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CONSTRUCTION OF CHIMERIC *CRY2A* GENE OF *BACILLUS THURINGIENSIS* BY DOMAIN SWAPPING AND ITS ANALYSIS

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ABSTRACT

A chimeric *cry2A* gene of *Bacillus thuringiensis* (Bt) was constructed by domain swapping. DNA fragment corresponding to domain I & II of Cry2Aa and domain III of Cry2Ac was amplified by PCR and cloned individually in a separate pBluescript vector. The cloned DNA fragments of *cry2A* genes were aligned to construct a chimeric *cry2Ax2* gene. The chimeric *cry2Ax2* gene was cloned in an *E.coli*-Bt shuttle vector in between *cry3Aa* promoter and *cry2Aa* terminator, for gene expression studies. Though the presence of chimeric *cry2Ax2* gene has been verified by PCR, its expression in the transformants of acrystalliferous Bt strain, 4Q7, was not obvious in SDS-PAGE analysis. The non-expression of the chimeric *cry2Ax2* gene may be attributed to the biasness of the new codons introduced during the process of its construction. Expression of the newly constructed chimeric *cry2Ax2* gene may be achieved in the recombinant Bt strain after site directed mutagenesis or by constructing the gene by a suitable promoter in *E. coli*. After getting the chimeric gene expressed, it can be used for further bioassays to assess its toxicity against lepidopteron pest.

Key Words: *Bacillus thuringiensis*, chimeric *cry2A* gene, domain swapping, *E. coli*-Bt shuttle vector.

INTRODUCTION

Bacillus thuringiensis (Bt), is a well-known gram-positive, spore-forming soil bacterium that forms parasporal insecticidal crystal proteins during the stationary phase of its growth cycle. These proteins are termed delta-endotoxins because of their intracellular location and have been used for many years as successful biological insecticides[1]. Cloning of the first crystal protein gene (*cry*) of Bt was reported by Schnepf & Whiteley[2], since then more than 270 *cry* genes have been cloned, characterized, and their classification based on amino acid sequence similarity of their proteins[3]. Cloning of *cry* genes provides an opportunity to express the cloned gene in acrystalliferous Bt or *E. coli* to find out

the insecticidal activities of their proteins. In the natural isolates of Bt the *cry2Aa* and *cry2Ac* genes are expressed as third *orf* in operon model whereas *cry2Ab* gene is cryptic in nature. Dankocsik *et al.* [4] achieved expression of *cry2Ab* gene by the promoter of *cry3Aa* gene in an acrystalliferous strain of Bt.

Transgenic crops that produce Cry1A toxin can control some key lepidopteran pests[5]. Continuous exposure to a single kind of Bt toxin can lead to resistance development in insect pests. Routine replacement of *cry* genes or pyramiding of *cry* genes could be useful for effective control of insect pests by transgenic technology. The Cry2A proteins of *B. thuringiensis* are promising candidates for management of resistance development in insects due to its differences from the currently used Cry1A proteins, in structure[6] and mode of action[7]. Combination of Cry1Ac and Cry2Ab is used in the second version of Bt cotton (Bollgard® II) in USA and Australia. But the Indian populations of *H. armigera* were thirty-five fold less susceptible to Cry2Aa than Cry1Ac[8]. Studies are insufficient on susceptibility of Indian population of *H. armigera* to different Cry2A proteins. Variation of a single amino acid can significantly influence the level of toxicity in Cry proteins[9,10]. New variants of the already known *cry* gene subgroups[11], mutated[12,13] and chimeric[13] *cry* gene sequences could encode crystal proteins with significant difference in the level of toxicity due to variation in their sequences. Hence, in the present study a new chimeric *cry2A* gene was constructed and analysed.

MATERIALS AND METHODS

Genomic DNA isolation and amplification of Cry2A domains

Plasmid DNA was isolated from recombinant E. coli strains, harbouring p2Aa and p2Ac plasmids. The p2Aa plasmid contains cry2Aa operon and p2Ac plasmid contains cry2Ac operon. The plasmid DNA isolated from p2Aa and p2Ac plasmids was used as a template for PCR amplification. DNA fragments of ~1465 bp and~441 bp was amplified using 2AFS & 2AR2C and 2CF2C & 2ARS primers (Table 1). The PCR was performed for 30 cycles as follows: 94 °C for 1 min, 60 °C for 45 sec and 72 °C for 1.0 min., the final extension was performed for 7 min at 72 °C. The amplicons were column purified and examined on 1.2 % agarose gel.

