Construction of a potent strain of *Bacillus thuringiensis* against the cotton leaf worm *Spodoptera littoralis*

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Abstract

This work has been carried out in order to construct a potent *Bacillus thuringiensis* (*Bt*) strain active against the Egyptian cotton leaf worm *S. littoralis*. Toward this target, the δ-endotoxin crystal protein genes *cry*1C (that encodes an insecticidal protein highly specific to *S. littoralis*) and *cry*1Ag (1Ac like) were used. *Hind*III digested *cry*1C was ligated into the *Hind*III site of the shuttle vector pHT7593 yielding the plasmid pHTNC3. *Hinc*II digested *cry*1Ag was ligated into *Sma*I site of pHT7593 and into *Sma*I site of pHTNC3. The three plasmid constructs were used to transform *E. coli* strain JM109. Colonies likely to contain these recombinant plasmids were screened for the production of toxin proteins. Positively identified transformants produced the expected size protein, detected by partial purification of protein, and is truncated upon trypsin digestion.

The pHT7593-bearing *cry*1C, pHT7593-bearing *cry*1Ag and pHTNC3-harboring-*cry*1Ag were transferred into the non-crystalliferous (Cry^-) Bt4 *Bt* strain. The introduction of the *cry*1Ag gene into Cry^- Bt4 resulted in the formation of bipyramidal crystals. The introduction of both *cry* genes 1C and 1Ag resulted in the multiplication of bipyramidal crystals. In bioassays, *cry*1Ag-expressing BT4 *Bt* strain caused mortality of *S. littoralis* larvae only slightly (the LC_{50} was 104 ppm). In the presence of only Cry1C, the LC_{50} was 64 ppm. In presence of Cry1C co-expressed with Cry1Ag the LC_{50} decreased to 2.2 ppm. Thus, a combination of the Cry proteins 1C and 1Ag could result in effective insect control. With this approach, a combination of Cry proteins can be designed rather than discovered.

Keywords: Bacillus thuringiensis, cry 1C gene, Cry 1C toxin protein, cotton leaf worm, Spodoptera littoralis, transformation of Bt

Zusammenfassung

Herstellung eines Stammes von *Bacillus thuringiensis* wirksam gegen *Spodoptera littoralis*

Zur Reduzierung des Aufwandes an Pestiziden im Baumwollanbau könnte die Herstellung virulenter Bakterienstämme gegen Insekten ein wirksames Mittel sein. Die Arbeit beschreibt die genetische Modifikation von *Bacillus thuringiensis* durch Implementierung des δ-endotoxin Protein Gens *cry*1C. Das modifizierte Bakterium zeigt Wirksamkeit gegenüber *Spodoptera littoralis*.

Schlüsselwörter: Bazillus Thuringiensis, cry1C, Spodoptera littoralis, Transformation von Bt

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1 Introduction

Bacillus thuringiensis (*Bt*) is a ubiquitous gram-positive, spore-forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle. *Bt* was initially characterized as an insect pathogen and its insecticidal activity was attributed largely or completely to the parasporal crystals and also to the vegetative insecticidal proteins (Vip) that *Bt* produce it during its vegetative growth stage (Fang et al., 2007). This character led to the development of bioinsecticides based on *Bt* for the control of certain insect species among the orders Lepidoptera, Diptera, and Coleoptera and other insect orders (Hymenoptera, Orthoptera and Mallophage), and against Nematodes, Mites, and Protozoa (Feitelson, 1993; Feitelson et al., 1992). *Bt* seems to be indigenous to many envioronments (Bernhard et al., 1997). Strains have been isolated worldwide from many habitats, including soils (Delucca et al.,1977), insects (Carozzi et al., 1991), stored-product dust (Burges and Hurst, 1997), and deciduous and coniferous leaves (Kaelin et al., 1994).

Bt is now the most widely used biologically produced pest control agent and the foliar sprys from it plays a role in the integrated pest management strategies (Crickmore, 2006). Although the use of synthetic chemical pesticides in agriculture still in the front of that of biological pesticides, but several environmental and safety considerations favor the future development of *Bt* Cry proteins that have been studied thus far are not pathogenic to mammals, birds, amphibians or reptiles but are very specific to the groups of insects and invertebrate pests against which they have activity. Cry-based pesticides generally have low costs for development and registration. Finally the mode of action for the Cry proteins differs completely from the modes of action of known synthetic chemical pesticides, making Cry proteins key components of integrated pest management strategies aimed at preserving natural enemies of pests and managing insect resistance. Natural isolates of *Bt* can produce several different crystal proteins, each of which may exhibit different target specificity (Hofte and Whiteley, 1989; Lambert and Peferoen, 1992). Certain compinations of Cry proteins have been shown to exhibit synergistic effects (Chang et al., 1993; Crickmore et al., 1995; Lee et al., 1996; Poncet et al., 1995; Wu et al., 1994).

According genetic manipulation of *Bt* to create combination of genes more useful for a given purpose than those known to occur in natural isolates. Engineering of *Bt* and *B. cereus* through electroporation technology to transform vegetative cells with plasmid DNA has been done (Belliveau and Trevors, 1989; Bone and Ellar, 1989; Lereclus et al., 1989; Mahillon et al., 1989; Masson et al., 1989; Schuter et al., 1989). These protocols differed in cell preparation methods, buffer components and electric pulse parameters. A wide variety of hosts and vectors was used, a variety of shuttle vectors, some employing *Bt* plasmid replicons (Arantes and Lereclus, 1991; Baum et al., 1990; Chak et al., 1994; Gamel and Piot, 1992) has been used to introduce cloned *cry* genes into *Bt* (Burke and Baum, 1991). Alternatively, integrational vectors have been used to insert *cry* genes by homologous recombination into resident plasmids (Adams et al., 1994; Lereclus et al., 1992) or the chromosome. Plasmid vector systems employing *Bt* site-specific recombination system have been developed to construct recombination *Bt* strains for new bioinsecticide products (Baum et al., 1996; Sanchis et al., 1996; Sanchis et al 1997).

Bt Cry IC δ-endotoxin is a lepidopteran-specific insecticidal protein with a toxic spectrum different from Cry IA δ-endotoxins, *Bt* toxine nomenclature, 2006, http://www.lifesci.sussex.ac.uk/home/neil_crickmore/*Bt*/. Cry IC has high activity against *Spodoptera* species, which are relatively resistant to Cry IA toxins. Cry IC remains active against several insect species that have acquired resistance to Cry IA toxins (Ferre et al., 1991; Tabashnik et al., 1994). Cry IC is released from *Bt* crystals as a 135 KDa protoxin and cleaved to a 62 KDa toxin in the alkaline insect midgut. Cry IC and Cry IA toxins recognize different sites in the larval midgut (Van Rie et al., 1990; Escriche et al., 1994). From literatures Cry IC and Cry IAc binding sites are functionally distinct. Cry IAc resistant insect species (*Plod interpunctella and Plutella xylostella*) still bind and are killed by Cry IC toxin (Luo et al., 1996).

