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Random Mutagenesis to Enhance the Toxicity of *Bacillus thuringiensis* Cry Proteins against *Earias vittella* (F.)

(Mutagenesis Rawak untuk Meningkatkan Ketoksikan Protein *Cry Bacillus thuringiensis* terhadap *Earias vittella* (F.))

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ABSTRACT

Insecticidal proteins derived from *Bacillus thuringiensis* (Bt) are widely utilized in a variety of insect control applications, including sprays and transgenic crops. The development of resistance in pests, on the other hand, can lessen the effectiveness of Bt toxins. In this study, we made efforts to enhance the toxicity of two cry proteins Cry1Ac and Cry2Aa through random mutagenesis against cotton bollworm *(Earias vitella*), one of the most destructive cotton pests in Pakistan. Random mutagenesis is an important tool for elucidating protein structure-function relationships and for modifying proteins to enhance or change their characteristics. We focused on whole cry proteins for random mutagenesis through error-prone PCR and constructed a recombinant library of *cry* proteins. Sequence analysis of eight mutants showed the mutations of 34 different nucleotides in Cry1Ac and Cry2Aa genes. All mutants were spared for toxicity bioassays against 2nd instar larvae of spotted bollworm. Cry1Ac mutant RM1AcM4 (D242E) and Cry2Aa mutants RM2AaM2 (T354A, T492R, F511L, G585E, D606Y) showed enhanced toxicity as compared to proteins without mutation. These two mutants comprise the mutations in domain-II of *cry* proteins important in specificity determining regions on midgut receptors in insect pests.

Keywords: *Bacillus thuringiensis*; error-prone PCR; random mutagenesis; *Earias vitella*

ABSTRAK

Protein racun serangga yang diperoleh daripada *Bacillus thuringiensis* (*Bt*) digunakan secara meluas dalam pelbagai aplikasi kawalan serangga, termasuk semburan dan tanaman transgenik. Perkembangan rintangan pada perosak sebaliknya boleh mengurangkan keberkesanan toksin *Bt*. Dalam kajian ini, kami berusaha untuk meningkatkan ketoksikan dua protein *cry* Cry1Ac dan Cry2Aa melalui mutagenesis rawak terhadap ulat bulu kapas (*Earias vitella*), salah satu daripada perosak kapas yang paling teruk di Pakistan. Mutagenesis rawak ialah alat penting untuk menjelaskan hubungan struktur-fungsi protein dan untuk mengubah suai protein untuk meningkatkan atau mengubah cirinya. Kami memberi tumpuan kepada keseluruhan protein *cry* untuk mutagenesis rawak melalui PCR yang terdedah kepada ralat dan membina perpustakaan rekombinan protein *cry*. Analisis jujukan lapan mutan menunjukkan mutasi 34 nukleotida berbeza dalam gen Cry1Ac dan Cry2Aa. Semua mutan telah dikecualikan untuk bioasai ketoksikan terhadap larva instar kedua ulat bulu. Mutan Cry1Ac RM1AcM4 (D242E) dan mutan Cry2Aa RM2AaM2 (T354A, T492R, F511L, G585E, D606Y) menunjukkan ketoksikan yang dipertingkatkan berbanding dengan protein tanpa mutasi. Kedua-dua mutan ini terdiri daripada mutasi dalam domain-II protein *cry* yang penting dalam kekhususan menentukan kawasan pada reseptor usus tengah pada perosak serangga.

Kata kunci: *Bacillus thuringiensis*; *Earias vitella*; mutagenesis rawak; PCR terdedah ralat

INTRODUCTION

Bacillus thuringiensis (*Bt*), is a Gram-positive soil bacteria that produce highly specific insecticidal proteins called delta-endotoxin. These toxins are produced as crystalline inclusions within the bacterial cell during sporulation. The crystal protein that is toxic against a wide variety of different insect pests (Adams, Eiteman & Hanel 2002; Koppenhöfer et al. 2000; Mannion et al. 2001). These

pest insects can deal with, avoid, or switch off natural plant defensive capabilities, which can include chemical toxins and proteins like protease inhibitors and lectins that stop insects from digesting food. Different physical, biological, and chemical methods used to control different insect pests in agriculture. As an alternative method, there has been a lot of progress in making control plans that use transgenic crops that are resistant to insects (Torres-Quintero et al. 2018).

It is impressive to note that agriculture contributes significantly to the country's GDP, and cotton is a vital crop for the economy. Within 1.3 million framers cultivating cotton over 3 million hectares, it shows that cotton farming is a crucial source of income and livelihood for many people in Pakistan. The country's position as a leading producer, exporter and consumer of cotton highlights its importance in the global cotton industry. However, it is worth noting that the cotton sector in Pakistan faces some challenges, like inefficient farming practices, limited access to modern technology and machinery and issues with water management. It is alarming to note that cotton is susceptibility to attack by more than 67 insects, with the bollworms being among the most significant threats to the crop. The fact that bollworms cause heavy damage to cotton is a concern for farmers, as it can result in significant yield losses and reduced profitability. Addressing these challenges could help to increase yields, improve quality, and boost the profitability of cotton farming for framers in Pakistan (Yunus, Makhdoom & Raza 2011).