Cloning of amplicons individually in pBKS vector

Two sets of pBKS vector were linearized by double digestion with *Kpn*I and *Hind*III for one set and *Hind*III and *Xba*I for another set. The amplicon of ~1465 bp (domain I & II of Cry2Aa) was double digested with *Kpn*I and *Hind*III. The amplicon of ~441 bp (domain III of Cry2Ac) was also double digested with *Hind*III and *Xba*I. The digested vector and the amplicons were resolved in agarose gel, excised from the gel and purified by gel extraction kit. Two sets of ligation mixture were prepared. The pBluescript vector digested with *Kpn*I and *Hind*III

restriction enzymes and the insert DNA fragment of ~1465 bp digested by the same enzymes were used in one set of ligation. In another set of ligation mixture, the pBluescript vector digested with *Hind*III and *Xba*I restriction enzymes and the insert DNA fragment of ~441 bp digested by the same enzymes were used. The vector and the insert DNA was used in 1: 3 ratio for ligation reaction. Ligation mixture was prepared and transformed to *E.coli* cells. The transformed colonies were selected on LB plate with XIA. The white colonies were screened by colony PCR with respective primers for checking the presence of insert. Restriction digestion was carried out as per the manufacturer's instruction. The recombinant pBKS plasmids (containing DNA fragment corresponding to domain I&II of Cry2Aa and DNA fragment corresponding to domain III of Cry2Ac) was double digested by *Kpn*I & *Hind*III and *Hind*III & *Xba*I, respectively to release the inserts. The digested product was kept at -20ºC to stop the reaction and analyzed by agarose gel electrophoresis.

Aligning of cloned DNA fragments to construct the chimeric gene, *cry2Ax2*

The recombinant pBKS containing DNA fragment corresponding to domain I & II of Cry2Aa was double digested by *Hind*III & *Xba*I for cloning domain III of Cry2Ac between *Hind*III & *Xba*I sites. The linearized recombinant pBKS harboring domain I & II of Cry2Aa and the insert (~441 bp of domain III of Cry2Ac) were excised from 0.8 % agarose gel. The DNA fragments were extracted using gel-extraction kit. Recombinant pBluescript vector and insert (DNA fragment of ~441 bp corresponding to domain III of Cry2Ac) were ligated used for transformation of *E. coli.* The transformants of *E. coli* colonies were screened by colony PCR and further confirmed by double digestion with *Kpn*I & *Xba*I restriction enzymes.

Cloning of the chimeric *cry2Ax2* gene in expression vector pHT3P2T

The recombinant pBKS plasmid carrying chimeric gene *cry2Ax2* was double digested by *Kpn*I and *Xba*I restriction enzymes to release the cloned DNA fragment. Simultaneously the expression vector pHT3P2T was also double digested using the same enzymes to linearize it. The expression vector pHT3P2T and insert (*cry2Ax2*) was ligated and transformed into *E. coli*. The presence of chimeric gene *cry2Ax2* was confirmed by restriction digestion of the recombinant pHT3P2T plasmid with the *Kpn*I and *Xba*I enzymes.

Transformation, screening and analysis of Bt strain 4Q7

Plasmid was isolated from recombinant *E.coli* colonies and transformed to acrystalliferous Bt strain, 4Q7 through electroporation[15]. Transformed Bt colonies were selected on LB agar plate containing erythromycin (50 µg/ml). The Bt transformants were screened for the presence of the chimeric gene *cry2Ax2* by PCR using 2AFS and

2AR2C primers (specific for DNA fragment corresponding to domain I and II of the chimeric gene *cry2Ax2*) and 2CF2C and 2ARS (specific for DNA fragment corresponding to domain III of the chimeric gene *cry2Ax2*). Spore crystal mixture was prepared from recombinant Bt strain, 4Q7 and analysed by SDS-PAGE.

RESULTS

Amplification and cloning of DNA fragment corresponding to domain I & II of Cry2Aa domain III of Cry2Ac

The DNA fragment of about 1465 bp encoding domain I & II of Cry2Aa was amplified from p2Aa with 2AFS and 2AR2C primers by PCR. The ~1465 bp amplicon was subjected for double digestion with *Kpn*I and *Hind*III. The double digested ~1465 bp amplicon was cloned into the vector pBKS. Recombinant *E. coli* clones were further screened for the presence of cloned DNA fragment by colony PCR using gene specific primers of the DNA fragment corresponding to domain I & II of Cry2Aa and by restriction digestion. The resultant plasmid is designated as pBKS2AA.