Spodoptera littoralis is a polyphagous insect, which is one of the major pests of cotton and other crops in Egypt and the Near East. This insect acquired resistance to several chemical insecticides, thus favoring the use of biological control. Many *Bacillus thuringiensis* strains possess insecticidal properties against different lepidopterous larvae. Several commercial preparations of *Bt*, mainly of serovars thuringiensis and kurstaki, are presently being employed throughout the world. However, these formulations have not yet succeeded to give good control of *S. littoralis* (Kalfon and De Barjac, 1985). In This study, we were interested in placing *cry*1C gene which encodes Cry1C protein, with other lepidopteran active gene into *Bt* strain to enhance the activity of *cry*1C against the cotton leaf worm *S. littoralis*. Moreover, the simultaneous production of two crystal proteins that act independently on the same insect, perhaps through the recognition of different receptors of larval midgut epithelial cells, might prevent or at least delay the appearance of insensitive insect population. To this end, construction of a *Bt* strain contained *cry*1C gene with other gene to enhance the toxicity and to be a potent *Bt* strain against the Egyptian cotton leaf worm *S. littoralis* was an aim.

2 Materials and methods

Bacterial strains and plasmids that were used in this work are illustrated in the Tables 1 and 2.

Table 1:
Bacterial strains used in this study

Bacterial strain	Description	Source
E. coli strains JM109		Contained in PGEM-T Easy vector system II kit. (Promega)
Bt strains *Kurstaki HD-73*	Cry1Ac	Pasteur institute, France
Entomocidus 60.5	Cry1C	
Bt4	Crystal negative	MMB lab, AGERI, Egypt
BtN1C	Cry1C	This study
BtNAg	Cry1Ag	This study
BtN1CAg	Cry1C/*cry*1Ag	This study
Transformed HD73	Cry1Ac/*cry*1C	This study

Table 2:
Plasmids used in this study

Plasmid	Vector/toxin gene	Promoter from which toxin gene was transcribed	Source	Accession No.
Cry1C	pTZ19R/1Cb	Cry1C	D.H.Dean, O.S.U	M97880
Cry1Ac	PKK223-3/1Ac	Cry1Ac	D.H.Dean, O.S.U	
Cry1Ag	pUC/1Ag	Cry1Ag	S.A.Mostafa, MMB lab, AGERI	AF081248
pHTNC3	pHT7593/*cry*1C	Cry1C	This study	
pHTNAg	pHT7593/*cry*1Ag	Cry1Ag	This study	
pHTN1CAg	pHT7593/ *cry*1C & *cry*1Ag	Cry1C/ *cry*1Ag	This study	

Construction of plasmids

The *cry*1C gene was generously obtained from Dr. Donald H. Dean's lab, Ohaio State University, USA; cloned into *Hind*III site of pTZ19-R. Cry1C in pTZ19-R plasmid was digested with *Hind*III to liberate the *Hind*III fragment that contains the coding region of *cry*1C gene with its regulatory region; at the same time the shuttle vector pHT7593 was digested with *Hind*III. The purified DNA corresponding to the *cry*1C gene and the vector were mixed in a 3:1 (insert: vector) molar ratio and ligated using T4 DNA ligase and transformed into *E. coli* cells JM109. Transformed colonies were screened by the rapid phenol/chloroform method (to an O/N 100 µl liquid culture of transformed colony, 50 µl phenol/chloroform and 10 µl loading day were added and then vortexed for 10 sec and spun for 3 min at 10,000 rpm). The same sequence of methods was used when the other 2 plasmids were constructed. The *cry*1Ag gene was obtained from Dr. Salah Mostafa, Microbial Molecular Biology lab (MMB), Agricultural Genetic Engineering Research Institute (AGERI), Giza, Egypt; cloned into pUC 18 vector. It was liberated by digestion of pUC 18/*cry*1Ag plasmid with *Hinc*II. The *Hinc*II fragment that coding for *cry*1Ag with its regulatory region was ligated with *Sma*I digested pHT 7593 vector. The third plasmid was constructed from the digestion of the 1st construct (*cry*1C in pHT 7593 shuttle vector) with *Sma*I and then mixed with the *Hinc*II fragment coding for *cry*1Ag gene and ligated together (Figure 1). The list of plasmids used is shown in (Table 1).

Transformation of Bt

Bt cells were transformed by electroporation as described by (Chang et al., 1992; Lecadet et al., 1992; Mettus and Macaluso, 1990 and Luchansky et al., 1988). 500 ng of the plasmid DNA, that derived from the recombinant *Bt* cells were added to 0.8 ml of *Bt* competent cells and placed in sterile, pre chilled electroporation cuvettes (0.4 cm interelectrode gap) and hold on ice for 5 min. Electroporation was carried out with a Bio-rad Gene pulser at a field strength 2.5 KV, resistance 150 Ω (Ohms) and capacitance 25 µf. The electroporated cells were added to 1.5 ml of LB medium, incubated for 1 h at 37 °C, plated on LB medium containing 100 µg/ml kanamycin and then incubated at 30 °C for 24 to 48 h. Kanamycin resistant (Kan^r) transformants were analyzed for the presence of the transformed plasmids.

Polymerase chain reaction (PCR)

PCR has been used to screen the transformed *E. coli* cells and the electroporated *Bt* clones for the presence of *cry*1C and *cry*1Ag, the lipedopteran toxin genes. The list of primers used is shown in (Table 3). The reaction conditions were performed according (Regev et al., 1996), where the PCR mixture was in a total volume of 25 µl contained 1 µg of total DNA, 50 pmol of each primer, 0.2 mM deoxy nucleoside triphosphates, 2.5 µl of the Taq polymerase enzyme, 2.5 µl of 10 X enzyme buffer and 2.5 µl Mg Cl_2. The amplification reaction was carried out using 35 cycles of 94 °C (45 sec), 48 °C (45 sec) and 72 °C (120 sec) and then a 7- min termination at 72 °C.

Expression of cry toxin genes in transformed cells

A total cellular protein of transformed *E. coli* cells/sporulated bacterial cells from the transformed *Bt* and the parent bacterial isolates were prepared. The bacterial cells from the transformed *Bt* were grown on T3 medium for 72 h in incubator shaker at 30 °C. SDS-PAGE was carried out as described by Laemmli, (1970). The transformed