Bollworms are the most destructive insect pests of cotton, to control them *Bt* biopesticide are used but suffers from a number of drawbacks, including poor virulence, a limited insecticidal spectrum, a short duration of action, and an increased likelihood of pest insect resistance (Kang et al. 2007). When it comes to the control of agricultural pests, the evolution of insect resistance may compromise the effectiveness of utilizing *Bt* toxins as a control method (Wei, Zhang & An 2019). As a result of the selection pressure that happens in the laboratory, certain insects have evolved resistance to toxins; hence, the modification of insecticidal proteins is an essential strategy for delaying the formation of insect resistance. When creating new proteins in the laboratory's controlled environment, scientists will sometimes use a method known as 'directed evolution'. This technique involves simulating the process of natural evolution without having prior knowledge of the protein's structure, active sites, or any other information (Romero & Arnold 2009).

Functional structure of *Bt cry* proteins has three domains (Domain-I, II & III), these domains have different functions: domain-I; the N-terminal region of the domain-I of *cry* protein comprising of six amphipathic helices with a central hydrophobic helix, it works by forming pores in the cell membrane, domain-II; consist of three antiparallel β-sheets, that oriented parallel to the α−helices of domain-I. Three surface exposed loops of variable length formed the domain-II apex in the hypervariable blocks identified as specificity determining regions. On ingestion by the insects, the *cry* proteins are solubilized and released protoxins in the alkaline midgut environment of the larvae. The protoxins (~130 kDa) are converted into toxic fragment (~ 66 kDa) by the gut proteases. Domain-III; it is composed of two antiparallel β-sheets into β-sandwich structure. Major function of this domain considered that maintaining the stability of the protein. Further studies showed the roles of β- sandwich such as stability of receptor binding, toxin specificity determination and control ion channel gating (Naqvi et al. 2017; Saraswathy & Kumar 2004).

Error-prone PCR is the *in vitro* evolution approach that is used the most frequently, and it is capable of generating combinatorial libraries that are founded on a single gene. It is possible to produce genetic variants by influencing the circumstances in which Taq polymerase amplifies DNA. Manipulating the environment may do this (Bleisch et al. 2022; Leung 1989; Van Dillewijn et al. 2004). Random mutagenesis and the advancement of technology that allows for *in vitro* gene recombination have both emerged as valuable tools in the effort to research and enhance the characteristics of proteins (Lutz & Patrick 2004; Vanhercke et al. 2005).

Mutation studies can be used to investigate the mechanism by which delta-endotoxin in *Bt* work and to generate *cry* toxin with enhanced toxicity. This approach involves introducing specific mutation into the gene encoding, the delta-endotoxin to alter the properties of the toxin (Manoj Kumar & Aronson 1999). These improved methods have the potential to be implemented in resistant management systems as potential replacements for toxins that are presently in use and to which insects may have developed resistance. Resistance development in insects is a major problem associated along with intensive utilization of *Bt* pesticides in agriculture, which decreases its effectiveness to control agricultural pests with genetically engineered transgenic crops and formulations designed to express genes coding for these *cry* proteins (Jan et al. 2015). Analysis of particularly biochemical and genetic studies of insects that have shown resistance to *Bt* toxins is specific to a subset of these toxins, and that reduced sensitivity to the toxins is linked to modifications in their binding sites on the brush

border membrane vesicles of the larval midgut (Gould et al. 1992; Talaei-Hassanloui et al. 2014; Wang et al. 2012).

The aim of this study was to create random mutations in Cry1Ac and Cry2Aa genes from *Bacillus thuringiensis* and analyzed the effect of these mutations on both the toxicity of the encoded proteins against major cotton pest in Pakistan; the cotton bollworms *Earias vitella*. We performed biotoxicity assays to further test the hypothesis that improved *Bt* toxins can be identified *in vivo* more effectively than in non-mutated toxins.

Materials and Methods

Materials

The Expression vector pGEM T-Easy7 and *E. coli* BL21 pLysS was procured from Promega, USA. Clones for CryIAc and Cry2Aa were obtained from Prof. Dr. Crickmore Laboratory (University of Sussex). *Bacillus thuringiensis* strains HD-73, HD-1 and *E. coli DH5α* were obtained from BGSC (Bacillus Genetic Stock Center).

Design & construction of cry1ac and cry2aa cloning vectors

Complete genes of Cry1Ac and Cry2Aa was obtained from wild type *Bacillus thuringiensis* strains, cloned and expressed both Cry1Ac and Cry2Aa genes from the T7 promoter in Promega's pGEM-TEasy cloning vector used *E. coli* BL21 pLysS as a host system (Table 1). Cry1Ac was expressed by the 130 kDa protoxin, and Cry2Aa was expressed by the 65 kDa toxin. Expressions of both proteins were induced by 0.5 mM IPTG overnight at 25 °C in 2xLB medium. Both toxins were expressed as inclusions, harvested, and purified from lysed cells by rounds of centrifugation and washing. The inclusions were treated with solubilization buffer (50 mM Sodium Carbonate, 10 mM dithiothreitol (pH: 10-12), to release soluble toxin. Proteins were verified by SDS-PAGE (Sodium dodecyl sulfate Polyacrylamide gel electrophoresis).

