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Metabolic Engineering of Bt for Enhanced Production of Insecticidal Proteins

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Abstract *Bacillus thuringiensis* (Bt) is a key bacterial strain used for producing bioinsecticides, with its insecticidal proteins being widely applied in agricultural pest control. However, traditional Bt production faces challenges such as low protein yield and high production costs. To address these issues, this study explores strategies to enhance Bt metabolic pathways through metabolic engineering, aiming to increase protein production. The research covers Bt's primary and secondary metabolic pathways, their relationship with protein synthesis, and case studies of enhanced protein production achieved through genetic modification and metabolic flux optimization. This study aims to provide feasible improvements for the commercial production and application of Bt products in agriculture by offering theoretical insights into the metabolic engineering of Bt.

Keywords *Bacillus thuringiensis*; Insecticidal proteins; Metabolic engineering; Genetic modification; Agricultural application

1 Introduction

Bacillus thuringiensis (Bt) is a Gram-positive, spore-forming bacterium widely used in biological pest control due to its ability to produce insecticidal Cry proteins. These proteins are highly toxic to various insect orders, including Lepidoptera, Coleoptera, and Diptera, making Bt a crucial tool in agricultural pest management (Akhtar et al., 2021). Bt's insecticidal properties have been successfully applied in sprayable biopesticides and transgenic crops, providing an environmentally friendly alternative to chemical pesticides (Li et al., 2022; Yamamoto et al., 2022). Moreover, Bt's effectiveness against pests such as mosquitoes, which are vectors of disease, further underscores its importance in integrated pest management (Nair et al., 2020).

In modern agriculture, Bt insecticidal proteins play a vital role in crop protection. They are widely used in transgenic crops like cotton and corn, significantly reducing the use of chemical pesticides and boosting crop yields. The specificity of Bt proteins, which target only harmful insects while being safe for humans and other non-target organisms, makes them an ideal choice for sustainable agriculture (Chen et al., 2021; Singh et al., 2021). However, the emergence of insect resistance to certain Bt proteins necessitates ongoing research to design and develop new insecticidal proteins with enhanced efficacy and broader insecticidal spectra (Yamamoto et al., 2022).

This study focuses on the latest advancements in metabolic engineering to enhance the production of Bt insecticidal proteins. It analyzes and discusses various strategies to improve Bt protein yield and efficacy, including genetic modifications, co-expression systems, and the environmental impact of engineered Bt strains. Through these analyses, this study aims to provide a theoretical foundation for the future development of Bt insecticidal proteins and to promote the advancement of more effective and sustainable pest management solutions.

2 Overview of Bt and Insecticidal Proteins

2.1 Bt strains and their characteristics

Bacillus thuringiensis (Bt) is a Gram-positive, spore-forming bacterium widely recognized for its ability to produce insecticidal proteins during the sporulation phase of its growth cycle. These proteins, primarily Cry, Vip, and Cyt, are highly specific to certain insect orders, making Bt a valuable tool in pest management (Bravo et al.,

2015; 2017). Bt strains have been utilized in both sprayable pesticide formulations and transgenic crops to protect against insect damage. The diversity of Bt strains and their respective insecticidal proteins allows for targeted pest control, reducing the need for broad-spectrum chemical insecticides (Li et al., 2022; Yamamoto, 2022).

2.2 Types of insecticidal proteins produced by Bt

Bt produces several classes of insecticidal proteins, each with distinct target specificities and modes of action. The primary types include:

Cry Proteins: These are the most extensively studied and utilized Bt toxins. They are crystalline proteins that target specific insect midgut receptors, leading to cell lysis and insect death. Examples include Cry1Ac, Cry1Ab, and Cry2Ab, which are used in transgenic crops like cotton and corn to control pests such as the tobacco budworm and European corn borer (Bravo et al., 2017; Rathinam et al., 2019; Yamamoto, 2022).

Vip Proteins: Vegetative insecticidal proteins (Vip) are produced during the vegetative growth phase of Bt. They have a different mode of action compared to Cry proteins and can target a broader range of insect pests.

Cyt Proteins: Cytolytic proteins (Cyt) are less commonly used but are effective against certain insect orders and nematodes. They work by forming pores in the cell membranes of the target insects (Bravo et al., 2015; 2017).