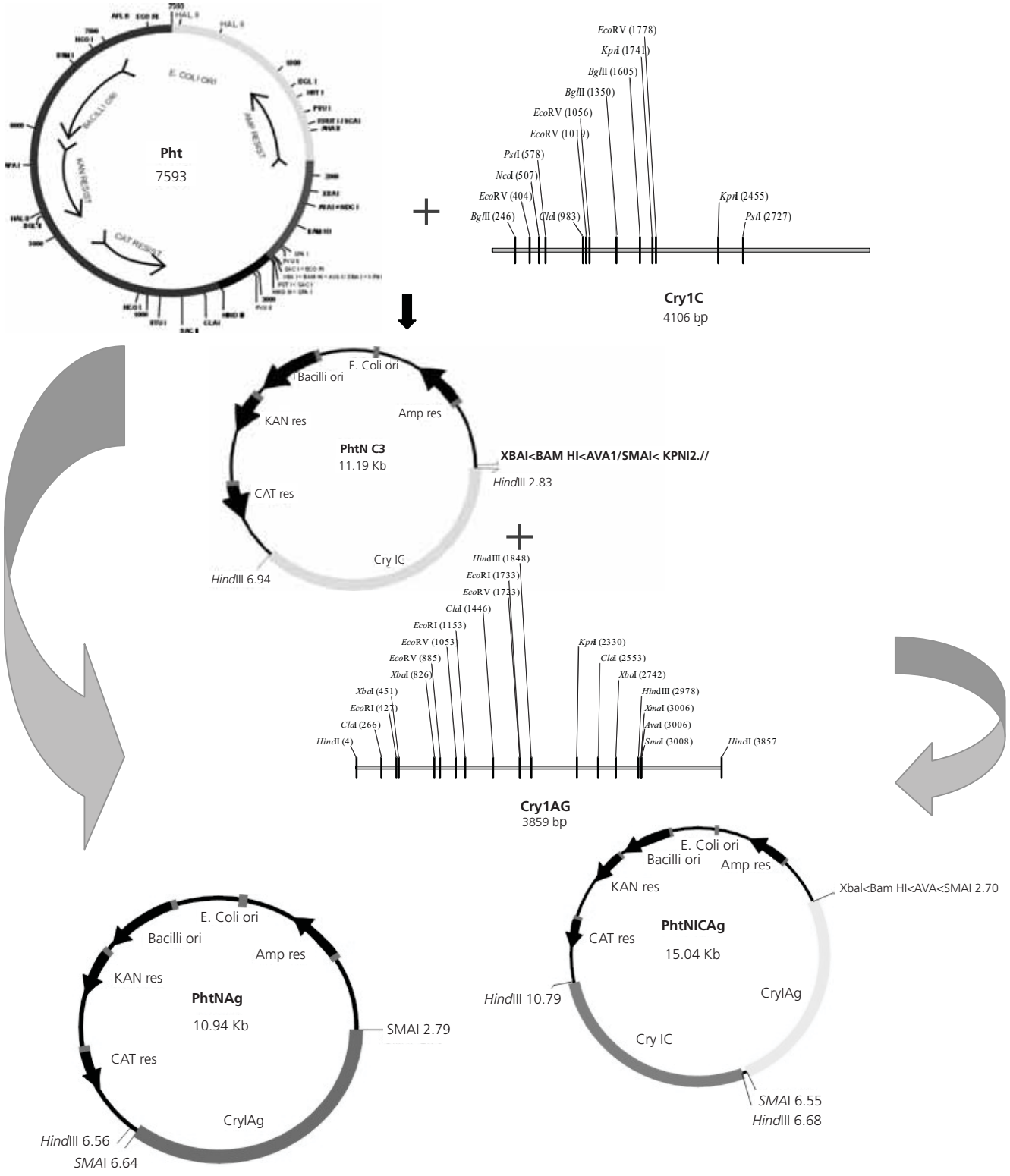


Figure 1:

Cry1C *Hind*III digested was cloned into the *Hind*III site of pHT7593 vector.
A HincII fragment carrying the full-length *cry*1Ag gene was ligated to *Sma*I digested pHT7593. Additionally, HincII-digested cry1Ag was ligated to *Sma*I-digested pHTNC3.

Table 3:
Primers used in the PCR reactions

Primer Pairs	Gene recog-nised	PCR expected product size in (bp)	Sequence of primers	Reference
Lep1A Lep1B	*Cry*1A (*Cry*1AC)	490	CCGGTGCTGGATTTGTGTTA AATCCCGTATTGTACCAGCG	Carozzi et al., 1991
Lep2A Lep2B		908	CCGAGAAAGTCAAACATGCG TACATGCCCTTTCACGTTCC	
IAF IAR	*Cry*1C	2247	ACGGAGGATCCATATGGAGG AAAATAATCAAAATC CTCTTGGATCCTAACGGGT ATAAGCTTTTAATTTC	Ragev et al., 1996
IAF ICR	*Cry*1C	3600	ACGGAGGATCCATATGGAGG AAAATAATCAAAATC TTATTCCTCCATAAGGAGTAAT TCC	This study
IACF IACR	*Cry*1Ac	2000	ATGGATAACAATCCGAACATC AAGTAAATTCCGCTCATCACT	This study

cells were collected by centrifugation and treated with sample buffer that composed of (50 mM Tris-HCl (pH 6.8), 2 % (W/V) SDS, 2 % (V/V) 2-mercaptoethanol, 10 % (v/v) glycerol and 0.0025 % (w/v) bromophenol blue) and boiled at 100 °C for 5 min. Samples were applied to a 10 % polyacrylamid gels and run at 200 V for 45 min at room temperature in mini protein Biorad cell. Protein bands on gels were visualized with coomassie brilliant blue R-250.

Purification of the expressed crystal δ-endotoxins

The various transformed *Bt* strains were grown in liquid T3 sporulation medium. Mixture of spore-crystal was harvested by centrifugation and the spore-crystal pellet was washed 6 times with deionized distilled ice-cold water containing 5 mM EDTA to get rid of sporangial debris. The cleaned pellet was resuspended in 50 mM Tris-HCl, 5 mM EDTA pH 7.5. Spores and crystals were separated using differential ultra centrifugation through a discontinuous sucrose density gradient of 55 %, 70 %, and 87 % W/V sucrose in 50 mM Tris-HCl/5 mM EDTA/10 mM KCl. The purified crystal protein was solubilized in pH 9.5 buffer of (50 mM Na_2CO_3/$NaHCO_3$) and 10 mM dithiothreitol at 37 °C for 4 h (Lu et al., 1994). The solubilized proteins were separated by centrifugation and dialyzed against ammonium bicarbonate buffer pH 8.3 and subjected to trypsinization (Lilly et al., 1980).

Western blot immunoassay

The presence of Cry1C, 1Ag and 1C-1Ag delta-endotoxins were detected in crude extracts of transformed cells by a western blot (immunoblot) analysis (Lampel et al., 1994). Total cellular proteins were prepared and solubilized by boiling in sample buffer and separated by electrophoresis on 10 % polyacrylamid gels. The gels were electrophoretically blotted onto pre wet PVDF membrane. The membranes were blocked in blocking buffer containing 1 % bovine serum albumin (BSA), then membranes were incubated in blocking buffer contained the toxins for 2 h. Anti-truncated 65 kDa from *Bt kur*- HD-1 serum (1-1000 dilution) was used as primary antibody and was incubated with membranes in the blocking buffer O/N at 4 °C. The membranes were incubated with alkaline phosphates conjugated secondary antibody (1-1000 dilution). CDP-chemiluminescent substrate was used and the emitted light was captured on X-ray film.

Insect Bioassay

Bacterial isolates were grown until sporulation in liquid T3 media for 72 h. Cultures were centrifuged and the pellets were washed once with Tris-HCl pH 8.00 containing 1 M NaCl, and lyophilized. The dried cells were used directly for bioassay. A stock concentration of 1000 ppm was made by dissolving 1 gm of lyophilized cells in 1000 ml H_2O (Dulmage, 1971). Different concentrations of 500, 250, 100, 50, 25, 15, 10, 5, 1, 0.5 and 0.2 ppm, were prepared and added to the surface of solidified artificial medium (dry powdered Lima beans 150 gm, dry yeast 15 gm, ascorbic acid 3 gm, Nipagin 3 gm, agar-agar 6 gm and 600 ml dd H_2O) (Loutfy, 1973) and kept for 2 h at room temperature. Ten neonate larvae of *Spodoptera littoralis* were added to each cup, the mortality was recorded every 24 h until 72 h.

3 Results

The constructed plasmids were transformed first into *E. coli* JM109 bacterial cells to test the expression of the plasmids. The transformed clones were screened and selected primarily according to their growth on ampicillin (100 mg/ml) plates after 18 h. The selected clones were tested for the presence of plasmids that contained the toxin genes and then by PCR.