TABLE 1. Specifically designed primers for full-length *cry*1Ac and *cry*2Aa genes

Primer's Name	Primer's Sequence 5'-3'
*cry*1Ac-F	5' GAATTCCCATGGAATAAAAGAGATGGAGGTAACTTATG 3'
*cry*1Ac-R	5' CCATGGGAATTCACCTGAGTTTGCATGAGAC 3'
*cry*2Aa-F	5' GAATTCCCATGGAAGGAGGAATTTTATATGAATAATGTATTG 3'
*cry*2Aa-R	5' CCATGGGAATTCTCACTAGTGATTCACTCAAACC 3'
RM1Ac-F	5' AATAAAAGAGATGGAGGTAACTTATG 3'
RM1Ac-R	5' ACCTGAGTTTGCATGAGAC 3'
RM2Aa-F	5' GAATTCGATTAAGGAGGAATTTTATATG 3'
RM2Aa-R	5' TCACTAGTGATTCACTCAAACC 3'
Int1Ac-F1	5' AATCAATTTAGAAGAGAATTAACACTAAC 3'
Int1Ac-F2	5'AATATAATTGCATCGGATAGTATTACTC 3'
Int1Ac-F3	5' ATTCAAATTTCAAAGACATTAATAGGC 3'
Int1Ac-F4	5' ACTCTCAATATGATCAATTACAAGC 3'
Int1Ac-R	5' CAGGAAACAGCTATGAC 3'
Int2Aa-F1	5' TGTATAAATACGTATCAAACTGCG 3'
Int2Aa-F2	5' AGAAATATAGAAAGTCCTTCGGG 3'
Int2Aa-R	5' CAGGAAACAGCTATGAC 3'

CREATION OF MUTAGENIZED TOXIN GENE LIBRARIES

For error prone (EP) random mutagenesis, we used the Gene Morph-II system from Agilent. Mutagenesis is carried out on a PCR product containing the active toxin portion of each toxin gene (i.e., domains I-III) using a PCR programme consisting of thirty cycles of denaturation followed by annealing at 55 °C for 30 s and extension at 72 °C for 1 min per kb. Using Agilent's EZ Clone, the resulting (randomly mutated PCR product) was then transferred back into the pGEM vector (The EZ clone system is a PCR based system that allows replacing a fragment in a plasmid with a PCR fragment of the same region (that may or may not contain some mutations). It avoided the need for restriction enzymes/ligation but only works where we are replacing one piece of DNA with a very similar one. It was ideal for this project where we replaced the non-mutated gene in the expression vector with the mutated ones. The mutant library was then introduced in *E. coli* BL21 for expression. DNA sequencing, and different bioinformatics tools were performed to identify the change in nucleotides of positive mutants.

PRELIMINARY CHARACTERIZATION

Initially prepared toxin from different mutants and optimized basic parameters of proteins expression, solubility of the crystal proteins in alkaline buffer and stability to trypsin to characterize the mutant library. Clones that showed the enhanced expression, solubilized in alkaline buffer were assayed against 2nd instar insect larvae. Initially a discriminatory dose was used just to detect large differences in activity. A second stage of preliminary characterization was involved in combing mutant forms of one toxin (Cry1Ac and Cry2Aa) with non-mutant forms of the other in order to detect change in toxicity.

DETAILED CHARACTERIZATION/ BIOTOXICITY ASSAYS

Biotoxicity assays were performed after the preliminary characterizations against spotted bollworms (*Earias vitella)*. Seed culture of spotted bollworms (*Earias vitella*) was collected from infested cotton fields and larvae were reared on artificial diets (Pinto beans flour 17%, Semolina 5%, Ravi yeast 2%, Vitamin C 0.3%, Sorbic acid 0.1%, Formaldehyde 0.15%, Parahydroxy Benzoic acid 0.2%, Agar 1.28%, and Distilled water 75%/L). Six different protein concentrations were used for biotoxicity assays ranged from 50 to 300 µg/gm. Each assay was performed in triplicate, and we used 2nd instar larvae for the biotoxicity assay. For a negative control, a comparable quantity of buffer was added to the diet and incubated at 28±2 ºC, 60±5 % relative humidity. We monitored mortality for 24 to 72 h and bioassays data was statistically analyzed using the Quantal Computer Program.

