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Protein Engineering Of *Bt* Genes *cry1Ab* And *cry1Ba* For The Development Of Chimeric Genes *cryAbabba*, *cryBabaab* And *cryAbbaab* Via Domain Swapping

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Abstract

Bacillus thuringiensis is renowned for its production of insecticidal cry proteins, widely utilized in crop protection to combat insects. However, the risk of insect resistance emerges due to the relatively loose binding of toxins to target sites on larvae's midgut brush boundary membranes. This resistance primarily stems from modifications in binding sites within midgut cells. To address potential threats, the discovery of new Cry proteins is imperative as insects continually evolve resistance against existing ones. Combining Cry toxins with diverse binding sites in larval midguts is proposed as an effective strategy to delay the onset of resistance. In this study, three chimeric *B. thuringiensis* proteins—CryAbAbBa, CryBaBaAb, and CryAbBaAb—were engineered via domain swapping, utilizing crystal proteins CrylAb and CrylB. Structural validation was conducted, confirming their integrity through Ramachandran Plots. The chimeric proteins can be used as additional resources in crop improvement programmes.

Keywords: Cry genes, Chimeric proteins, Insect resistance, Domain swapping

1. INTRODUCTION

Cry toxins, also referred to as δ-endotoxins, are produced by *Bacillus thuringiensis* (*Bt*) and are invaluable insecticidal proteins with applications in managing agricultural pests. These toxins find utility in insecticidal sprays or are engineered into transgenic plants to effectively target a diverse array of insect species. Several studies[1] underscore the versatility of cry toxins in combating insect pests, particularly those belonging to the order Lepidoptera. Both sprayable products and transgenic plants utilize Cry1- and Cry2-class proteins to control lepidopteran pests. Notably, Cry1Ab and Cry1Ac toxins demonstrate potent insecticidal activity against various Lepidoptera insects, including key pests affecting maize (such as *Helicoptrapa zea* and *Ostrinia nubilalis*) and the primary pests of cotton (including *Helicothis virescens*, *Pectinophora gossypiella*, and *Helicoverpa armigera*)[2]. While second-generation transgenic cotton varieties typically incorporate combinations of Cry1Ac and Cry2Ab proteins, the predominant *Bt* crops currently cultivated predominantly express either Cry1Ab or Cry1Ac. However, the potential for insect tolerance to Bt toxins raises concerns regarding their sustained effectiveness over the long term.

The conventional model explaining the sequence of events leading to insect death by Bt proteins, referred to as the Bt mode of action, is both clear-cut and extensively acknowledged, having been widely embraced for over four decades [3,4,5,6]. This model delineates six fundamental processes: ingestion, proteolysis, receptor binding, membrane insertion, pore development, and the resultant damage to the epithelium, culminating in insect demise. Thus, detailed investigations into the general mode of action of new Bt proteins, particularly Cry proteins, may not be necessary. Instead, emphasis should be placed on identifying the receptors involved in the binding process, as numerous studies have highlighted that resistance to Bt proteins often correlates with alterations in receptor binding [7,8,9,10]. Therefore, specific binding represents a critical step in the mode of action of Cry proteins against target insect pests.

Considerable evidence suggests that, following binding, Cry1A proteins form oligomers and penetrate the midgut epithelium, leading to cell lysis. However, there have been suggestions of involvement of a signaling pathway [11]. Similarly, Cry2A toxins exhibit binding mechanisms akin to Cry1A toxins [12]. Nevertheless, the mode of action may vary among different toxins. For instance, Cry1Ab and Cry1Ba exhibit specific and saturable binding to *Pieris brassicae* BBMV, whereas only Cry1Ab binds to BBMV from *Manduca sexta* [13,6]. Bioassay results indicate variations in toxicity among different Cry toxins for each species. For instance, toxicity rankings were Cry1Ac>Cry1Ab>Cry2Ab for *H. armigera*, Cry1B>Cry1C>Cry2Ab for *Spodoptera exigua*, and Cry2Ab>Cry1B>Cry1C for *S. litura*. Only Cry2Ab was toxic to *Agrotis ipsilon*.