The DNA fragment of about 441 bp encoding domain III of Cry2Ac was amplified from p2Ac with 2CF2C and 2ARS primers by PCR. The amplicon (~441 bp) was subjected for double digestion with *Hind*III and *Xba*I and cloned into the pBKS vector. Recombinant *E. coli* clones were further screened for the presence of cloned DNA fragment by colony PCR using gene specific primers of the DNA fragment corresponding to domain III of Cry2Ac and by restriction digestion. The resultant plasmid is designated as pBKS2AC.

Aligning of DNA fragments corresponding to domain I & II of Cry2Aa and domain III of Cry2Ac

The recombinant pBKS2AA and pBKS2AC plasmids were double digested with *Kpn*I & *Hind*III and *Hind*III & *Xba*I, respectively. The released insert of ~441 bp from pBKS2AC plasmid was cloned into the linearized recombinant plasmid, pBKS2AA. The recombinant colonies were screened by colony PCR for the presence of gene specific primers of the DNA fragment corresponding to domain I & II of Cry2Aa and domain III of Cry2Ac and by restriction digestion (Fig. 1A). The constructed chimeric gene and its plasmid is named as *cry2Ax2* and pBKS2Ax2, respectively. Alignment of nucleotide sequence data obtained from chimeric *cry2Ax2* gene with that of *cry2Aa1* and *cry2Ac1* genes indicated fusion of domain I & II of Cry2Aa and domain III of Cry2Ac in the expected manner.

Cloning of chimeric *cry2Ax2* gene in expression vector, pHT3P2T

The recombinant pBKS2Ax2 and expression vector pHT3P2T were double digested with *Kpn*I and *Xba*I restriction enzymes. The released insert of ~1.9 kb from pBKS2Ax2 plasmid was cloned into the linearized recombinant plasmid, pHT3P2T. The recombinant colonies were confirmed by restriction digestion (Fig. 1B). The

recombinant pHT3P2T plasmid harboring *cry2Ax2* is named as p2Ax2.

Transformation of Bt strain 4Q7 with p2Ax2

The p2Ax2 plasmids (containing *cry2Ax2* gene under the transcriptional control of *cry3Aa* promoter and *cry2Aa* terminator) was transformed into the acrystalliferous Bt strain 4Q7 and selected on LB agar plate containing erythromycin (50 µg/ml). Transformants of Bt strain, 4Q7 were screened by PCR using gene specific primers. The amplification of DNA fragments corresponding to domain I & II of Cry2Aa (~1.5 kb) and domain-III of Cry2Ac (~441 bp) was observed in the transformants of Bt strain 4Q7 and there was no amplification in the case of negative control, 4Q7.

Comparison of protein profile of recombinant 4Q7 strains harboring *cry2Aa* and *cry2Ax2* gene expression plasmids

The spore-crystal mixtures obtained from the recombinant Bt strain, 4Q7 harbouring *cry2Aa* and *cry2Ax2* genes under the transcriptional control of *cry3Aa* promoter and *cry2Aa* terminator were subjected to SDS-PAGE analysis. The transformants of Bt strain 4Q7 harboring p2Ax2 plasmid did not show any prominent band equivalent to the size of Cry2Aa protein (~65 kDa) where as other positive control, recombinant Bt strain harbouring pHT3P2AT plasmid (Cry2Aa protein), showed the prominent band of ~65 kDa. The protein profile of the recombinant 4Q7 (pHT2Ax2) harboring the chimeric *cry2Ax2* gene was similar to that of negative control 4Q7 as shown in fig. 2.

DISCUSSION

A chimeric gene was constructed by replacing the whole DNA fragment of 441 bp encoding domain III of Cry2Ac with that of Cry2Aa. De Maagd *et. al.,*[18] had shown that several Cry1 toxins (e.g. Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ea) with low or no activity against beet armyworm (*Spodoptera exigua*) become active when their domain III is replaced by that of Cry1Ca. Alternatively, Malvar and Gilmer [19] showed that hybrids of Cry1Ac and Cry1Fa have a wider target spectrum than either of the parental toxins from which they were derived. Rang *et al*., [20], demonstrated that domain-III appears to modulate the activity of the chimeric toxins. They also explained that combination of domain -III from Cry1Ab with domain-I and II of Cry1C showed strong activity against insect cell lines than wild type Cry1C. Domain-III of Cry1Ac is involved in the specificity of binding to the putative Cry1Ac receptor from *Manduca sexta*, aminopeptidase, as well as in the binding to intact membranes [18].