STATISTICAL ANALYSIS

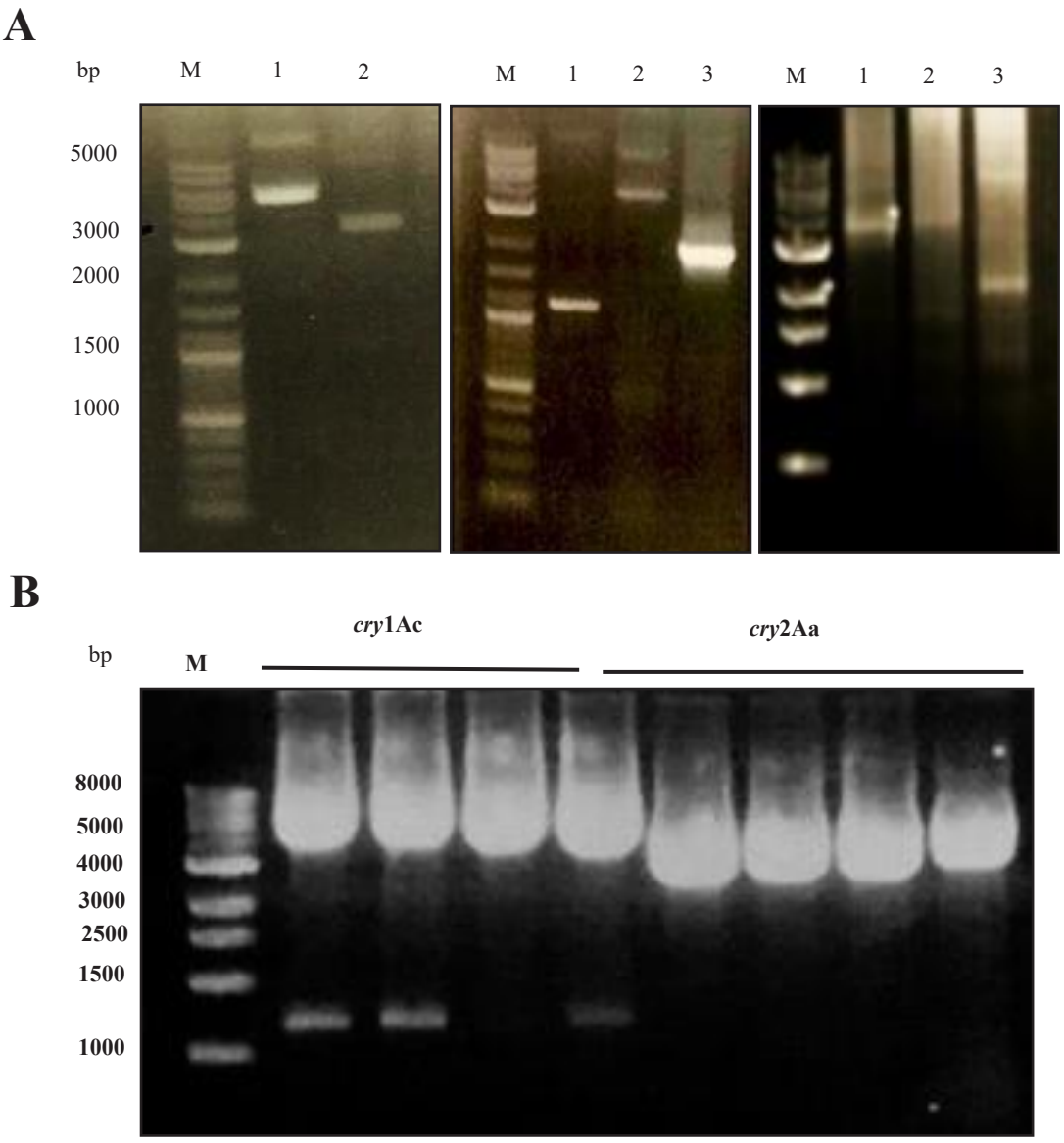
The statistical analysis was performed through SPSS, Quantal Program and Probit analysis to find out the mortality rate and LC_{50} values of mutants and non-mutant clones.

RESULTS

Full-length crystal protein genes for Cry1Ac and Cry2Aa were cloned from wild type *Bacillus thuringiensis* strains and expressed in pGEM T easy vector (Invitrogen) in *E. coli* BL21 pLyss host system. The clones Cry1Ac/pGEM and Cry2Aa/pGEM were spared for random mutagenesis with specifically designed primers through Gene Morph-II mutagenesis kit (Agilent). The amplified PCR products for both *cry* genes of size of 4.1 kb of Cry1Ac and 2.0 kb for Cry2Aa were cloned and transformed into the *E. coli* BL21 pLyss host system (Figure 1). Agarose gel electrophoresis performed for plasmids from randomly picked clones.

For mutation confirmation, we randomly picked twenty-eight clones for sequencing and selected ten mutants that showed change in different bases in three domains of *cry* genes (Figure 2). Out of ten, eight mutants showed alterations in their base sequences, and they were named as RM1Ac1–RM1Ac5 and RM2Aa1–RM2Aa5, respectively, according to the location of mutation and domain. In Table 2, all those mutants displayed the statistical information for change in nucleotides and amino acids. Eight mutants out of ten showed nucleotides altered at thirty-four different locations in three domains of *cry* gene for Cry1Ac and ry2Aa. By using sequence demarcation tool (SDT), we compared pairwise distance matrix of all mutant sequences aligned with original sequence of non-mutants through CLUSTAL W, where different color intensity showed the percentage of nucleotides mutation in both *cry* genes (Figure 2). Cry proteins were expressed and solubilized from those eight mutants for Cry1Ac and Cry2Aa proteins with 133 kDa and 68-72 kDa, respectively. Sodium dodicylesulfate polyacrylamide gel electrophoresis of mutant showed the bands size comparable to without mutation bands of protein.

In qualitative bioassays, the percentage mortality of Cry1Ac mutants RM1AcM1, RM1AcM2, RM1AcM3, RM1AcM4, and RM1AcM5 at concentration of 300 µg/mL was 33.3%, 47.0%, 63%, 73.0%, and 70.0%, respectively, when compared to non-mutants Cry1Ac strain, which was 60.0%. Significant difference was observed between the Cry1Ac mutants (Table S1 & S2). Whereas, the mortality of Cry2Aa mutants RM2AaM1, RM2AaM2, RM2AaM3, and RM2AaM4 at concentration of 300 µg/mL were 56.0%, 70.0%, 53.0%, 47.0%, and 67.0%, respectively, which showed the significant