Understanding the mechanism of resistance to Cry toxins in insect species is crucial for developing resistance management strategies. The three-dimensional structure of protease-activated Cry1Aa toxin, closely related to Cry1Ab, has been elucidated, revealing three structural domains [14]. Domain I, comprised of a bundle of seven α-helices, is involved in pore formation and oligomerization of the toxin. Domain II, consisting of antiparallel β-sheets forming a β-prism structure with exposed loops, interacts with midgut proteins of susceptible insects; domain III, an antiparallel β-sheet sandwich, is also involved in binding interactions with midgut receptors [15,16]. X-ray crystallography has determined the three-domain structures of Cry1Aa [14], Cry1Ac [17,18], Cry2Aa [19], Cry3Aa [20], Cry3Ba, Cry3Bb [21], Cry4Aa [22], Cry4Ba [23], and Cry8Ea [24]. These proteins share a striking three-dimensional similarity, leading researchers to speculate that the domains may serve similar functions.

The toxicity of Cry1A against insect pests is contingent upon its interaction with various larval midgut proteins, such as cadherin (CAD), aminopeptidase N (APN), or alkaline phosphatase (ALP). It is theorized that Cry1A toxins initially bind to the highly abundant APN or ALP proteins to concentrate them in the midgut epithelium [25,26]. Subsequently, Cry1A toxins bind to CAD, facilitating the removal of α-helix 1 from their amino-terminal regions and inducing toxin oligomerization [27]. Oligomers then bind again to APN or ALP, resulting in toxin insertion into the midgut cell membrane, leading to osmotic shock and cellular death (references [25,28].

Cry1Ab, with a mutation at position 16 (L511A), affects ALP binding but not APN binding, indicating that ALP binding serves as a limiting step for Cry1 toxicity across different insect species. The low sensitivity of certain insect pests to Cry1A toxins may be attributed to limited receptor binding and/or low stability against midgut proteases [25,26].

Though numerous Cry-expressing varieties have been successfully commercialized to date, they raise concerns about the likelihood of insect populations developing resistance to the insecticidal proteins from these crops. Various methods have been proposed to address this issue, including using high-dose Cry proteins in combination with a refuge [29] and mixing different toxins in one crop [30]. Exploring new insect-resistance proteins is another crucial approach to counter emerging resistance [31,32,33,34,35]. Implementing Bt application systems in transgenic crops within containment facilities to control target pests is essential. A multi-toxin deployment system can enhance insecticide efficacy and delay the adaptive resistance of cotton bollworms to Bt toxins. In the present study, we devised three combinations of Cry toxins (Cry1AbBaBa, Cry1BaBaAb, Cry1AbBaAb) targeting Lepidopteran pests like *H. armigera*. Our aim was to control different insect pests with various toxin combinations to delay the evolution of resistance in target insects.

2. Materials and Methods

2.1. Materials

Luria-Bertani (LB) medium [36], Ampicillin, Agarose, tris(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), The Plasmid pBinAR Bt6 and the sequences of Cry1Ab and Cry1B

genes, were obtained from ICAR-National institute for Plant Biotechnology, New Delhi. The pGEM-T vector and Escherichia coli (DH5α) was used in this study.

2.2.Methods

2.2.1. Protein engineering of chimeric genes

We constructed three chimeric genes via *cryAbBaBa*, *cryBaBaAb* and *cryAbBaAb* by exchanging domain-coding fragments (D1,D2,D3) between *cry1Ab* and *cry1Ba* genes.

2.2.1.1 Designing domain specific PCR primers

Parental genes *cry1Ab* and *cry1Ba* were named as *Ab1D-Ab2D-Ab3D* and *Ba1D-Ba2D-Ba3D* to represent domains I, II and III of *cry* genes, respectively. The chimeric gene constructed by swapping the domains I and II of *cry1Ba* with domain III of *cry1Ab* was named as *Ba1D-Ba2D-Ab3D (BaBaAb)* and the hybrid made by exchanging domain I of *cry1Ab* with domains II and III of *cry1Ba* was named as *Ab1D-Ba2D-Ba3D (AbBaBa)*. Another hybrid was constructed by exchanging the domain II of *cry1Ab* with that of *cry1Ba* and was named as *Ab1D-Ba2D-Ab3D* (*AbBaAb*). Primer sequences were designed at the domain junctions (Table 1). To facilitate cloning of the domain fragments, restriction sites were created without altering the amino acid sequences. Polymerase chain reaction (PCR) conditions standardized for the amplification of the domain(s) are given in Table 2.

