achieve more effective pest control, leading to higher crop yields and reduced reliance on chemical pesticides. This is particularly important for major crops such as soybean and maize, where pest resistance has been a significant challenge. The use of genetically engineered *Bt* strains can also contribute to more sustainable agricultural practices by reducing the environmental impact of pest control measures and promoting the use of eco-friendly biopesticides. Overall, the integration of CRISPR technology with *Bt* insecticides holds great promise for improving pest management strategies and supporting global food security.

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Conflict of Interest Disclosure

The author affirms 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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