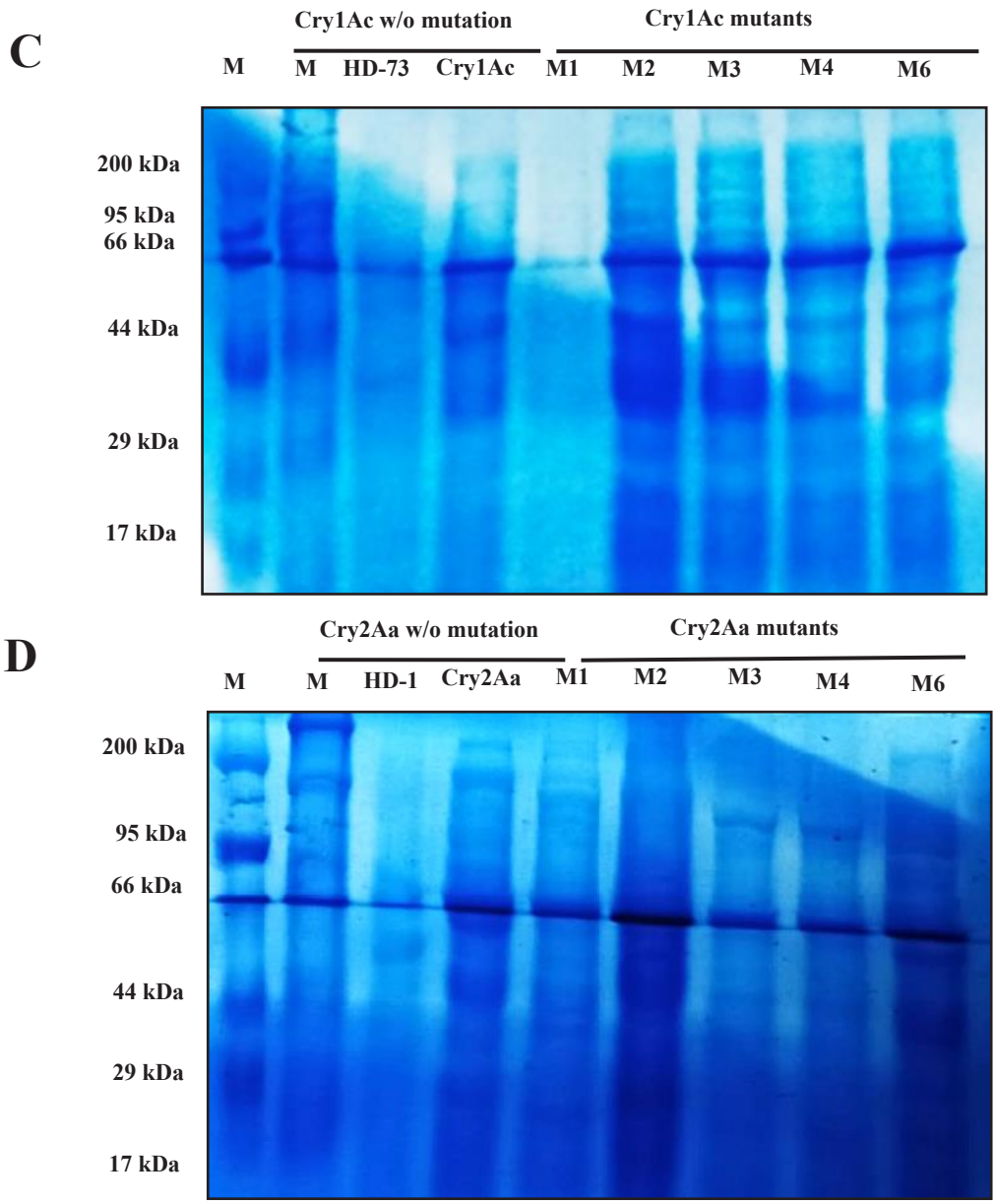


FIGURE 1. Random mutagenesis PCR for *cry*1Ac and *cry*2Aa full-length genes (A) Amplification of *cry*1Ac ~4.1 kb gene and *cry*2Aa ~2.0 kb gene through Gene Morph-II kit. (B) Plasmid extraction (Miniprep) from *cry*1Ac and *cry*2Aa mutants. (C) SDS-PAGE analysis for Cry1Ac mutant proteins expressed in *E. coli.* (D) SDS-PAGE analysis for Cry2Aa mutant proteins expressed in *E. coli*

difference between the mutants of Cry2Aa (Figure 3; Table S3).

DISCUSSIONS

Pakistan is an agriculture-based country and cotton is a major cash crop, which contribute in the major part of economy of Pakistan. One of the major factors that contribute important role for reducing cotton quantity and quality is the attack of different insect pests. Cotton insect pests divided into two types: sucking insects and chewing insects. The 80% damage was caused by the chewing

insects i.e., bollworms includes spotted bollworm (*Earias vitella, Earias insulana*), armyworm (*Spodoptera litura*), and American bollworm (*Helicoverpa armigera*). They damage many parts of cotton crop and most of the times it found boring in the reproductive and vegetative parts of cotton. The biological pesticide has a great potential for the control of various lepidopteran, dipteran and coleopteran insects. The most widely used biopesticide worldwide are those based on preparation of the bacterium *Bacillus thuringiensis*, which is an aerobic, spore forming soil bacterium that produces highly specific insecticidal crystal (*cry*) protein termed as delta-endotoxin, which is toxic against different insects (de Oliveira et al. 2023).