Table 1. Primers used for the amplification of domain(s) from *cry1Ab* and *cry1Ba* genes

S.No	Primer	Domain(s)	Nucleotide sequences
1.	Ba-N-1D2DF	Dom1,2cry1Ba	5'CGGGATCCATGGTTACCTCCAACCGTAAG3' BamH1
2.	Ba-N-1D2DR	Dom1,2cry1Ba	5'ACGCGTCGACAGACCTGTGAGTCCAAGAG 3' Sal1
3.	Ba-N-2D3DF	Dom2,3-cry1Ba	5' GAAGATCTTACCCCATCAACACCTCTG 3' Bgl II
4.	Ba-N2D3DR	Dom2,3-cry1Ba	5' CGAGCTCTTACCTCTCCAAGTCGTAC 3' SacI
5.	Ab-N-1DF	Dom1-cry1Ab	5'CGGGATCCATGGACAACAACCCAAAC 3' BamH1
6.	Ab-N-1DR	Dom1-cry1Ab	5'GAAGATCTGGAGTCATACTTCGGGAAG 3' BglII
7.	Ba-N-2DF	Dom2-cry1Ba	5'GAAGATCTTACCCCATCAACACCTCTG 3' BglII
8.	Ba-N-2DR	Dom2-cry1Ba	5'ACGCGTCGACAGACCTGTGAGTCCAAGAG 3' SalI
9.	Ab-N-3DF	Dom3-cry1Ab	5'ACGCGTCGACTTCAACAATATCATTCCTTC3' SalI
10.	Ab-N-3DR	Dom3-cry1Ab	5' CGAGCTCTCAGTACTCAGCCTCGAAG3' SacI

Table 2. PCR conditions standardized for the amplification of the domain(s)

S. No.	Domain	Annealing temperature	No. of cycles	Minutes
1.	Initial denaturation	94 ^{0}C	1	5
2.	Denaturation	94 ^{0}C	30	1
3.	Annealing			
	cry1Ab Domain I cry1Ba Domains I and II cry1Ab Domain III cry1Ab Domain II cry1Ba Domains II and III	52 ^{0}C	30	1
4.	Extension			
	cry1Ab Domain I	72 ^{0}C	30	1
	cry1Ba Domains I and II			2
	cry1Ab Domain III			2
	cry1Ab Domain II			1
	cry1Ba Domains II and III			3
5.	Final extension	72 ^{0}C	1	10

2.2.1.2. Cloning of domain fragments onto pGEMT vector

The *cry1Ba* fragment in coding domains I and II was amplified (1500 bp) using domain-specific primers. This was further purified and digested with *BamHI* and *Sal1*, and ligated into the pGEMT vector. The amplified PCR product containing domain III of *cry1Ab* (0.45 kb) was digested with *Sal1* and *Sac1* and cloned into pGEMT::*Ba1D-Ba2D*, which was restricted with the same enzymes to get the final construct of pGEMT::*Ba1D-Ba2D-Ab3D*. Domain I encoded by cry1Ab was amplified with domain-specific primers and resolved in a 1% agarose gel. The amplified product of 750 bp was obtained and purified. The purified product was restricted with BamH1 and BglII and ligated into pGEMT vector. Domain II of *cry1Ba* gene was amplified

using PCR primers. The amplified product (600 bp) was purified and restricted with *Sal1* and *BglII*, and cloned into the *Sal1* and *BglII* sites of pGEMT vector carrying the domain I of *cry1Ab*. Furthermore, domain III of *cry1Ab* was amplified using specific primers. The purified product (0.45 kb) was restricted with *SalI* and *Sac1* and cloned into pGEMT ::*Ab1D-Ba2D*, which was restricted with the same enzyme.

2.2.2.Molecular Modelling and Validation of the chimeric *Bt* genes

The SWISS-MODEL was employed to explore suitable templates for homology modeling of three chimeric proteins. Utilizing the modeller server, 3D structures were predicted based on templates exhibiting high similarity. The selection of the best models for each chimeric protein was guided by the stereochemistry quality report generated through PROCHECK, an assessment tool that evaluates overall structure quality and highlights areas for further scrutiny. To verify the structures, the ERRAT tool was applied, focusing on non-randomly distributed atoms, which are considered more reliable due to their energetic and geometric effects. Models with less random atom distributions are deemed more reliable. Additionally, VERIFY-3D was utilized to assess the compatibility of the developed 3D models with their respective amino acid sequences.

