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ORIGINAL ARTICLE

# Recombinant Cry1Ia protein is highly toxic to cotton boll weevil (*Anthonomus grandis Boheman*) and fall armyworm (*Spodoptera frugiperda)*

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## Abstract

**Aims:** To evaluate the activity of *cry1Ia* gene against cotton pests, *Spodoptera frugiperda* and *Anthonomus grandis*.
**Methods and Results:** Had isolated and characterized a toxin gene from the *Bacillus thuringiensis* S1451 strain which have been previously shown to be toxic to *S. frugiperda* and *A. grandis*. The toxin gene (*cry1Ia*) was amplified by PCR, sequenced, and cloned into the genome of a baculovirus. The Cry1Ia protein was expressed in baculovirus infected insect cells, producing protein inclusions in infected cells. The Cry1Ia protein has used in bioassays against to *S. frugiperda* and *A. grandis.*
**Conclusions:** Bioassays using the purified recombinant protein showed high toxicity to *S. frugiperda* and *A. grandis* larvae. Molecular modelling of the Cry1Ia protein translated from the DNA sequence obtained in this work, showed that this protein possibly posses a similar structure to the Cry3A protein. Ultrastructural analysis of midgut cells from *A. grandis* incubated with the Cry1Ia toxin, showed loss of microvilli integrity.
**Significance and Impact of the Study:** The results indicate that the *cry1Ia* is a good candidate for the construction of transgenic plants resistant to these important cotton pests.

## Introduction

*Bacillus thuringiensis* (Bt) is a Gram-positive bacterium, characterized by the production of protein crystals, some with known insecticidal activity, during sporulation. These crystals are composed of proteins with high toxicity to larvae of different insect orders and to some species of the orders Nematoda, Protozoa and Acari (Feilteison 1994). These proteins are known as $\delta$-endotoxins, because of their intracellular location, and as proteins Cry because of their crystal nature (Kostichka *et al.* 1996; Monnerat and Bravo 2000). Nowadays, more than 300 *cry* genes have been sequenced and their encoded proteins classified into 51 groups organized in different subgroups, according to their levels of amino acid identity. The update information of these data can be seen at the website: http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/.

Tailor *et al.* (1992) described the first *cry1Ia* gene, coding for a protein with 81 kDa and 62% identity to the *cry1Ba* gene. Upon activation by proteolysis these proteins are cleaved into active toxins of about 65 kDa, with insecticidal activity to *Ostrinia nubilalis* (Lepidoptera: Pyralidae) and to *Leptinotarsa decimlineata* (Coleoptera: Chrysomelidae) (Tailor *et al.* 1992; Tounsi *et al.* 2003).

Cotton is one of the most important crops cultivated worldwide. The cotton productive chain is one of the most important in Brazil and in the world, as it generates thousands of direct and indirect jobs and annually only the Brazilian cotton industry generates around US$1·5 billion (Martins *et al.* 2007). Several insect pests are present in cotton and among them, the cotton boll

 

weevil, *Anthonomus grandis* (Coleoptera: Curculionidae), is an important one, because of its destructive power and difficult control (Martins *et al.* 2007). If this insect is not controlled, the damages to cotton fibre production can reach 75% (Martins *et al.* 2007). *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is also a difficult pest to control and a polyphagous insect which attacks several important crops in many countries. In Brazil, this insect is found in different cultures such as corn, sorghum, rice, wheat, alfalfa, beans, peanut, tomatoes, cotton, potato, cabbage, spinach, pumpkin and kale (Monnerat *et al.* 2007).

*Bacillus thuringiensis* is a natural candidate as a source of insect resistance genes to control insect pests and decrease the excessive use of pesticides, great responsible for polluting and contaminating the environment. This work describes the cloning, sequencing, and for the first time, insecticidal activity of a Cry1Ia protein, from a Brazilian strain of *B. thuringiensis* towards *A. grandis* and *S. frugiperda*. Furthermore, the three-dimensional modelling of the Cry1Ia protein showed that the structure of the protein is probably similar to the Cry3A protein.