To improve the effectiveness of *bt* biopesticide against cotton bollworm, different strategies were used, one of them is the enhancement of the crystal protein toxicity through random mutagenesis (Jurat-Fuentes, Heckel & Ferré 2021; Sena da Silva et al. 2021; Vílchez, Jacoby & Ellar 2004; Zhang et al. 2013) to avoid resistance development in insect pests. We reported previously, the possible synergism between two proteins Cry1Ac and Cry2Aa against spotted bollworm for the effectiveness in pest management (Reisig et al. 2018; Yunus, Makhdoom & Raza 2011). The aim of this study was to enhance/ improve the toxicity of these synergistically acting *cry* proteins against *Earias vitella*. The error-prone PCR technique is an efficient method of mutagenesis that allows the mutation frequency to be controlled (Rasila, Pajunen & Savilahti 2009). The mutations in Domain III of Cry1Ac, and more especially changes located in the loop that links β16 and β17, are necessary for the enhancement of insecticidal activity against insect pests to increase the *cry* toxin binding affinity for the insect receptor (Shan et al. 2011).

TABLE 2. Nucleotide and amino acid sequence mutations of the random library

Mutants	Mutations		
	Nucleotides	Amino Acids	Domain
RM1Ac M1	T2853A, T2884A, T3234A	L962I, D1078E	I, II
RM1Ac M2	G2381T	G794V	I
RM1Ac M3	A1660T, A1758T, G3049T	T789A, T554S	I, II
RM1Ac M4	No result		
RM1Ac M5	A3666G	D242E	II
RM2AaM1	A858T, T1038C, A1110G, C1463A, A1562G	Q286H, T354A, P488Q, D512G	I,II
RM2AaM2	T1038C, A1060G, C1475G, T1533A, G1751A, A1790T, G1866T	T354A, T492R, F511L, G585E, D606Y	I,II
RM2AaM3	T1026C, T1038C, A1060G, C1232A, T1286A, C1316T, G1378T, T1398G, G1399A, T1626A, C1731T	D411A, D429V, I439T, S459A, M466V, R542S	II
RM2AaM4	T1038C, A1060G, C1584T	A354T	II
RM2AaM5	No result		

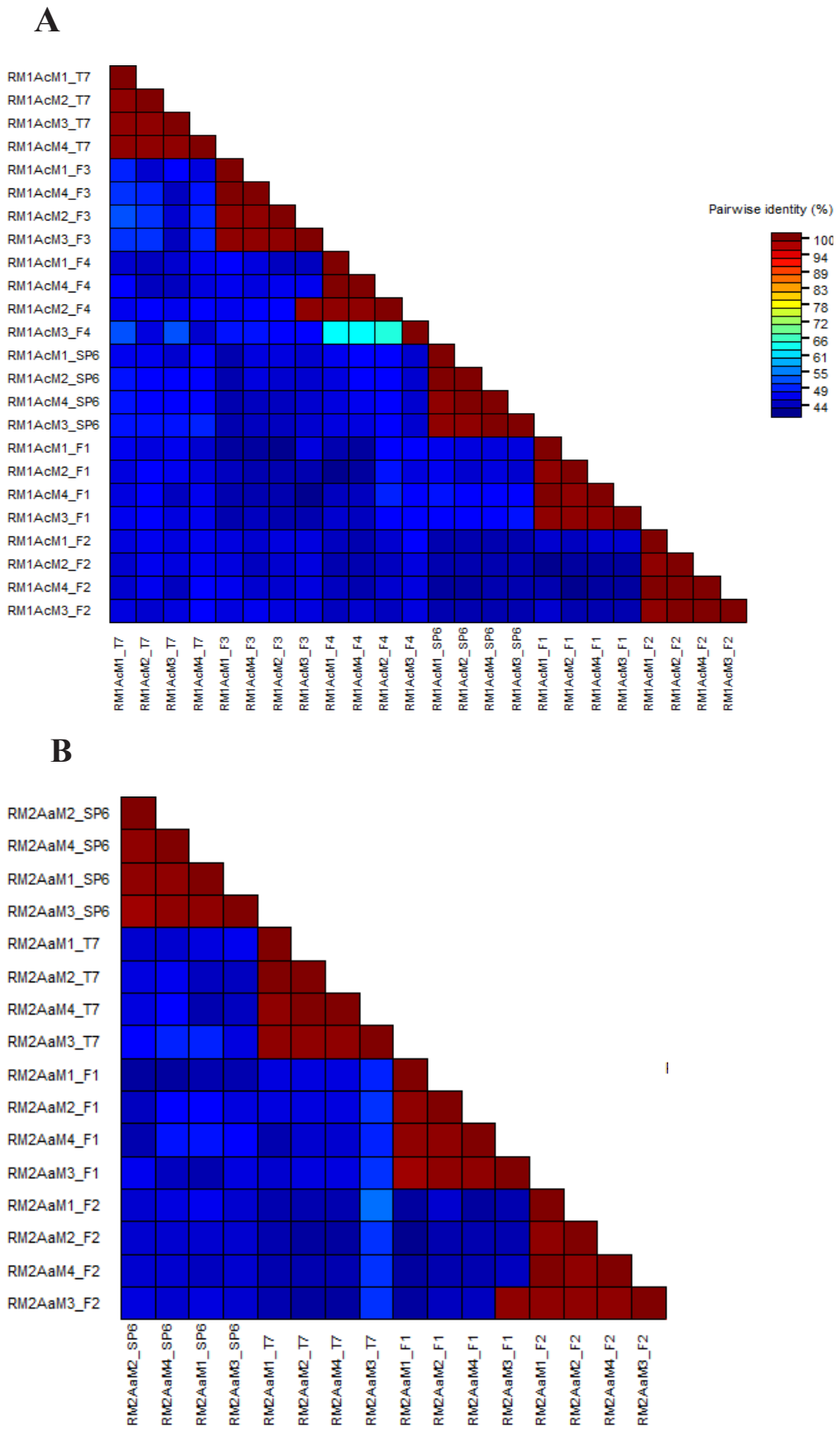


FIGURE 2. Pairwise distance matrix of all mutant sequences aligned with original sequence of non-mutants through CLUSTAL W using sequence demarcation tool (SDT). (A-B) Comparison of percentage identity of mutants and non-mutants clones of *cry*1Ac and *cry*2Aa, respectively