PCR

The two sets of primers IAF & IAR and IAF & ICR specific for *cry*1C, were used with several recombinant clones and gave the ~ 2.2 kb and ~ 3.7 kb expected PCR products, respectively. From that clones; one clone (clone 3) was selected and its amplified PCR products were electrophoresed on agarose gel electrophoresis (Figure 2A). The results obtained from the gel revealed that clone 3 was contained *cry*1C gene. The two specific primers IACF and IACR were used to detect the *cry*1Ag. The data in (Figure 2B) showed the expected 2 kb PCR product amplified from clones 9 & 10 and from the positive control *cry*

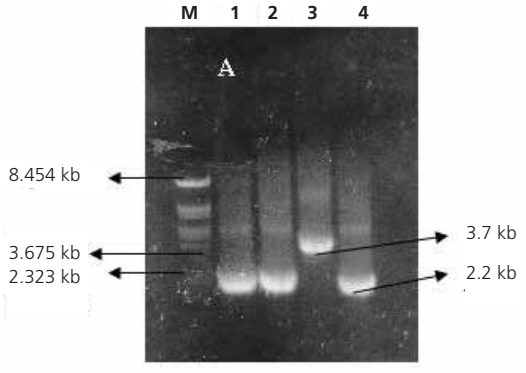


Figure 2A:

Gel electrophoresis for amplified PCR products from DNA of recombinant clone 3 compared with that from DNA of the original plasmid pTZ19-R/*cry*1C (*cry* 1C specific primers were used).
M: Lambda *Bst*E II DNA marker, Lanes 1 & 2: pTZ19-R/*cry* IC plasmid with IAF & IAR, Lane 3: clone 3 with IAF & ICR, Lane 4: clone 3 with IAF & IAR.

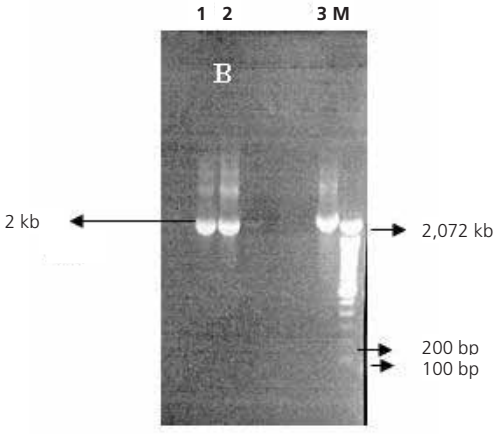


Figure 2B:

Agarose gel electrophoresis for PCR amplified products from clone 10 compared with pUC 18/*cry* IAg plasmid, the primer set used was, IACF and IACR.
Lanes 1 & 2: clones 9 & 10, Lane 3: pUC 18/*cry* IAg plasmid, M: 100 bp ladder DNA marker (2,072, 1,500, 600, 200, and 100 bp are the main bands).

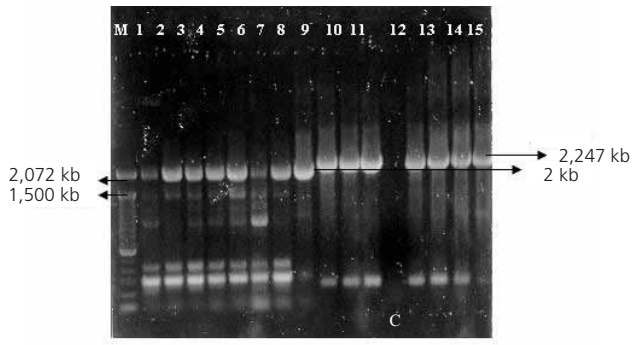
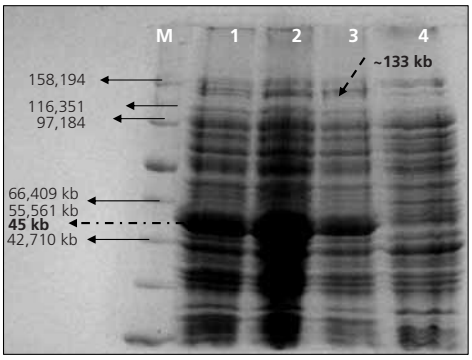


Figure 2C:

Gel electrophoresis for PCR amplified products from clone 12 compared with clone 3 and pUC 18/*cry*1Ag. The primer sets used were: IAF & IAR that give 2,247 kb PCR product with *cry*IC and IACF & IACR that give 2 kb PCR products with *cry*1Ag.
M: 100 bp DNA ladder marker, Lanes 1 to 6: are clones 1, 2, 3, 30, 31 and 40 with primer set IACF & IACR, Lane 7: clone 12 with primer set IACF & IACR, Lane 8: *pUC* 18/*cry*1Ag plasmid with primer set IACF & IACR, Lanes 9 to 13: are clones 2, 3, 30, 31 and 40 with primer set IAF & IAR, Lane 14: clone 12 with primer set IAF & IAR, Lane 15: clone 3 with primer set IAF & IAR.



This pointer refer to the expressed toxin protein ~ 133 kd
This arrow refer to the expressed protein from the pHT vector

Figure 3:

Expression of cry1C, cry1Ag, and cry1C/cry1Ag genes in E. coli strain JM109. A coomassie-stained SDS-polyacrylamide gel showing the expressed ~ 133 kd toxin protein.
M: Protein marker, broad range (212, 158.19, 116.35, 97.18, 66.40, 55.56, 42.71, and 36.48 kd are the main bands), Lane 1: clone 3 expressing Cry1C toxin protein, Lane 2: clone 10 expressing Cry1Ag toxin protein, Lane 3: clone 12 expressing Cry1C/Cry1Ag toxin protein, Lane 4: non transformed JM109 *E. coli* strain that lacks the ~ 133 kd and the ~ 45 kd.

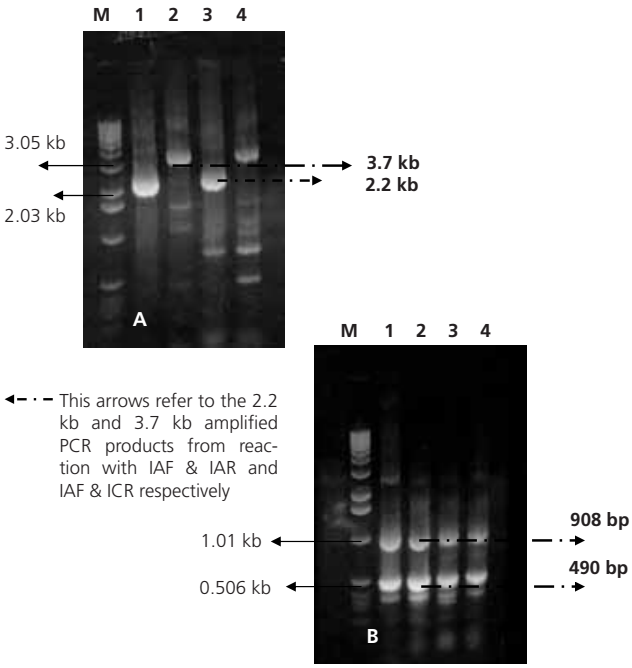


Figure 4:

A:
Agarose gel electrophoresis for amplified PCR products from DNA of transformed *Bt* strains (Bt4 and HD73) with the recombinant plasmid pHTNC3.
M:1 kb ladder DNA marker (12.2, 11.19, 10.18, 9.16, 8.14, 7.12, 6.1, 5.09, 4.07, 3.05, 2.03, 1.63, 1.01, 0.506, and 0.396 kb are the M.wt(s) of its bands), lane 1: transformed Bt4 with *cry*1C with IAF & IAR, lane 2: transformed Bt4 with *cry*1C with IAF & ICR, lane3: transformed HD73 with *cry*1C with IAF & IAR, lane 4: transformed HD73 with *cry*1C with IAF & ICR.