## Material and methods

### Virus and cells

*Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) and the recombinant viruses vSynVI-gal (Wang *et al.* 1991; O'Reilly *et al.* 1992; Aguiar *et al.* 2006) and vSynBtCry1Ia (constructed in this work) were propagated in *Trichoplusia ni* (BTI-Tn5B1-4) cells, in culture (Granados *et al.* 1994). The cells were grown in TC-100 (Gibco-BRL, Grand Island, NY), supplemented with 10% of foetal bovine serum at 27°C. Cells of *Escherichia coli* DH5-α (Invitrogen, Carlsbad, CA) were used as hosts to plasmids in this work.

### Cloning and sequencing of the *cry1Ia* gene

Molecular cloning techniques employed in this work were performed as described in Sambrook *et al.* (2001). All oligonucleotides used in this work were purchased from Invitrogen. The *cry1Ia* gene of *B. thuringiensis* S1451 strain from the entomopathogenic *Bacillus* strain collection at Embrapa Genetic Resources and Biotechnology (Brazil), was amplified by PCR using the oligonucleotides Cry1IaF (5′-GTATAAGTGGAG*GGATCC*ATATG-3′) and Cry1IaR (5′-*GGATCC*CTACATGTTACGCTT-3′), which were designed from the published *cry1Ia* gene sequence (Genbank accession number: X62821) (Tailor *et al.* 1992). *Bam*HI restriction sites were added to sequence of the oligos (letters in italics). The PCR program employed for the amplification was 94°C/5 min, three cycles of 95°C/1 min, 49°C/1 min and 30 s, 72°C/1min and 30 s, and 27 cycles of 95°C/1 min, 52°C/1 min and 30 s, 72°C/1 min and 30 s, as well as an final extension of 72°C/7 min. The amplified fragment was cloned into the vector pGEM-T easy (Promega, Madison, WI) according to the manufacturer's instructions, and three positive clones were sequenced (Mega BACE 1000, Amersham Biosystems, Freiburg, Germany), following the manufacturer's instructions. The sequences from the three clones were analysed using the open reading frame finder (ORF finder) and BLAST programs (Altschul *et al.* 1990) at the NCBI home page (http://www.ncbi.nlm.nih.gov).

### Recombinant baculovirus construction

The plasmid containing the *cry1Ia* gene (pGEMBtCry1Ia) was partially digested with *Eco*RI, and a 2·1 kb fragment was separated by electrophoresis in an 0·8% agarose gel, eluted from the gel (Sephaglass™ Bandprep Kit, Amersham Pharmacia, Frieburg, Germany), according to the manufacturer's instructions. The purified fragment of 2·1 kb was cloned into the vector pSynXIVX3+ (Wang *et al.* 1991), previously digested with *Eco*RI, producing the recombinant plasmid pSynBtCry1Ia, (Fig. 1). One micro-