2.3 Mechanisms of action of insecticidal proteins

The insecticidal proteins produced by Bt operate through several mechanisms to exert their toxic effects on target pests:

Cry Proteins: These proteins bind to specific receptors in the insect midgut, such as cadherin-like proteins, leading to pore formation in the gut epithelial cells. This results in cell lysis, gut paralysis, and ultimately, insect death. The specificity of Cry proteins to their receptors is a key factor in their effectiveness and safety (Bravo et al., 2017; Liu et al., 2018; Rathinam et al., 2019).

Vip Proteins: Vip proteins also target the insect midgut but have a different binding mechanism compared to Cry proteins. They can be effective against insects that have developed resistance to Cry proteins, providing an alternative mode of action (Bravo et al., 2015; 2017).

Cyt Proteins: These proteins form pores in the cell membranes of target insects, leading to cell lysis and death. Their mode of action is similar to that of Cry proteins but involves different target sites within the insect (Bravo et al., 2015; 2017).

The continuous evolution of insect resistance to Bt proteins necessitates ongoing research and development of new proteins and engineering of existing ones to maintain their efficacy. Strategies such as protein engineering, domain swapping, and bioconjugation have been employed to enhance the insecticidal activity and broaden the spectrum of Bt proteins (Pan et al., 2019; Rathinam et al., 2019; Yamamoto, 2022).

3 Metabolic Pathways in Bt

3.1 Primary metabolic pathways

Bacillus thuringiensis (Bt) primarily relies on standard bacterial metabolic pathways for its growth and survival. These include glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation, which are essential for energy production and biosynthesis of cellular components. The primary metabolism in Bt is crucial for providing the necessary precursors and energy required for the synthesis of secondary metabolites, including insecticidal proteins.

3.2 Secondary metabolic pathways

3.2.1 Pathways leading to toxin production

The production of insecticidal proteins in Bt, such as Cry and Vip toxins, is a hallmark of its secondary metabolism. These proteins are synthesized during the sporulation phase of Bt and are encoded by specific genes

that are activated under certain environmental conditions. The Cry proteins, for instance, are produced as protoxins that require activation to exert their insecticidal effects (Tabashnik et al., 2015). The Vip proteins, on the other hand, are secreted during the vegetative growth phase and have distinct mechanisms of action compared to Cry proteins (Jin et al., 2022).

3.2.2 Regulatory networks

The regulation of toxin production in Bt involves complex networks of genetic and environmental factors. Key regulatory genes and proteins control the expression of toxin genes in response to specific signals. For example, the expression of Cry proteins is tightly regulated by sporulation-specific sigma factors and other regulatory proteins that respond to nutrient availability and other environmental cues (Figure 1) (Li et al., 2022). Additionally, the interaction between primary and secondary metabolic pathways plays a significant role in modulating toxin production (Tabashnik et al., 2015).