B:
Agarose gel electrophoresis of PCR products amplified from transformed *Bt* strains that harboring pHTNC3, pHTNAg, pHTN1CAg, and tranformed HD73 with pHTNC3 with primers specific for *cry*1-type toxin genes (Lep1A, Lep1B, Lep2A, and Lep2B).
M: 1 kb ladder DNA marker, lane 1: transformed *Bt* harboring pHTNC3, lane 2: transformed *Bt* harboring pHTNAg, lane 3: transformed *Bt* harboring pHTN1CAg, lane 4: transformed HD73 harboring pHTNC3.

1Ag plasmid revealing that clones 9 & 10 contained the recombinant plasmid that harbors *cry*1Ag. The two specific set of primers IACF & IACR and IAF & IAR were also used to detect both the *cry*1Ag and *cry*1C in the recombinant clones that were transformed with the 3rd construct. The data in (Figure 2C) showed the expected PCR products, 2 kb and 2.2 kb, indicating the presence of *cry*1Ag and *cry*1C genes in its transformed cells. From 7 clones, clone 12 was selected to complete the work on it.

Protein expression

Cellular proteins from the recombinant clone 3, clone 10, and clone 12 were analyzed on SDS-polyacrylamid gel electrophoresis (Figure 3). The data on the gel revealed the expression of the ~ 133 kd of toxin protein of Cry1C, Cry1Ag, and Cry1C/Cry1Ag respectively. The results were compared to the protein pattern from *E. coli* free (non-transformed) which didn't show the 133 kd protein. The three clones showed a protein band at ~ 45 kd that did not appear with the protein from the non-transformed *E. coli*. This data confirmed the expression of the recombinant plasmids in the transformed *E. coli* cells.

Electrontransformation

The three prepared plasmids, pHTNC3 (harboring *cry*1C gene), pHTNAg (harboring *cry*1Ag) and pHTN1CAg (harboring both *cry*1C and *cry*1Ag) were electroporated separately into the Cry negative (Cry-) *Bt* local strain Bt4. The transformed cells were selected primarily according to their growth on 100 µg/ml kanamycin plates and they were subjected to screening via PCR using the same specific primers that were used with transformed *E. coli* JM109 cells. Additionally, the set of primer pairs Lep1A & 1B and Lep2A & 2B that are specific for *cry*1A were used for detection of *cry*1Ag The results of PCR revealed the presence of the toxin genes in the different transformed *Bt* cells (Figure 4A and 4B).

Protein expression in Bt bacterial cells

Total cellular proteins of the sporulated cells of transformed *Bt* isolates were fractionated on denaturing gels by SDS-PAGE. The protein banding pattern of the transformed *Bt* were compared to the protein banding pattern of the parent *Bt* strain Bt4 (Figure 5). Examination of the protein pattern showed the highly expressed protein at ~ 133 kd and this 133 kd protein did not appear in the parent strain Bt4, revealing the high expression of the *cry*1C, *cry*1Ag, and *cry*1C/*cry*1Ag genes in the recombinant plasmids pHTNC3, pHTNAg, and pHTN1CAg respectively that were transformed into the acrystalliferous (Cry-) *Bt* strain Bt4. The data also showed that the Bt4 parent strain was not able to show any insecticidal crystal protein (ICP), revealing that it did not contain any *cry* genes. A protein band at ~ 45 kd appeared with all the transformed *Bt* strains but did not appear neither with the protein of the parent strain Bt4 nor with the positive control *kur* HD73 *Bt* strain that naturally contains *cry*1Ac, revealing that this protein may be resulted from the expression of the pHT 7593 vector.

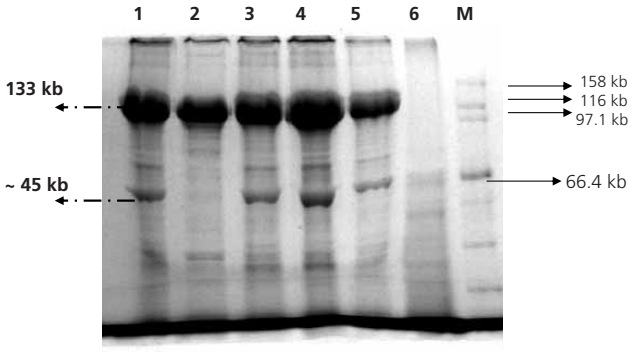


Figure 5:

SDS-PAGE (polyacrylamid gel electrophoresis) of the transformed *Bt* strains expressing the toxin protein Cry1C, Cry1Ag, and Cry1C/Cry1Ag.
Lane 1: transformed HD73, lane 2: *kur*-HD73 *Bt* strain, lane 3: transformed Bt4 with pHTN1CAg plasmid, lane 4: transformed Bt4 with pHTNIAg plasmid, lane 5: transformed Bt4 with pHTNC3 plasmid, lane 6: Bt4 strain, M: protein marker, broad range (2 - 212 Kd).

Crystal purification, solubilization and trypsinization

The crystals produced by the transformed Bt4 *Bt* cells were purified on sucrose gradients, solubilized in a buffer of pH 9.5 and trypsinized with pure trypsin. The molecular masses of the crystal proteins and the activated toxins were analyzed by SDS-PAGE. Figure 6 showes the purified and solubilized protoxins (at ~133 kd) banding patteren

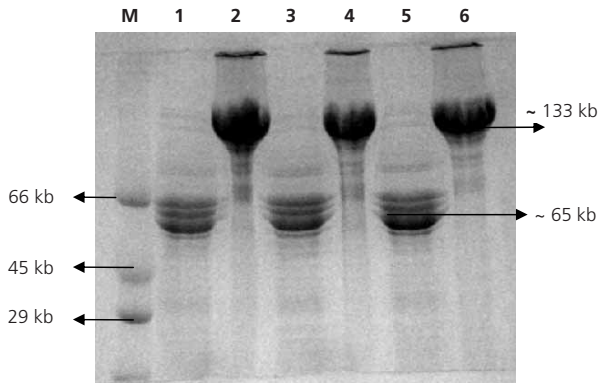


Figure 6:

Protein analysis of crystals synthesized by the transformed *Bt* strains harboring *cry*1C, *cry*1Ag and *cry*1C/*cry*1Ag.
M: Low range molecular protein marker, lane 1: activated toxin of Cry1C/Cry1Ag, lane 2: protoxin of Cry1C/Cry1Ag, lane 3: activated toxin of Cry1Ag, lane 4: protoxin of Cry1Ag, lane 5:activated toxin of Cry1C, lane 6: protoxin of Cry1C.