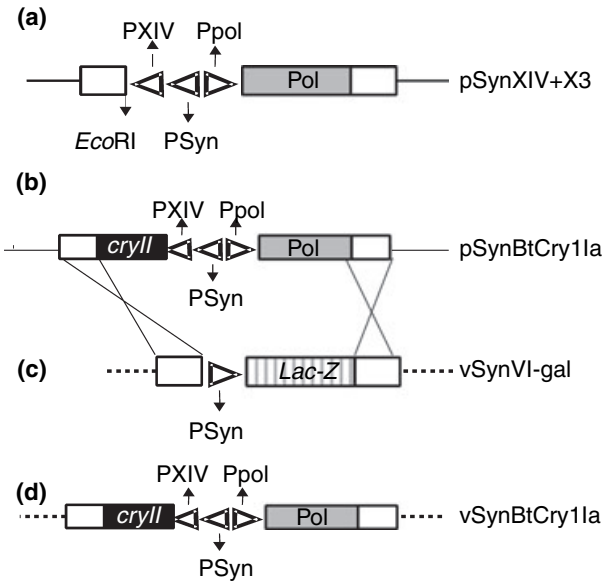


**Figure 1** Scheme showing the polyhedrin gene locus in the plasmids PSynXIVX3+, pSynBtCry1Ia, and viruses SynVI-gal and vSynBtCry1Ia. The plasmid pSynXIV+X3 was used to clone the *cry1Ia* gene into the *Eco* RI site for the construction of the transfer vector pSynBtCry1Ia, which was co-transfected in insect cells with the DNA of the vSynVI-gal virus to produce the recombinant virus vSynBtCry1Ia. The Pol (polyhedrin) *lac-Z*, (*β*-galactosidase) and *cry1Ia* genes are indicated in the figure. Besides, the promoters PXIV, Ppol and Psyn, which control the expression of these genes, are also indicated in the figure.

gram of DNA from the pSynBtcry1Ia plasmid and 0·5 μg of DNA of vSynVI-gal, previously digested with *Bsu*36I, were co-transfected into BTI-Tn5B1-4 cells ($1 \times 10^6$) by using liposomes and following the manufacturer's instructions (Cellfectin®, Invitrogen). The co-transfected cells were incubated for a week at 27°C and the supernatant used to purify the recombinant virus by serial dilution in 96 well plates (O'Reilly *et al.*1992; Aguiar *et al.* 2006). The presence of the *cry1Ia* gene into the genome of the recombinant virus (vSynBtCry1Ia) was checked by PCR using the oligonucleotides described above.

### Production of polyclonal antiserum against the recombinant Cry1Ia protein

Thirty third-instar *S. frugiperda* larvae were infected with the recombinant vSynBtCry1Ia by injection of virus ($5 \times 10^5$ PFU) into the hemocel using a microsyringe (Hamilton). At 120 h.p.i., dead larvae were collected and kept at −80°C. Viral occlusion bodies (protein occlusions containing virus characteristic of baculovirus) and protein crystals were purified following the protocol described elsewhere (O'Reilly *et al.* 1992; Aguiar *et al.* 2006). The purified occlusions and crystals were fractionated in a 12% SDS-PAGE (Laemmli 1970) using a Mini-Protean apparatus and following the manufacturer's instructions (Bio-Rad, Hercules, CA). After electrophoresis, a protein band of 65 kDa was removed from the gel and homogenized in a mixture of 200 μl of PBS and 200 μl of Freund's incomplete adjuvant. A rabbit was subcutaneously injected with the suspension. Immunizations were performed three times in a 15 days interval and the bleedings (10 ml) started 40 days after the first injection, according to the protocol described by Harlow and Lane (1988). The collected blood samples were centrifuged at 5·000 ***g*** for 20 min and stored in aliquots with 50% glycerol at −20°C (Harlow and Lane 1988; Sambrook *et al.* 2001).

### Recombinant Cry1Ia expression in insects

Third-instar *S. frugiperda* larvae were infected with the recombinant vSynBtCry1Ia, as described above, and at 120 h.p.i. dead insects were collected, viral occlusion bodies and protein crystals were purified following the protocol described by O'Reilly *et al.* (1992). Mock-infected (infected with only cell culture medium), and purified crystals and viral occlusion bodies were analysed in two 12% SDS-PAGE gels, according to Laemmli (1970). One of them was stained with Comassie blue. The other was used to transfer the proteins to a PVDF (Millipore) membrane using the Trans-Blot® SD – Semi Dry Transfer Cell (Bio-Rad), according to the manufacturer's instructions. The membrane was processed and revealed by using the Western Breeze® kit (Invitrogen), using the anti-Cry1I antibody in a 1 : 50 dilution, according to the manufacturer's instructions.