3. Results

3.1.Cloning and expression of parental and chimeric toxins

The isolation of *cry1Ba* gene fragment from plasmid pBin Bt6 has been performed using plasmid minikit. Cloning of *cry1Ba* in expression plasmid pGEMT was facilitated with the addition of restriction sites at the 5' end of the primers. Cry1Ab, Cry1Ba domain-specific primers were designed (Table 1) and PCR was carried out to amplify the gene sequence following the standardized conditions (Table 2). The amplified PCR product was purified and cloned into pGEMT- vector, and transformed into *E. coli* DH5α. A single colony was inoculated in 5 ml LB medium and grown overnight at 37 ^{0}C, at 220 rpm. The overnight grown culture was subcultured into 100 ml Luria Bertani medium (LB) and incubated at 37 ^{0}C, 220 rpm until the OD600 reached 0.6. All parental and chimeric genes were transformed into BL21(DE3) cells.

3.1.2. PCR mediated Cloning of different domains of *Cry1Ab* and *Cry1Ba* onto pGEMT vector

3.1.2.1 Cloning of AbD1 from pGEMT into pGEMT containing BaD2D3

The gene construct pGEMT::*Ab1D-Ba2D-Ba3D* was developed by PCR amplification of the domain I of cry1Ab with specific primers. The amplified product showing a size of 750 bp was purified (Figure 02) and restricted with *BamH1* and BglII, and ligated into pGEMT . Domains II and III of cry1Ba (1.0 kb) were amplified using domain-specific primers, purified, restricted with BglII and SacI, and ligated in BamHI and BglII sites of pGEMT::Ab1D. The final construct pGEMT ::Ab1D-Ba2D-Ba3D was confirmed by restriction analysis.

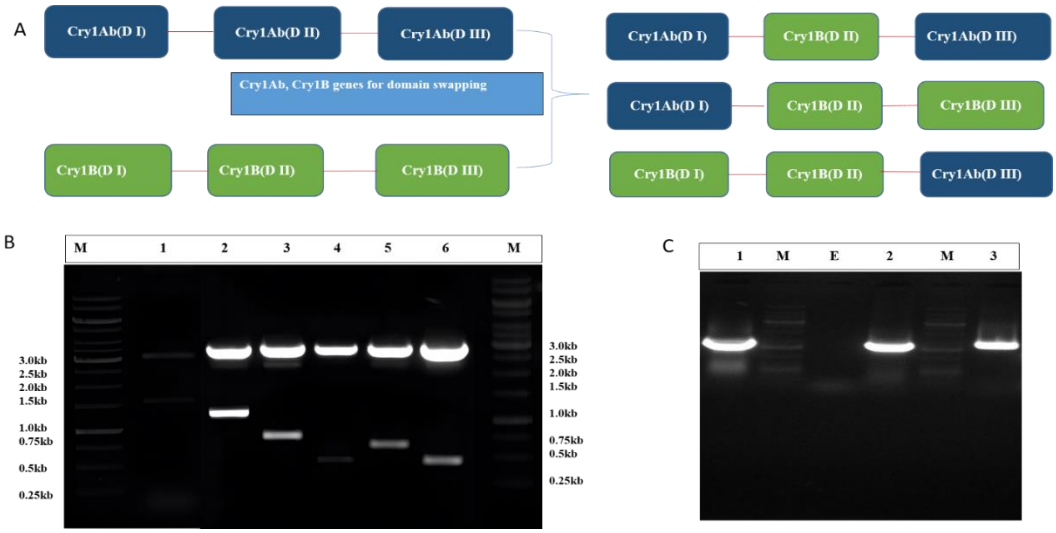

Figure 1 A: Strategy for domain swapping of Cry1Ab and Cry1B toxins.

Figure 1B M:1kb ladder; Amplified PCR products of domain fragments (Lane1-6) Lane1:BaD1D2; Lane2:BaD2D3; Lane3:AbD1; Lane4:BaD3; Lane5:BaD2; Lane6:AbD3

Figure 1C M:1kb ladder; Lane1:Colony confirmation for AbBaBa; Lane2: Empty; Lane3:Colony confirmation for AbBaAb; Lane4:Colony confirmation for BaBaAb

3.1.2.2. Cloning of AbD3 from pGEMT onto pGEMT containing BaD1D2

The gene construct pGEMT::Ba1D-Ba2D-Ab3D was developed by PCR amplification of the domain III of cry1Ab with specific primers. The amplified product showing a size of 450 bp was purified (Figure 02) and restricted with Sal1 and SacI, and ligated into pGEMT . Domains II and III of cry1Ba (1.0 kb) were amplified using domain-specific primers, purified, restricted with BglII and SacI, and ligated to SalI and SacI sites of pGEMT::Ab3D. The final construct pGEMT ::Ba1D-Ba2D-Ab3D was confirmed by restriction analysis.