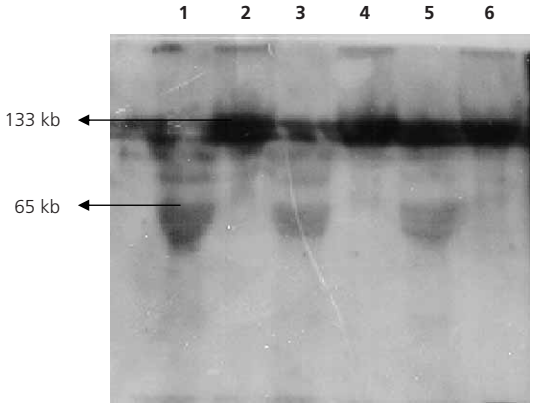


Figure 7:

Immunodetection of crystal proteins in transformed *Bt*
Lanes 1 & 2: active toxin and protoxin from *Bt* transformed with *cry*1C/*cry*1Ag
Lanes 3 & 4: active toxin and protoxin from *Bt* transformed with *cry*1Ag
Lanes 5 & 6: active toxin and protoxin from *Bt* transformed with *cry*1C.

simultaneously with its activated toxin (trypsinized protoxin) at ~ 65 kd from the transformed Bt4 with pHTNC3, pHTNAg, and pHTN1CAg respectively. The data obtained on the gel (Figure 6) revealed the high expression of the Cry toxins that were transferred into the *Bt* strains cloned in the pHT vector indicating; the high stability of that vector in *Bt* strains.

Western immunoblotting

Antibody-antigen immunoreaction was detected between anti-60 kd toxin antiserum and antigen expressed by the *Bt* transformed with pHTNC3, pHTNAg, and pHTN1CAg (Figure 7). The data revealed that the recombinant proteins from all the transformed *Bt* strains gave sharp bands where the homologous antiserum reacted strongly to its respective homologous toxin protein, and the resulted bands of the protoxin were at the expected molecular masses of protoxin protein ~133 Kd and that of the activated protein were at the expected kd of the activated toxin ~ 65 kd.

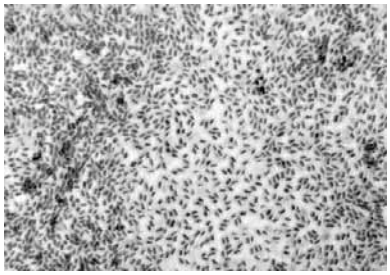


Figure 8:

The microscopic examination of sporulating cells of *Bt* Bt4 strain.
Bt4 has been grown on T3 sporulation media for more than five days at 28 - 30 °C, cells tended to give spores but not crystals.

Microscopic examination of spore-crystal stained smears from parent strain, Bt4 compared to the transformed strains were illustrated. Figure 8 showed the spores from sporulating cells of *Bt* Bt4, which gave no crystals when grown on T3 sporulation media at 28 - 30 °C for 3 - 7 days Figure 9A, 9B and 9C showed the bipyramidal crystals in the transformed Bt4 with pHTNC3, pHTNAg, and pHTN1CAg respectively. The three transformed *Bt* strains were grown on T3 sporulating media at 28 - 30 °C for 3 days. The data revealed and confirmed all the previous experiments (in this study) that the different *cry* genes (*cry*1C, *cry*1Ag, and *cry*1C/*cry*1Ag) cloned into the pHT7593 vector were expressed in the native host bacteria *Bt*.

Bioassay

The spore-crystal complexes from transformed *Bt* strains were examined for their toxicity against lepidopteran insects. In this assays, Bt4 transfomed with pHTNC3, pHTNAg, and pHTN1CAg were used against the larvae of *S. littoralis*. The toxicity of the transformed Bt4 isolates was compared to that of the parent strain Bt4 (the spores of Bt4 were used in this assays) (Figure 10). Table 4 shows the LC_{50} values from the different toxins used in these assays against the cotton leaf worm *S. littoralis*. The bioassay of dried-pellet culture from up to 2000 ppm of Bt4, did not

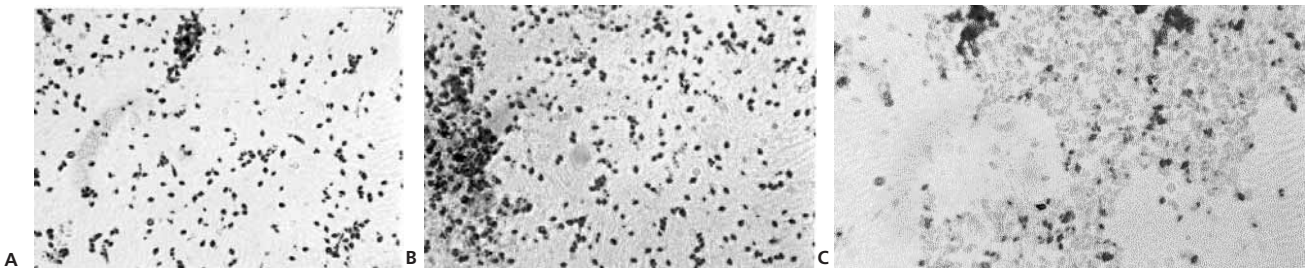


Figure 9:

A: The microscopic examination of sporulating cells from the constructed *Bt* that harboring *cry*1C gene. He figure shows the bipyramidal crystals as a result of expression of Cry1C toxin protein.
B: The microscopic examination of sporulating cells from the constructed *Bt* that harboring *cry*1Ag gene. The figure shows the bipyramidal crystals as a result of expression of Cry1Ag toxin protein.
C: The microscopic examination of sporulating cells from the constructed *Bt* that harboring *cry*1C/*cry*1Ag genes. The figure shows the bipyramidal crystals as a result of expression of Cry1Ag/Cry1C toxin proteins.

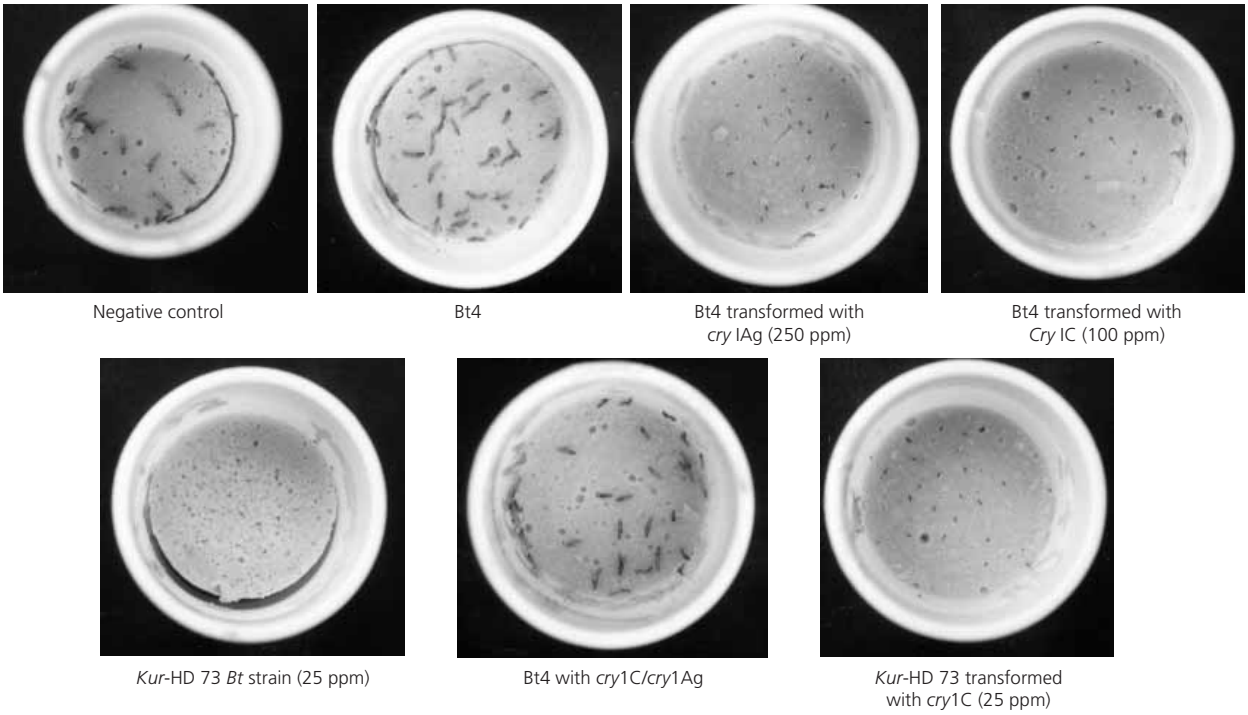


Figure 10:

The effect of toxicity of *Bt* Bt4 Cry$^-$ and Bt4 transformed with *cry*1C, *cry*1Ag and *cry*1C/1Ag on larvae of *S. littoralis* compared with the effect of *Bt* strain *kur*-HD73 and its transformed one (*kur*-HD73 harboring *cry*1C).