The cloning of DNA fragment of 1465 bp encoding domain I & II of Cry2Aa in pBKS was achieved by domain I & II specific primers by introducing *Kpn*I and *Hind*III restriction sites at 5’ and 3’ end respectively. The cloning of DNA fragment of 441 bp encoding domain III of Cry2Ac in pBKS was achieved by its domain specific primers by introducing *Hind*III and *Xba*I restriction sites 5’ and 3’

respectively. *Hind*III restriction site was created by changing the codons at 485 and 486 positions (CATTTG → AAGCTT) there by altering the amino acids at 485th position (His →Lys). The chimeric gene of ~1.9 kb was constructed by aligning domain III of *cry2Ac* at 5' end between *Hind*III and *Xba*I sites of recombinant pBKS containing domain I & II fragment of *cry2Aa*. The construction of chimeric gene was verified by sequencing and named as *cry2Ax2.*

The *cry3Aa* gene, isolated from the coleopteran active *B. thuringiensis* var. *tenebrionis*[19], is a typical example of a non-sporulation-dependent *cry* gene. It has been shown that the *cry3Aa* promoter is weak, but significantly expressed during vegetative phase of growth unlike the *cry1A* promoter, which is sporulation dependent. The expression of *cry3Aa is* not dependent on sporulation-specific sigma factors either in *B. subtilis* [21] or in *B. thuringiensis* [22]. The *cry3A* promoter, although located unusually far upstream of the start codon (position - 558), resembles promoter recognized by the primary sigma factor (σ^A) of vegetative cells. Moreover, *cry3A* expression is increased and prolonged in mutant strains, which are unable to initiate sporulation [23].

In the present study the constructed chimeric gene *cry2Ax2* was cloned into expression shuttle vector pHT3PT between *cry3Aa* promotor and *cry2A* terminator. In the previous studies the vector has been tested for expression of *cry2Aa* gene[24]. The recombinant shuttle vector containing the chimeric gene *cry2Ax2* was transferred to Bt strain 4Q7 by electroporation. SDS-PAGE analysis was done for spore crystal mixture harvested from the recombinant clones for Bt strain, 4Q7. Expression of the chimeric gene, *cry2Ax2* was not seen as the prominent band of ~65 kDa. Protein profile of the recombinant 4Q7 was same as that in the case of negative control 4Q7. Positive control including recombinant 4Q7 containing *cry2Aa* gene in the same cassette showed expression as a prominent band of ~65 kDa.

The non-expression of the chimeric gene, *cry2Ax2*, may be attributed to usage of the codons that was introduced to create *Hind*III restriction site (CATTTG → AAGCTT) for cloning DNA fragments. The usage for these two codons was compared with the codon usage table for *B. thuringiensis* from CODON USAGE DATABASE (http://www.kazusa.or.jp/codon/). The usage for CAT codon, which is coding for histidine amino acid was 12.6 and 16.1 per 1000 codons for *cry2Aa* and *cry2Ac* genes, respectively. For TTG codon, which is coding for leucine amino acid, the usage was 14.2 and 9.6 per 1000 codons for *cry2Aa* and *cry2Ac* genes, respectively. The usage of codons present in *Hind*III site showed that AAG codon, coding for lysine amino acid, was 1.6 per 1000 codons for both *cry2Aa* and *cry2Ac* genes. Another codon, CTT present in the *Hind*III site, coding leucine amino acid, showed usage of 18.9 and 28.9 per 1000 codons for *cry2Aa* and *cry2Ac* genes, respectively.

Previous studies on codon usage showed that the frequencies with which

individual synonymous codons are used to code their cognate amino acids is quite variable from genome to genome and within genomes, from gene to gene. There is general agreement that codons are translated at different rates[25]. Hence codon usage has been identified as the single most important factor in prokaryotic gene expression[26]. In the present study, the introduced codons in the *Hind*III site of the chimeric *cry2Ax2* gene is less preferred for Bt. Therefore, tRNA recognizing the anti-codons in mRNA during translation may not be present in sufficient amount for successful expression of the chimeric gene in the recombinant Bt strain.

CONCLUSION

The expression studies of the newly constructed chimeric *cry2Ax2* gene may be done after changing the introduced codons back to the most preferred one (CATTTG → AAGCTT) by site directed mutagenesis. In previous study, silent mutation of a less preferred codon for an amino acid to more preferred one have increased the expression to four fold[27]. Expression of the newly constructed chimeric *cry2Ax2* gene may be achieved in the recombinant Bt strain after site directed mutagenesis. The *cry2A* genes are known to be expressed in recombinant *E. coli* strains[28,29]. The codons introduced in the present study are not less preferred by *E. coli*. Therefore, expression of the newly constructed gene by a suitable promoter in *E. coli* may also be successful. After getting the chimeric gene expressed, it can be used for further bioassays to assess its toxicity against lepidopteron pest.