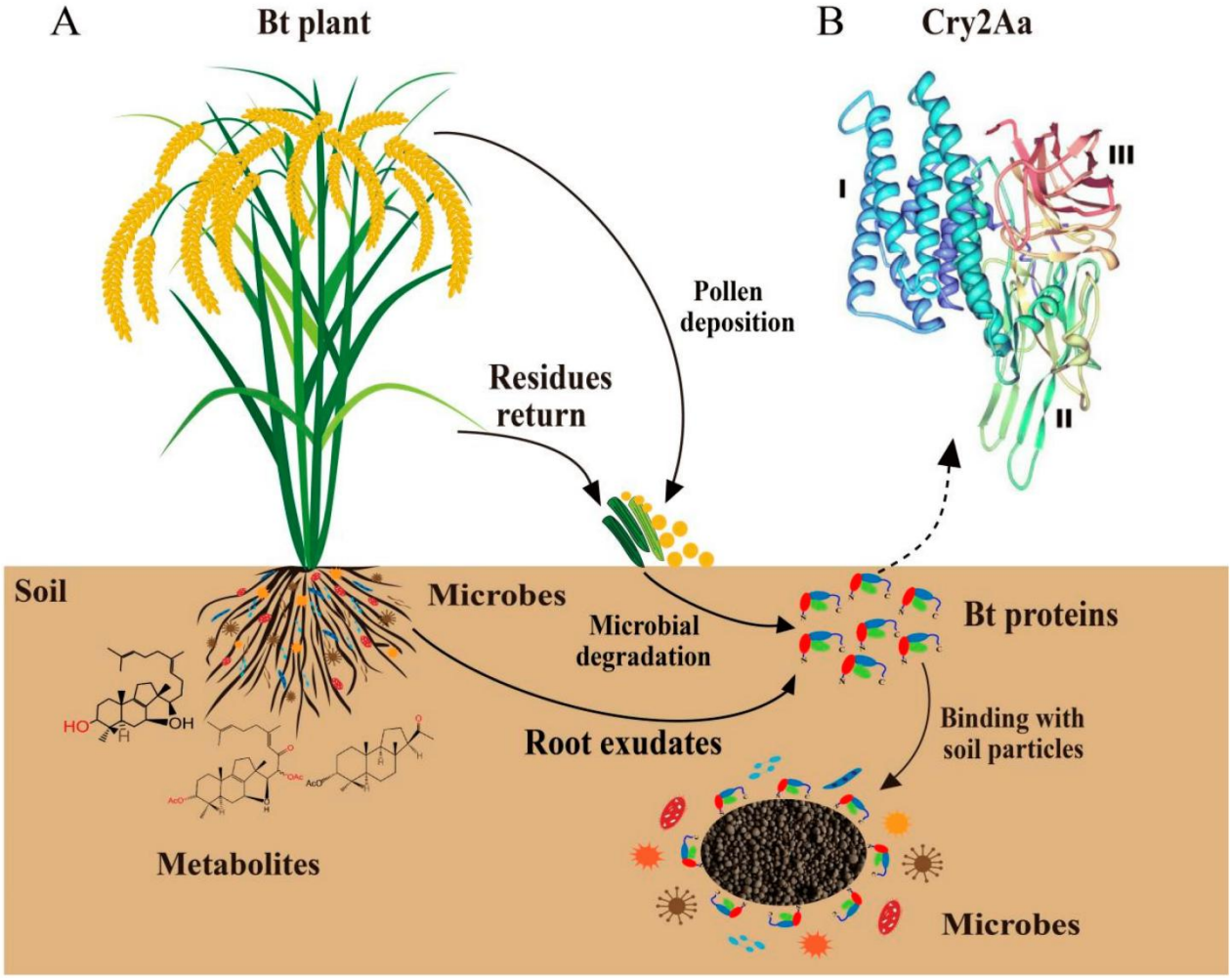

Figure 1 Environmental behaviors of Bt protein (A) and its three-dimensional structures (B). I, II, and III: domains I, II, and III (Adopted from Li et al., 2022)

The production of Bt toxin is influenced by both genetic regulation and environmental factors. At the genetic level, the expression of specific genes and regulatory networks play a key role in Bt toxin production, while environmental factors such as temperature, pH, and nutrients significantly affect the synthesis and release of the toxin. Figure 1 illustrates how high-throughput screening methods systematically assess these genetic and environmental variables to optimize Bt toxin production. This approach is crucial for improving the efficiency and stability of Bt toxin production and is an important tool in Bt metabolic engineering research.

3.2.3 Interactions with primary metabolism

The synthesis of insecticidal proteins in Bt is intricately linked to its primary metabolism. The energy and precursors required for the production of these proteins are derived from primary metabolic processes. For instance, the amino acids and nucleotides necessary for protein synthesis are generated through the central metabolic pathways. Moreover, the regulation of primary metabolic pathways can influence the efficiency and yield of toxin production, highlighting the interconnectedness of primary and secondary metabolism in Bt (Tabashnik et al., 2015; Li et al., 2022).

3.3 Role of metabolism in protein synthesis

Metabolism plays a crucial role in the synthesis of insecticidal proteins in Bt. The metabolic state of the bacterium determines the availability of resources required for protein synthesis. During the sporulation phase, when Cry proteins are produced, Bt undergoes significant metabolic shifts to allocate resources towards the synthesis of these proteins. Similarly, the production of Vip proteins during the vegetative phase is influenced by the metabolic activity of the bacterium. Understanding the metabolic pathways and their regulation in Bt is essential for optimizing the production of insecticidal proteins through metabolic engineering (Tabashnik et al., 2015; Jin et al., 2022; Li et al., 2022).