3.1.2.3. Cloning of AbD3 from pGEMT onto pGEMT containing AbD1BaD2 obtained by subcloning of BaD2 from pGEMT onto pGEMT containing AbD1

The gene construct pGEMT::Ab1D-Ba2D-Ba3D was developed by PCR amplification of the domain I of cry1Ab with specific primers. The amplified product showing a size of 750 bp was purified (Figure 02) and restricted with BamH1 and BglII, and ligated into pGEMT . Domains II of cry1Ba (600 bp) was amplified using domain-specific primers, purified (Figure 02), restricted with BglII and SalI, and ligated in BamHI and BglII sites of pGEMT::Ab1D. Domain III of cry1Ab(450bp) was amplified using domain-specific primers, purified and restricted with SalI and SacI and ligated to BglII and SalI sites of pGEMT::Ab1D-Ba2D. The final construct pGEMT ::Ab1D-Ba2D-Ab3D was confirmed by restriction analysis.

3.2.Molecular Modelling and Validation of Chiameric proteins

The SWISS-MODEL server was utilized to predict the protein models for Cry1AbBaBa, Cry1AbBaBa, and Cry1BaBaAb using selected PDB templates: 1ciy (61.3%), 1ciy.1.A (75%), and 6owk.1.A (74.5%), respectively (Figure 2). Validation of the predicted models was performed using the structural evaluation program PROCHECK, as illustrated in Figure 2 and detailed in Table 3. Ramachandran plots were employed to analyze the peptide dihedral angles, classifying them into allowed and non-allowed regions. The models exhibited favorable backbone conformations, with over or near 90% of amino acid residues falling within the favorable region (Figure 2 B, D, C). Further, quality validation was carried out using the ERRAT score, resulting in scores of 93.5, 94.5, and 91.1 for Cry1AbBaBa, Cry1AbBaBa, and Cry1BaBaAb, respectively (Table 3). These scores indicated non-randomly distributed atoms, emphasizing the reliability of the models. Verify-3D analysis supported the dependability of the proposed models, with 91.14% (Cry1AbBaBa), 87.22% (Cry1AbBaBa), and 90.66% (Cry1BaBaAb) of amino acids having an average 3D-1D score of >0.2 (Table 4).

Table 3. Ramachandran plot statistics of predicted protein models of three chimeric proteins

Plot Statistics	**CRY1AbBaBa**		**CRY1AbBaAb**		**Cry1BaBaAb**	
Residues in most favoured regions [A,B,L]	466	91.0%	478	92.6%	459	88.4%
Residues in additional allowed regions [a,b,l,p]	41	8.0%	34	6.6%	52	10.0%
Residues in generously allowed regions [~a,~b,~l,~p]	3	0.6%	2	0.4%	7	1.3%
Residues in disallowed regions	2	0.4%	2	0.4%	1	0.2%
Number of non-glycine and non-proline residues	512	100%	516	100%	519	100%

Table 4. Validation of selected models of all the chimeric proteins

Protein Name	ERRAT	VERIFY3D
CRY1AbBaBa	93.5	91.14%
CRY1AbBaAb	94.5	87.22%
Cry1BaBaAb	91.1	90.66%