Table 4:

The LC_{50} values in ppm of toxins and combination of toxins used against the cotton leaf worm *S. littoralis*

Strain/toxin	LC_{50} (ppm)	*95 % confidential limits*	Slope/SE
Kur-HD73 Cry1Ac	197.42	(150.42 - 321)	1.79 ± 0.36
Bt/NC3 Cry1C	63.23	(221.15 - 22.07)	1.67 ± 0.391
Bt/NAg Cry1Ag	103.69	(122.71 - 87.71)	2.94 ± 0.40
Bt/N1C1Ag Cry1C & Cry1Ag	2.216	not determined	0.87 ± 0.38
Entomocidus Cry1C	41.48	not determined	3.9 ± 1.016
Transformed-HD73 Cry1C & Cry1Ac	6.65	not determined	1.89 ± 1.059
Mixture of HD73 & *entomocidus*	31.32	(33.49 - 29.23)	9.13 ± 0.926
Mixture of *Bt*N1Ag & *Bt*N1C	6.6	not determined	1.89 ± 1.059

[a] Bioassays were performed on spore-crystal preparations from T_3 liquid cultures
[b] LC_{50} is a concentration of toxin required to kill 50 % of 1st instar larvae
[c] LC_{50}s were calculated by probit analysis
[d] Probit model is $Y = a + b * x$

where Y = probit value
a = intercept, probit value for x = 0
b = slope, regression coefficient of y on x
x = log (dose)
[e]SE is the standard error

show any toxicity against *S. littoralis* (Figure 10) revealing that the parent strain Bt4 does not express or may be not contain any *cry* genes. On the other hand the bioassay of transformed Bt4 with *cry*1C (Figure 10) showed high specificity against *S. littoralis* comparable to that from *entomocidus Bt* strain that were used as positive control which naturally contains only the delta-endotoxin Cry1C. Bioassay from transformed Bt4 with *cry*1Ag showed also positive results of toxicity against *S. littoralis* (Figure 10).

The toxicity of transformed Bt4 with *cry*1C/*cry*1Ag was 18 time more than that of *entomocidus* against *S. littoralis* and 28 time more than that of transformed *Bt* with only *cry*1C. The mixture of equal concentrations of spore-crystal complex from the transformed *Bt* with *cry*1C

and transformed *Bt* with *cry*1Ag showed similar toxicity against *S. littoralis* comparable to that from transformed *Bt* with *cry*1C/*cry*1Ag. On the other hand the transformed HD73 showed LC_{50} against *S. littoralis* similar to that from transformed *Bt* with *cry*1C/*cry*1Ag which was 6.6 ppm and 2.2 ppm respectively (Table 4).

4 Discussion

This study aimed to construct a potent *Bt* strain against *S. littoralis*. Toward this end, both *cry*1C and the local (Egyptain) *cry*1Ag genes were chosen to construct a genetically modified *Bt* strain. The Egyptian Cry$^-$ *Bt* strain Bt4 was used for that aim. The *Bt* strain *entomocidus* was also used as a positive control, which it is one of the most important strains that used against Noctuidae. It produces potential δ-endotoxin and shows high potential activity against *S. littoralis* (Salama and Foda, 1982), it produces Cry1C only.

Several strategies have been used to introduce and stably maintain cloned *cry* genes in *Bt*. In this work the gene transfer system was the *E. coli-Bt* shuttle vector pHT7593. This vector made from *E. coli* ori joined with bacilli ori, which showed high stability in *Bt* strains.

A 4.1 kb DNA fragment of *cry*1C was liberated from pTZ19R/*cry*1C by digestion with *Hind*III and cloned into the *Hind*III site of pHT7593 vector, then used to transform *E. coli* JM109 strain. Analysis of recombinant clones predictive of cloned *cry*1C was carried out using PCR. The specific primers IAF & IAR (Regev et al., 1996) and IAF & ICR were used and they gave the expected PCR products 2.2 kb and 3.7 kb respectively to confirm the presence of *cry*1C in the selected clone 3 (Figure 2A). When clone 3 was digested with the restriction enzyme *Hind*III it gave the ~ 4.1 kb fragment of *cry*1C and the ~ 7.1 kb fragment of the pHT vector and when digested with *Sma*I it gave the ~ 11 kb of the linear form of clone 3 (data not shown). A *Hinc*II fragment carrying the full-length *cry*1Ag gene was ligated to *Sma*I digested pHT7593. Additionally, *Hinc*II-digested *cry*1Ag was ligated to *Sma*I-digested pHTNC3 (Figure 1). Screening of the recombinant *E. coli* cells harboring *cry*1Ag by polymerase chain reaction was performed. The primer set specific for *cry*1Ac (1ACF & 1ACR) was used and gave the expected 2 kb PCR product (Figure 2B). Accordingly, clone 10 was selected as recombinant *E. coli* harboring *cry*1Ag. In addition, screening of recombinant *E. coli* cells harboring both the *cry*1C and *cry*1Ag by polymerase chain reaction was done. The primer sets 1ACF & 1ACR and 1AF & 1AR specific for *cry*1Ag and *cry*1C respectively were used. Both of them detected the presence of *cry*1Ag and *cry*1C, which gave the 2 kb and 2.2 kb PCR products respectively (Figure 2C). Clone 12 was selected as a recombinant *E. coli* harboring *cry*1Ag and *cry*1C.

Expression of *cry*1C in *E. coli* (clone 3) was examined and analysed on SDS-PAGE and the total protein profile showed the ~ 132 kd corresponding to Cry1C toxin protein (Figure 3), the same results were revealed by (Kalman et al., 1993) who worked with the same *cry*1C in a similar experiment. The protein profile also showed a protein band in the region between 55 and 42 kd, this band might belong to the expressed protein from the plasmid vector, which was not found in the non-recombinant *E. coli* cells. Protein expression profile for clone 10 and clone 12 was also analyzed on SDS-PAGE. Cry1Ag-harboring and *cry*1C/*cry*1Ag-harboring *E. coli* cells showed the ~ 133 kd and ~ 135 kd respectively (Figure 3). Thus the construction and gene expression of clones 3, 10, and 12 were performed in *E. coli* strain JM109 host cells. The next step was to transform these constructs to the native *Bt* host cells. Toward this end, pHTNC3, pHTNAg, and pHTN1CAg were separately electroporated into the Cry$^-$ Bt4 recipient cells. Plasmid profiling was conducted to select for the recombinant Bt4 cells (data not shown).