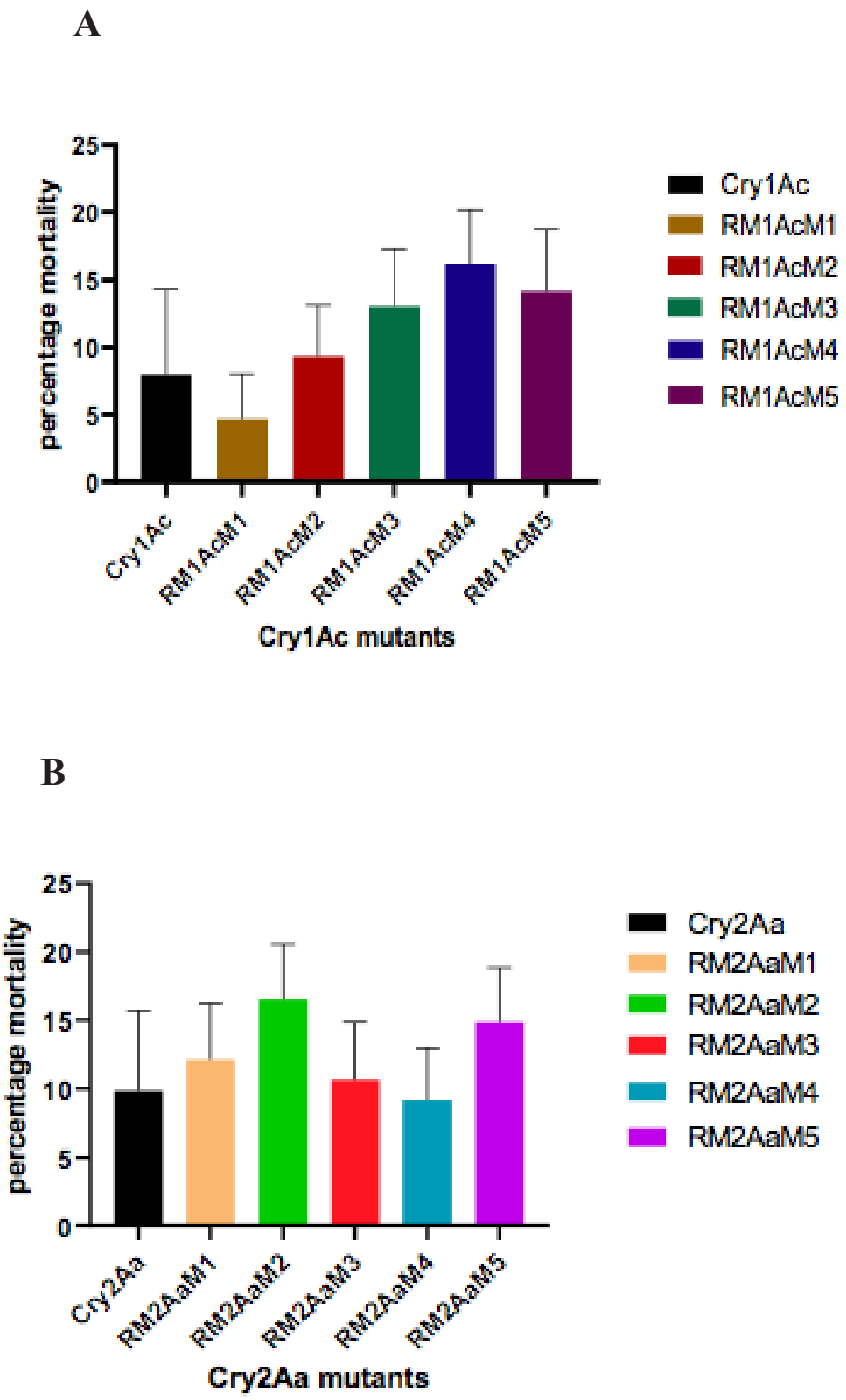


FIGURE 3. Graphical representation of LC_{50} values for biotoxicity assays against *Earias vitella* compared Cry proteins with and without mutations (A) Comparison of LC_{50} values of Cry1Ac mutants with original. (B) Comparison of LC_{50} values of Cry2Aa mutants with original

Using the pooled clone method, it was possible to successfully isolate a number of cry2 toxin genes from different strains of *B. thuringiensis*, one of which was a novel gene. There are now 11 members of the Cry2A family of toxins, and all of them share at least 70% of their sequence with one another. Despite this, they all have fairly different spectra of action. Cry2Ac is an anomaly since it does not have any action toward *H. punctigera* or *H. zea*, despite the fact that all of the Cry2A toxins that have been studied to this point have shown toxicity towards *H. armigera* and the majority of them have shown toxicity towards *H. zea* and *H. punctigera*. Cry2Aa is poisonous to *Spodoptera exigua*, despite the fact that the majority of Cry2A poisons are ineffective against this insect. Cry2Aa and Cry2Ag have activity against *Aedes aegypti*, but Cry2Ab and Cry2Ac do not; hence, the cross-order activity against dipteran species has been the subject of a significant amount of research (Liao, Heckel & Akhurst 2002; Van Frankenhuyzen 2009; Zheng et al. 2010). In contrast, both Cry2Aa and Cry2Ab have been shown to be active against the *Anopheles gambiae* mosquito, and a significant amount of mutagenesis research has been conducted in an effort to identify the areas of the Cry2A toxins that are responsible for this activity (McNeil & Dean 2011; Morse, Yamamoto & Stroud 2001).