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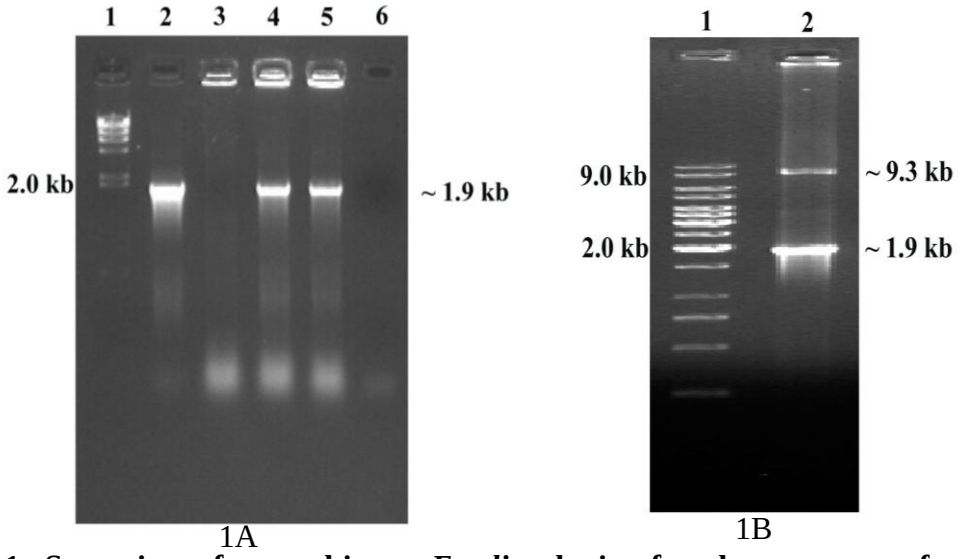
Table 1. Primers used for cloning DNA fragments corresponding to domain I & II of Cry2Aa and domain III of Cry2Ac

S.No.	Primer name	Sequence (5' 3')[a] →	Amplicon (size in bp)
1	2AFS	AT**GGTACC**ATGAATAATGTATTGAATAGTGGAA	DNA fragment corresponding to Domain I&II of *cry2Aa* (~1465 bp)
2	2AR2C	GG**AAGCTT**GATCATAGTACCATTTTCATTAGC	
3	2CF2C	AG**AAGCTT**GCGCCAAATGACTATACAGGAT	DNA fragment corresponding to Domain III of *cry2Ac* (~441 bp)
4	2ARS	GT**TCTAGA**CTCAAACCTTAATAAAGTGGTG	

[a]Primer sequences containing restriction endonuclease recognization sites for *Kpn*I (GGTACC), *Hind*III (AAGCTT) and *Xba*I (TCTAGA) are in bold faces.



1A 1B

Fig. 1. Screening of recombinant *E.coli* colonies for the presence of chimeric *cry2Ax2* gene by PCR and restriction digestion

1A: Lane 1, 2, 3, 4 & 5 - λ /*Hind*III marker**,** positive control, negative control, recombinant *E.coli* colonies harbouring *cry2Ax2* gene

1B: Lane , 2 : 1 kb marker, Digestion of p2Ax2 plasmid with *Kpn*I and *Xba*I enzymes

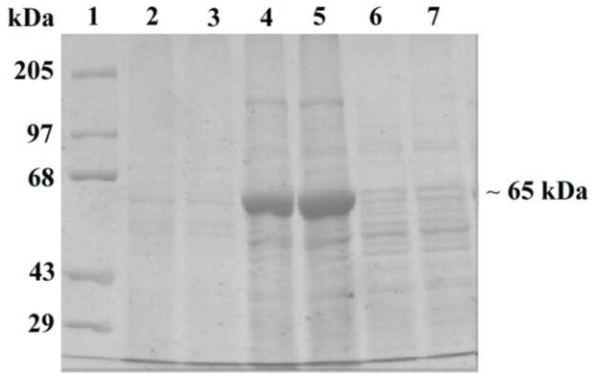


Fig. 2. SDS - PAGE analysis of spore - crystal mixture isolated from Bt strains

Lane 1 : Protein molecular weight marker

Lane 2&3 : Acrystalliferous Bt strain 4Q7

Lane 4&5 : Transformant of Bt strain 4Q7 harbouring pHT3P2AT

Lane 6&7 : Transformant of Bt strain 4Q7 harbouring p2Ax2