Polymerase chain reaction was used to detect the presence of the *cry*1C and *cry*1Ag genes in the recombinant *Bt* strains. The specific primer sets IAF & IAR was used to obtain a 2.2 kb *cry*1C coding region starting from the translation start site and containing the sequence of the 1st 756 amino acids as described previously. Moreover the reverse primer ICR (designed in this study) was used with the forward primer IAF to get the full length of *cry*1C gene (~ 3.7 kb). When used with the recombinant strains that harboring pHTNC3, they gave the expected 3.7 kb PCR product (Figure 4A), indicating that *cry*1C was fully cloned into the pHT vector. The Lep1A & 1B and Lep2A & 2B primer sets specific for detection of *cry*1A toxin genes, were used to detect the *cry*1Ag (1Ac like) in the recombinant *Bt* strains and they gave the expected PCR products 490 bp & 908 bp (Figure 4B).

The expression of the cloned genes were examined and analyzed on SDS-PAGE (Figure 5). Our results revealed that the cloned crystal protein genes *cry*1C, *cry*1Ag, and *cry*1C-Ag were highly expressed in the Bt4 Cry$^-$ strain. This finding was similar to the results obtained by Lecadet et al., 1992 who introduced the *cry*IIIA gene cloned in pHT7911 into the 407 Cry$^-$ isolate of *Bt* by electroporation. Very large rhomboid crystals were seen as a result of the high level expression of the *cry*IIIA gene

In this study, crystal proteins purified from the genetically modified *Bt* strains were solubilized in pH 9.5 and analyzed on SDS-PAGE. The results showed a major band that was migrated at ~ 133 kd. The trypsinization of ~ 133 kd with TPCK trypsin gave the ~ 65 kd of activated toxin (Figure 6).

Western immunoblotting assay using polyclonal antibody raised in rabbit against the activated toxins of *Bt kur-*

HDI was performed. However, molecular mass of Cry1C, 132.8 kd, is slightly different from *cry*1Ag, 133 kd. Our results showed that the antiserum reacted with the proteins from all the recombinant *Bt* strains (Figure 7). The immunoblotting showed a thick band appeared as more than one protein in both the protoxin and activated toxin from the recombinant *Bt* strain that harboring pHTN1CAg. The thickness of the protein band might indicate the presence of more than one protein as a result of transformation. Our results agreed with the results obtained by (Lereclus et al., 1992), which introduced *cry*IIIA cloned in pHT5132 into the *Bt* strain *kur*-HD73 by electroporation. The *cry*1Ac formed bipyramidal crystals, whereas *cry*IIIA formed flat rectangular crystals. This recombinant HD73-II produced a great total quantity of protein than the parental strain. Furthermore, it showed insecticidal activities against both the two orders of insect, coleoptera (resistance to CryI-IIA) and lepidoptera (resistance to Cry1Ac). Accordingly, microscopic examinations of the Bt4 (Figure 8) and the recombinant *Bt* strains harboring the pHTNC3 (Figure 9A), pHTNAg (Figure 9B), and pHTN1CAg (Figure 9C) were performed. All *Bt* strains were grown on the same sporulating media, incubated for the same period of time and at the same temperature. They showed spores from the parent strain *Bt* Bt4 and the expressed Cry1C, 1Ag, and 1C & 1Ag as insecticidal crystal proteins from the transformed ones. The bipyramidal shaped of crystals were detected clearly in all the recombinant *Bt* strains. These microscopic examinations confirmed the molecular findings represented by presence of transferred plasmids, PCR-amplification products, expressions of cloned *cry* genes, and western blotting. The ability of the recombinant strains to sporulate under normal growth conditions and in the presence of selectable antibiotic (100 µg/ml kan) was investigated. When cultures of Bt4 Cry$^-$ and the transformed *Bt* strains were grown until they reached the sporulating phase, the Bt4 remained for up to 10 days as spores and never gave any crystals. On the other hand the transformed strain cultures showed after 3 days, spores and crystals. It was also observed that part of the spore population degenerates whereas crystals were in extreme high numbers. Similar observations have been reported by (Donvan et al., 1988) and (Lecadet et al., 1992), who electroporated the cloned *cry*IIIA in pHT7911 into the lepidoterans active *Bt* strain *aizawai*. Accordingly, transformants displayed dual specificities with levels of activity identical to those of the parental strains. Our results also agreed with those obtained by (Sanchis et al., 1997), who worked on the same gene combinations (introduced recombinant *cry*1C into *kur*-HD73) and proposed that the expression of these two genes is summed in the recombinant strain, presumably because they do not compete for rate-limiting elements of the gene expression since the expression systems of the two genes are different. Our results also show the same observation with the *Bt* strain that harbors both the *cry*1C and 1Ag although they are found on the same vector, but each under its own promoter and regulatory region.

In the present work, bioassay clearly confirmed that the expression of *cry*1C in the Cry$^-$ Bt4 recipient strain make it active against *S. littoralis* (Table 4). The toxicities of the transformant and of the parent strains against *S. littoralis* were determined. The *Bt cry*1C-Ag harboring showed high activity against *S. littoralis*, whereas the parental recipient strain showed no toxicity to it. Chang et al., 1992, who transformed the *cry*1VD cloned in pHT3101 into a Cry$^-$ *Bt* strain, obtained similar results. The obtained protein was comparable in size, shape, and toxicity to that produced by parental *Bt* subsp *morrisoni*. Baum et al., 1990 who cloned *cry*1Ac and introduced it into the *Bt* strain subsp *aizawai*. Accordingly, a recombinant strain exhibiting good isecticidal activity against *S. exigua* and having an improved spectrum of insecticidal activity was obtained. In comparison with all the deduced studies, our study was unique which it targeted the potency and the high toxicities of the constructed *Bt* strains against the Egyptian cotton leaf worm *S. littoralis*. We were able to construct a *Bt* recombinant strain containing a unique combination of *cry*1 genes, *cry*1Cb and *cry*1Ag cloned in the pHT shuttle vector.

In this study *Cry*1C-harboring HD73 was a good model, for comparison with the pHTN1CAg-harboring *Bt* strain. *Cry*1C/*cry*1Ag expressing Bt4 *Bt* strain had LC_{50} approximately twenty fold lower than that of *cry*1C expressing Bt4 strain and fifteen fold lower than that showed by *entomocidus*. It was also 47 fold lower than that obtained from *cry*1Ag-expressing *Bt* strain. In conclusion, we suggest that the increase in toxicity against *S. littoralis* when *cry*1Ag was introduced with *cry*1C, a gene which is highly active and potent against *S. littoralis*, is additive. Lereclus et al., 1992 who used different gene combinations against species of coleoptera and species of lepidoptera, described that broaden in the activities was additive, agreed with our suggestion.

When crystal-spore complexes from *entomocidus Bt* strain (Cry1C) were mixed with equal concentrations, of crystal-spore complexes from *kur*-HD73 (Cry1Ac), it showed toxicity against *S. littoralis* 2 fold more than that caused by crystal-spore complexes from *entomocidus Bt* strain alone (Table 4). This result also reinforces our opinion that the increase in toxicity in this study was additive. In a similar attempt, to increase the insecticidal effect of *cry*1C on the larvae of *S. littoralis*, Regev et al., 1996, used a combination of *cry*1C and endochitinase. They used it in only a transgenic system in which the Cry protein is not expressed in crystalline form.

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