4 Metabolic Engineering Strategies for Bt

4.1 Genetic modifications for enhanced protein production

4.1.1 Overexpression of key genes

Overexpression of key genes in *Bacillus thuringiensis* (Bt) has been a pivotal strategy to enhance the production of insecticidal proteins. For instance, the introduction of a late embryogenesis abundant (LEA) peptide co-expression system has shown significant promise. By using the expression vector pHT01 with a strong σA-dependent promoter, the production of crystal proteins was enhanced threefold after 12 hours of induction with IPTG (Akhtar et al., 2021). This method was further optimized by using lactose as an inducer, which provided a more cost-effective and efficient alternative to IPTG, leading to enhanced Cry protein expression through intermittent induction (Akthar et al., 2022).

4.1.2 Gene knockouts to remove bottlenecks

Gene knockouts have been employed to remove metabolic bottlenecks that hinder the efficient production of insecticidal proteins. By targeting and knocking out specific genes that compete for resources or produce inhibitory byproducts, the metabolic flux can be redirected towards the synthesis of desired proteins. This approach has been instrumental in increasing the yield and efficacy of Bt toxins, although specific examples in the context of Bt require further elucidation in the literature.

4.1.3 Use of synthetic biology tools

Synthetic biology tools have revolutionized the metabolic engineering of Bt. Techniques such as CRISPR-Cas9 and advanced gene editing have enabled precise modifications to the Bt genome, facilitating the creation of strains with enhanced insecticidal properties. For example, the engineering of Bt toxin Cry2Ab30 and its bioconjugation with 4″-O-succinyl avermectin (AVM) resulted in a bioconjugate with significantly higher insecticidal activity and binding affinity compared to the native Cry2Ab30 (Pan et al., 2019). This demonstrates the potential of synthetic biology in creating more potent and efficient Bt strains.

4.2 Optimization of metabolic flux

Optimizing the metabolic flux within Bt is crucial for maximizing the production of insecticidal proteins. This involves fine-tuning the metabolic pathways to ensure that precursors and energy are efficiently channeled towards the synthesis of target proteins. The use of stable isotope labeling, as demonstrated in the production of 13C/15N-labeled Cry1Ab/Ac proteins, provides insights into the metabolic fate of these proteins and helps in understanding and optimizing their production (Wang et al., 2020). Additionally, the co-expression of LEA peptides has been shown to enhance the metabolic flux towards crystal protein production, further highlighting the importance of metabolic optimization (Akhtar et al., 2021; Akthar et al., 2022).

4.3 Enhancing precursor availability

Enhancing the availability of precursors is another critical strategy in the metabolic engineering of Bt. By ensuring a steady supply of essential building blocks, the production of insecticidal proteins can be significantly increased. For instance, the use of molecular docking and bioinformatic analysis to engineer chimeric proteins like Cry1AcF has shown broad-spectrum efficacy against pests, indicating that optimizing precursor availability can lead to the development of more effective Bt strains (Rathinam et al., 2019). Moreover, the bioconjugation of Cry2Ab with AVM not only improved binding affinity but also suggested potential mechanisms for enhancing precursor availability through structural modifications (Pan et al., 2019).

The metabolic engineering of Bt for enhanced production of insecticidal proteins involves a multifaceted approach, including genetic modifications, optimization of metabolic flux, and enhancing precursor availability. These strategies collectively contribute to the development of more potent and efficient Bt strains, thereby improving their efficacy in pest management.

5 Case Studies of Successful Engineering in Bt

5.1 Enhanced Cry protein production

By employing various genetic engineering strategies, the production of Cry proteins in *Bacillus thuringiensis* (Bt) has been significantly enhanced. For example, the developed chimeric protein Cry1AcF exhibits broad-spectrum insecticidal effects. This chimeric protein is engineered to effectively interact with aminopeptidase 1 receptors from different insect species, thereby enhancing its insecticidal activity (Rathinam et al., 2019). Studies have shown that genetically engineered Bt proteins have higher stability and effectiveness, showing significant resistance enhancement against multiple pests, effectively addressing the issue of resistance to traditional Bt proteins (Figure 2). Furthermore, the seed industry is also optimizing the activity of Bt insecticidal proteins by modifying the protein structure to improve their effectiveness against target pests (Yamamoto et al., 2022).