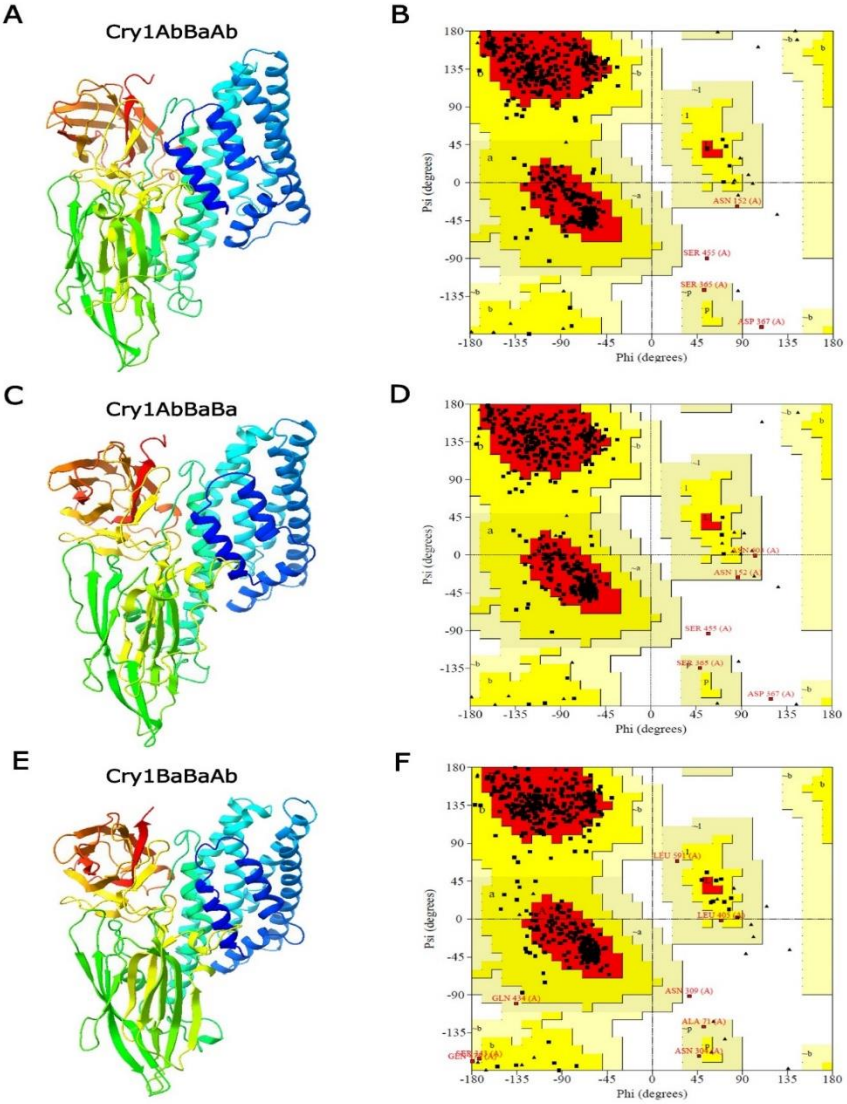

Figure 2 Structure prediction and validation of the chimeric proteins. (A, C, E) 3-D structures of the respective chimeric proteins; (c) Ramachandran plot for the chimeric proteins

4.DISCUSSION

A significant amount of research is underway to develop transgenic crops with enhanced market value, with the insertion of genes like the Bt toxin genes to control insects being a prominent strategy [37]. Recent advancements include the development of "pyramid" cultivars incorporating multiple cry genes (*cry1Ac* and *cry2Ab*), offering stronger resistance against Lepidoptera target insects [37]. According to English et al. [38], Cry1A and Cry2A toxins exhibit distinct binding sites in the larval midgut, suggesting that understanding the mode of action of Cry toxins is crucial for their optimization. It has been hypothesized that the insecticidal selectivity of Cry toxins against target pests is significantly influenced by the receptors on the midgut epithelial cell membranes of respective pests [37].

The mechanism of action of Cry proteins appears to involve a two-step process: initial binding to specific receptors followed by integration into the membrane, leading to membrane disruption [39]. However, research indicates that toxicity is only partially dependent on receptor binding, suggesting that post-binding events such as protein integration into the membrane or pore formation may be more significant [40].

Seven new domain mutants were created by replacing Cry1Ab's domain III with seven distantly related Cry proteins [41]. Dietary exposure studies on the Asian corn borer revealed varying levels of insect resistance among these mutants, indicating that not all domain III exchanges enhance insecticidal activity [41]. Additionally, transgenic rice expressing Cry1A/Cry1I-like fusion protein effectively suppresses lepidopteran pests, highlighting the potential of hybrid toxins in pest management strategies [41].

The present study aimed to produce chimeric Bt genes from *cry1Ab* and *cry1Ba* through domain swapping, with the developed genes exhibiting the canonical Bt gene structure. These chimeric genes hold promise for designing introgression studies in crop improvement programs [37].

5.References

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