In the present study, we focused to enhance the toxicity of two *cry* proteins, which was reported to be effective against *Earias vitella* (Yunus, Makhdoom & Raza 2011) by constructing a random recombinant library of these *cry* proteins using error prone PCR with specifically designed primers. We randomly picked twenty-eight colonies and screened them for any alteration in three domains of crystal protein. After sequencing, eight mutants showed the change in nucleotides at thirty-four different locations on three domains for both *cry* proteins (Table 2). We used laboratory biotoxicity assay for the screening of eight mutants that showed mutations in three domains of *cry* protein against 2nd instar larvae of *Earias vittella.* Cry1Ac mutant, RM1AcM4 showed 73% mortality, which was significantly higher as compared to standard Cry1Ac toxins which shows 60% mortality. Whereas, Cry2Aa mutant RM2AaM2 toxins expressed 70% mortality that was also significantly higher as compared to standard Cry2Aa toxin displayed 56% mortality. LC_{50} values of RM1AcM4 and RM2AaM2 were 122.3 µg/gm and 117.3 µg/gm, respectively. These two mutants showed lowest LC_{50} values as compared to all other mutants as well as standard toxins and the change of nucleotides reported in these mutants was in domain-II. Domain-II showed the correlation between toxicity of *cry* protein and binding of toxin to specific receptors on the mid-gut of insect pest. LC_{50} values indicated the degree of toxicity of the toxins; decrease in LC_{50} values depicts the high effectiveness of toxins on spotted bollworm (Saraswathy & Kumar 2004).

To overcome the resistance development problem in insect pests against *Bt*, we can use this strategy to mutate a few nucleotides/amino acids within three domains of crystal protein that could be more toxic against insect pests as compared to without mutation proteins. Enhancements in toxicity slow down the resistance development in insect pests. Toxins' activities may vary depending on factors including their affinity for a certain binding site and the number of binding sites present, so toxins selection should be based on less sharing of the same domain-II structure (Jenkins et al. 2000; Saraswathy & Kumar 2004; Shikano & Cory 2014).

Conclusions

The current findings of the research directed evolution were used as a method to enhance the insecticidal action of *cry* proteins. As a result, it was possible to create mutant proteins with an increased level of insecticidal activity. It serves as a reference for the research of the important locations of insecticidal action of *cry* protein as well as the unique mechanism of insecticidal activity. In addition to this, it offers genetic resources for the management of spotted bollworm pests, which has considerable theoretical relevance as well as valuable practical applications. These mutagenic proteins have the potential to serve as a useful tool for the generation of novel mutagenic *cry* genes for transgenic crops that have a high level of insecticidal action.

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TABLE S1. LC_{50} values of *cry*1Ac and *cry*2Aa mutants

Mutant	LC_{50} value	Slope	Upper n lower limit 95% confidence
	cry 1Ac		
RM1Ac M1	195.9	2.38 ±0.32	170.6 -235.0
RM1Ac M2	222.1	2.73±0.37	194.3-267.6
RM1Ac M3	189.8	2.56±0.33	167.0-223.2
RM1Ac M4	296.3	2.86±0.45	249.1-397.5
	cry 2Aa		
RM2Aa M1	188.2	2.76±0.34	167.1-218.1
RM2Aa M2	171.6	2.42±0.31	150.7-200.2
RM2Aa M3	211.4	2.69±0.35	185.6 - 252.1
RM2Aa M4	215.5	2.61±0.35	188.2-259.9
Standard strains			
*cry*1Ac	257.3	1.8±0.5	185.5 -638.0
*cry*2Aa	161.4	1.8±0.5	120.8 – 243.7

TABLE S2. Mortality percentage of cry1Ac mutant proteins with different concentrations

Conc.	Protein in µg/g of diet	Control	RM1AcM1	RM1AcM2	RM1AcM3	RM1AcM4	RM1AcM5
1.	50 µg/g	0	4/90 (4.4%)	17/90 (19%)	22/90 (24.4%)	32/90 (34.4%)	22/90 (27%)
2.	100 µg/g	0	6/90 (7%)	23/90 (26%)	28/90 (31%)	36/90 (40%)	31/90 (34.4%)
3.	150 µg/g	0	8/90 (9%)	27/30 (7%)	33/90 (37%)	40/90 (42%)	36/90 (40%)
4.	200 µg/g	1	10/90 (11%)	34/90 (38%)	40/90 (44.4%)	45/90 (50%)	43/90 (48%)
5.	20 µg/g	2	14/90 (16%)	36/90 (40%)	44/90 (49%)	54/90 (60%)	49/90 (54%)
6.	300 µg/g	2	18/90 (20%)	41/90 (46%)	58/90 (64.4%)	67/90 (74%)	63/90 (70%)