The study by Rathinam et al. (2019) demonstrates that through genetic engineering strategies, the production of Cry proteins in *Bacillus thuringiensis* (Bt) has been significantly improved. Specifically, these strategies include protein structure optimization and gene combination modifications, resulting in engineered Bt with higher insecticidal efficiency and broad-spectrum resistance. This enhanced production capability effectively addresses the challenge of pest resistance, providing a more powerful tool for agricultural pest management.

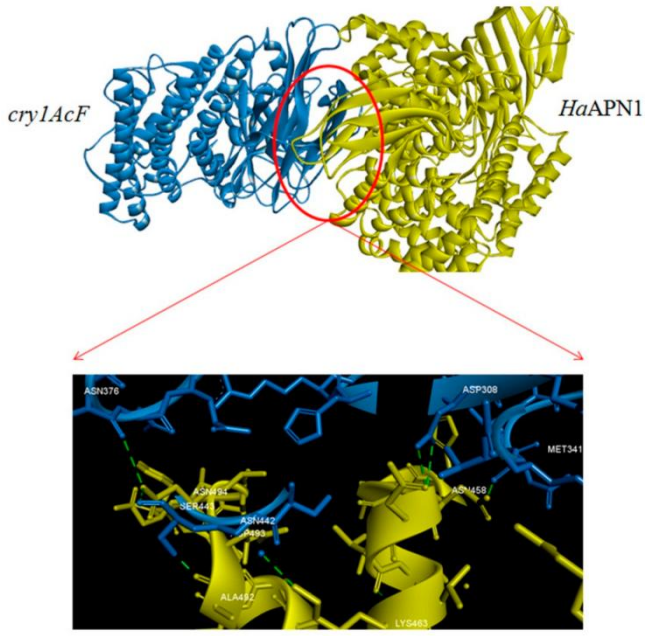

Figure 2 Molecular interaction between Cry1AcF and SlAPN1 (insect: specific region of interaction between the two proteins) (Adopted from Rathinam et al., 2019)

5.2 Improved stability and yield

Improving the stability and yield of Bt insecticidal proteins is crucial for their effectiveness in agricultural applications. One innovative approach involves the use of magnesium hydroxide nanoparticles (MHNPs) to control the loss of Cry1Ac protein. These nanoparticles enhance the adhesion of Cry1Ac to plant surfaces, thereby increasing its stability and insecticidal activity. The Cry1Ac-loaded MHNPs showed a significant increase in pest mortality and remained stable during the adsorption process, making them a promising adjuvant for biopesticides (Rao et al., 2018). Furthermore, studies have shown that the environmental behavior of Bt proteins, including their adsorption, retention, and degradation in soils, can impact their stability and efficacy. Understanding these behaviors helps in designing strategies to maintain the activity of Bt proteins in field conditions (Li et al., 2022).

5.3 Integration of multiple engineering strategies

The integration of multiple engineering strategies has been pivotal in enhancing the production and effectiveness of Bt insecticidal proteins. For example, the combination of different Cry proteins in transgenic crops has been employed to delay resistance and broaden the spectrum of pests controlled. Second-generation Bt crops producing multiple toxins, such as Cry1Ac and Cry2Ab, have been developed to combat resistance in pests like *Helicoverpa armigera*. This approach has shown success in maintaining the efficacy of Bt crops over time (Tabashnik et al., 2015). Additionally, the creation of chimeric proteins by combining domains of different Cry proteins has led to enhanced insecticidal properties and broader pest control (Koch et al., 2015). These integrated strategies not only improve the effectiveness of Bt proteins but also contribute to sustainable pest management practices.

6 Challenges and Future Directions

6.1 Technical challenges in metabolic engineering

Metabolic engineering of *Bacillus thuringiensis* (Bt) for enhanced production of insecticidal proteins faces several technical challenges. One significant issue is the optimization of Bt insecticidal proteins to overcome resistance in target pests. For instance, pests such as *Heliothis virescens* and *Ostrinia nubilali*s have developed resistance to Cry1Ac and Cry1Ab proteins, necessitating the engineering of new Bt proteins with higher activity levels (Yamamoto et al., 2022). Additionally, the structural complexity of polyketide insecticidal agents poses challenges for chemical modification, which is essential for enhancing their efficacy and production (Yi et al., 2023). The metabolic pathways involved in the production of these proteins are intricate, and any genetic modifications can have unintended effects on bacterial growth, sporulation, and protein formation, as seen with the *gabD* and *sucA* gene knockouts.

6.2 Ecological and safety concerns

The ecological and safety concerns associated with Bt proteins are paramount. Bt insecticidal proteins, when released into the environment through transgenic plants or biopesticides, can persist in the soil and potentially affect non-target organisms. The structural and functional differences between naturally occurring Bt proteins and those expressed in genetically modified organisms (GMOs) necessitate thorough biosafety evaluations before field deployment (Li et al., 2022). Moreover, the evolution of resistance in pests not only reduces the efficacy of Bt crops but also raises concerns about the long-term sustainability of Bt technology (Jurat-Fuentes et al., 2021; Tabashnik et al., 2023). While extensive studies have shown that current Bt crops do not adversely affect non-target species, continuous monitoring and assessment are essential to ensure environmental safety (Koch et al., 2015; Romeis et al., 2019).

6.3 Scaling up production and commercialization

Scaling up the production of Bt insecticidal proteins for commercial use involves overcoming several hurdles. The production of stable isotope-labeled Cry proteins, for instance, requires precise recombinant expression protocols and purification processes, which can be technically demanding and costly (Wang et al., 2020). Additionally, the optimization of Bt proteins for higher insecticidal activity, as required for commercial viability, involves complex protein engineering and metabolic engineering strategies (Rathinam et al., 2019; Yamamoto et al., 2022). The development of high-yield strains through metabolic engineering and combinatorial biosynthesis is crucial for the

economical production of these proteins (Yi et al., 2023). Furthermore, the commercialization of Bt crops and biopesticides must navigate regulatory frameworks that ensure their safety and efficacy, adding another layer of complexity to the scaling-up process (Koch et al., 2015).

While the metabolic engineering of Bt for enhanced production of insecticidal proteins holds great promise, it is fraught with technical, ecological, and commercial challenges. Addressing these challenges through innovative research and stringent safety evaluations will be key to the successful application and sustainability of Bt technology in pest management.

7 Advances in Biotechnology and Tools

7.1 CRISPR-Cas and genome editing

The advent of CRISPR-Cas technology has revolutionized the field of metabolic engineering, providing a precise and efficient method for genome editing. This technology allows for targeted DNA cleavage, enabling various genome engineering modes such as insertions, deletions, and chromosomal rearrangements (Nishida and Kondo, 2020). The versatility of CRISPR systems has led to the development of derivative technologies, such as deaminase-mediated base editing, which introduces point mutations with reduced cytotoxicity. Additionally, CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) enable temporary control of gene expression without altering the genomic sequence, further expanding the toolkit available for metabolic engineering (Nishida and Kondo, 2020).

CRISPR-Cas systems have been successfully applied to a wide range of organisms, including industrially relevant microbes like Escherichia coli and Saccharomyces cerevisiae (Mougiakos et al., 2016). These systems have facilitated the rapid development of microbial cell factories by enabling high-efficiency genome editing and transcriptional regulation (Jakočiūnas et al., 2016). For instance, CRISPR-Cas9 has been used to introduce multiple mutations simultaneously in E. coli, optimizing metabolic pathways for the overproduction of valuable compounds such as β-carotene (Li et al., 2015). The discovery of novel Cas9-like systems from diverse microbial environments continues to broaden the range of organisms that can be engineered using CRISPR technology (Mougiakos et al., 2018).

7.2 High-throughput screening methods

High-throughput screening methods are essential for the efficient evaluation of genetic modifications and the identification of optimal metabolic pathways. CRISPR-Cas technology has significantly enhanced the capabilities of high-throughput screening by enabling multiplexed genome editing and the construction of guide RNA (gRNA) libraries (Figure 3) (Ding et al., 2020). These advancements allow for the comprehensive discovery and evaluation of metabolic pathways, accelerating the development of engineered strains with desired traits (Nishida and Kondo, 2020).

The integration of CRISPR-Cas systems with high-throughput screening has been particularly impactful in the field of microbial biotechnology. For example, CRISPR-based tools have been used to perform genome-wide perturbations and model-guided genome editing strategies, enabling the systematic exploration of metabolic networks (Jakočiūnas et al., 2017). This approach has been applied to both model organisms and non-model microbes, facilitating the development of novel production hosts for biotechnologically relevant products (Mougiakos et al., 2018).

7.3 Computational modeling and simulation

Computational modeling and simulation play a crucial role in the design and optimization of metabolic engineering strategies. These tools enable researchers to predict the effects of genetic modifications on metabolic pathways and to identify potential bottlenecks and targets for further engineering. The integration of CRISPR-Cas technology with computational modeling has further enhanced the precision and efficiency of metabolic engineering efforts (Jakočiūnas et al., 2017).

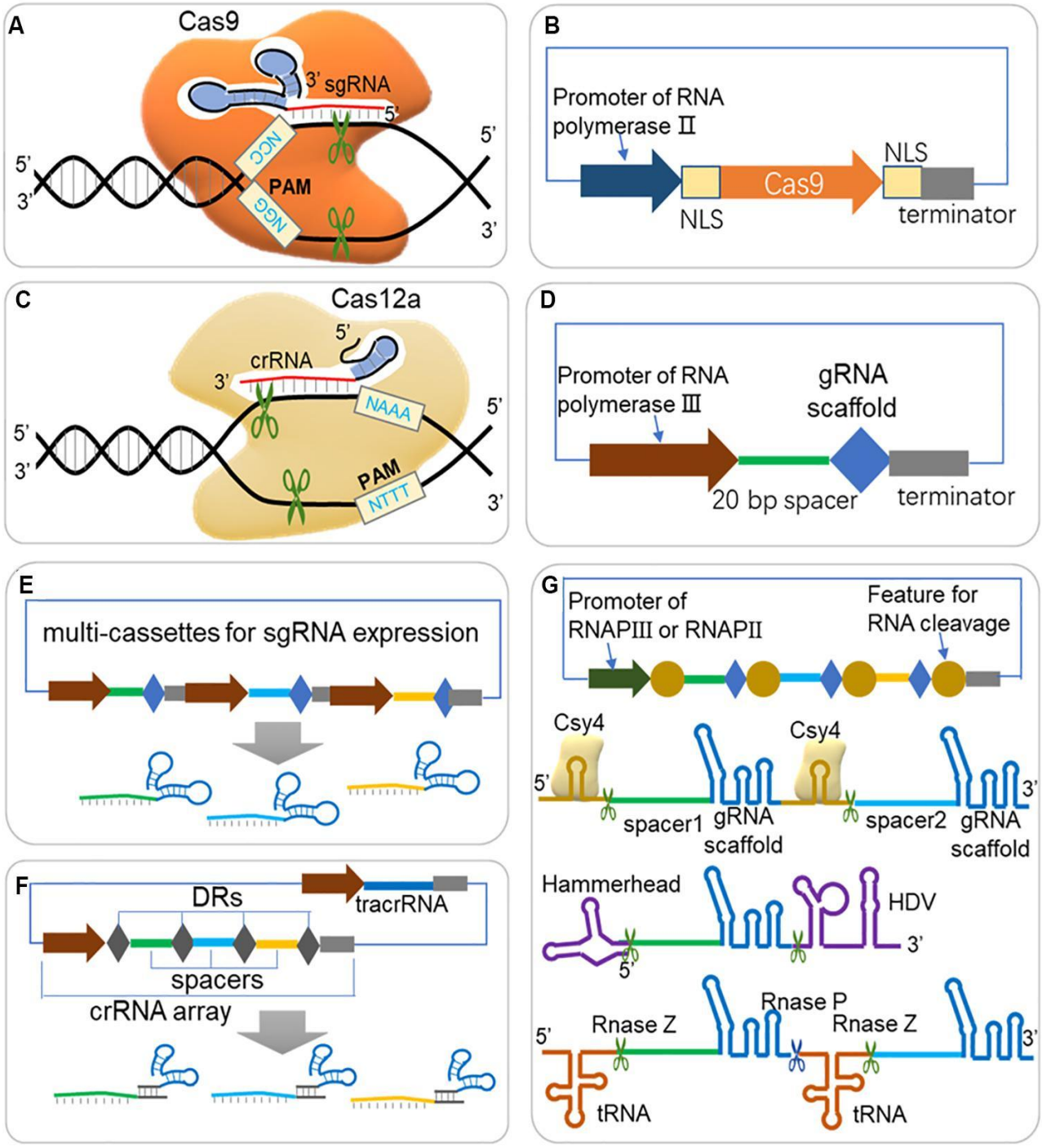

Figure 3 Guidelines for expression of Cas protein and sgRNA in CRISPR/Cas system (Adopted from Ding et al., 2020)

Image caption: (A) Scheme of CRISPR/Cas9 system. The Cas9-sgRNA (or Cas9-crRNA-tracrRNA) complex binds to DNA target arising from Watson-Crick base pairing of spacer sequence, and triggers double strand break (DSB) when next to a short protospacer adjacent motif (PAM, 'NGG' for Cas9 from *S. pyogenes*). (B) Expression cassette for Cas9. For efficient targeting to nucleus in eukaryotes, the Cas9 should be fused to NLS (nuclear localization sequence) at one end or both ends. (C) Scheme of CRISPR/Cas12a (Cpf1) system. Cas12a triggers DSB through a similar scheme of Cas9, but depends on different PAM ('NTTT') and less folded crRNA, and creates a sticky end at 18–23 bases away from the PAM. (D) Expression cassette for sgRNA. A promoter of RNA polymerase III (RNAP III) is usually required for directing sgRNA in nucleus and with less modification. (E) Multi-sgRNA expression through multi-cassettes. Repeated elements, such as promoters, gRNA scaffold and terminators are repeated for different spacer sequences. (F) Multi-sgRNA expression through crRNA array and tracrRNA (HI-CRISPR system). (G) gRNA multiplexing strategies. Both RNAP II and RNAP III promoter can be used for expression the sgRNA array, where sgRNAs are separated by features for RNA cleavage (Adopted from Ding et al., 2020)

Model-guided genome editing strategies, supported by CRISPR-Cas systems, allow for the rational design of metabolic pathways and the systematic testing of hypotheses (Jakočiūnas et al., 2017). Computational tools for the design of gRNAs and the prediction of off-target effects are also essential for the successful application of CRISPR technology in metabolic engineering (Jakočiūnas et al., 2016). These tools help to minimize unintended genetic modifications and to ensure the accuracy and efficiency of genome editing efforts.

The advances in CRISPR-Cas technology, high-throughput screening methods, and computational modeling have significantly enhanced the capabilities of metabolic engineering. These tools have enabled the precise and efficient modification of microbial genomes, facilitating the development of engineered strains with improved production of insecticidal proteins and other valuable compounds. The continued integration of these technologies promises to drive further innovations in the field of metabolic engineering.

8 Concluding Remarks

The systematic review of the metabolic engineering of *Bacillus thuringiensis* (Bt) for enhanced production of insecticidal proteins has highlighted several key findings. Bt proteins, particularly Cry and Vip toxins, have played a crucial role in pest management through their use in genetically engineered (GE) crops such as corn, cotton, and soybean. These proteins are highly specific to target pests while remaining safe for non-target organisms, including humans. However, the emergence of resistance in some pest populations presents a significant challenge, emphasizing the need for the development of new Bt proteins and strategies to manage resistance. Advances in protein engineering have led to the creation of chimeric and optimized Bt proteins with enhanced insecticidal activity and broader spectrum efficacy. Environmental and biosafety assessments of Bt crops generally support their safety, though ongoing monitoring and evaluation are essential.

The metabolic engineering of Bt for enhanced production of insecticidal proteins holds substantial potential for agriculture. Developing Bt proteins with higher potency and broader spectrum activity can improve pest control, reduce reliance on chemical insecticides, and promote sustainable agricultural practices. Enhanced Bt proteins can also help mitigate the issue of pest resistance, ensuring the long-term efficacy of Bt crops. Integrating Bt crops into integrated pest management (IPM) strategies can support the conservation of natural enemies and overall ecosystem health. The economic benefits for farmers, including increased crop yields and reduced pest management costs, further underscore the positive impact of metabolic engineering on agriculture.

Future research should focus on maximizing the benefits of metabolic engineering of Bt. Continued development and optimization of new Bt proteins are necessary to stay ahead of evolving pest resistance. Exploring novel protein engineering techniques and bioconjugation methods to enhance the efficacy and stability of Bt proteins is important. Comprehensive biosafety evaluations should be conducted to assess the environmental impact of new Bt proteins and ensure their safety for non-target organisms. Investigating the mechanisms of resistance in pests is essential for developing more effective resistance management strategies. Integrating Bt crops into broader IPM frameworks and promoting the use of refuges can help sustain the long-term effectiveness of Bt technology.

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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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