### Bioassays with the recombinant Cry1Ia protein

One thousand and five hundred third-instar *S. frugiperda* larvae were infected with vSynBtCry1Ia as described above and 5 days later, viral occlusion bodies and protein crystals were purified as above. The total protein content of purified occlusion bodies and crystals was measured with the Bradford reagent (Bradford 1976). Of the purified occlusions and crystals, 15 μl were fractionated in a 12% SDS-PAGE as described above. The gel was scanned and the amount of Cry1Ia protein quantified by using the Image Phoretix 2D program (Amersham Pharmacia), using different concentrations of BSA as standards. Five doses of recombinant Cry1Ia protein (100, 50, 20, 10 and 5 $\mu g\ ml^{-1}$) were separately added to *A. grandis* artificial diet as described by Martins *et al.* (2007). The *B. thuringiensis* strains were grown in NYSM (Yousten 1984) medium for 72 h and then, cultures (600 ml) were centrifuged at 12 800 ***g*** for 30 min, at 4°C, the spore/crystal mixture pellets were frozen for 16 h and lyophilized for 18 h in Labconco model Lyphlock 18 freeze-dryer. Afterwards, the material was weighted for use in the bioassay. The required weight of powder for each dilution was taken up in 1 ml 0·01% Tween 20 to achieve a more homogeneous suspension and this was added to 4 ml of the artificial diet before it was poured out into six well plates. Five holes were punched in each well and each hole received a neonate larva. The bioassay was kept in an incubator with photoperiod of 14 : 10 at 27°C. A week later, the bioassay was assessed and the $LC_{50}$ was determined through Probits analysis (Finney 1971). We used the Btt (S1122) and S811 strains for comparison reasons as they have been recently shown to be toxic to *A. grandis* (Martins *et al.* 2007). Furthermore, the Btt strain (S1122) express the Cry3A and the S811 contain the *cry1Ab*, *cry1I* and *cry8* genes and produce major proteins of 130 and 65 kDa (Martins *et al.* 2007). The total culture was used as the Cry1Ia protein in this strain does not crystallize and probably would be lost during crystals purification. *Spodoptera frugiperda* bioassays were carried out according to Monnerat *et al.* (2007). Ten doses (from $10^{-1}$ to $10^{-10}$) from a recombinant Cry1Ia protein stock (20 μg protein/ml) and from total culture from *B. thuringiensis* S1450 (HD-1) strain were used. Mortality was recorded 7 days later, and the $LC_{50}$ obtained by Probits analysis (Finney 1971). The bioassays were repeated three times and the $LC_{50}$ were compared by ANOVA using the Sigma stat program (Kuo *et al.* 1992).

### Ultra structural analysis of putative Cry1Ia crystals

One hundred microlitre of the purified viral occlusion bodies and protein crystals from extracts of dead *S. frugiperda* larvae infected with vSynBtCry1Ia was spread out on a metal base, dried at room temperature, covered with gold for 180 s using a sputter coater Emitech K550 (Ashford, Kent), visualized in a scanning electron microscope (DSM 962; Zeiss, Thornwood, NY) and photographed.

### Ultra structural analysis of *Anthonomus grandis* midguts inoculated with the recombinant Cry1Ia protein

Fifteen fourth-instar *A. grandis* larvae were fed with artificial diet containing 115 $\mu$g of recombinant protein Cry1Ia per millilitre of diet. Inoculated larvae were collected at 24, 48 and 72 h after diet ingestion and the midguts were removed and processed for transmission electron microscopy, according to protocol described by Bozzola and Russel (1992). Ultra thin sections were carried out in an ultramicrotome (Leika ultracut UCT) and visualized in a transmission electron microscope (Jeol 1011, Brookvale, Australia).

### Molecular modelling of the Cry1Ia protein

The sequence of the *cry1Ia* gene was translated and the protein sequence used to submit to @TOME (Labesse and Mornon 1998) server, to identify the most suitable structural template for homology modelling to proteins of known structure. Followed by threading for conserved domains in the same server, the protein sequence was submitted to TITO server to evaluate the compatibility between the sequence of CryI1a and probable templates. A rough 3D model was constructed from the sequence alignment between Cry1Ia and the best template protein using MODELLER 8v0 (Depts of Biopharmaceutical Sciences and Pharmaceutical Chemistry, Univ. California San Francisco, CA, USA) with parameters of energy minimization value. In the last step of molecular modelling was the refined structure where the model was subjected to a series of tests for its internal consistency and reliability. Backbone conformation was evaluated by the inspection of the Psi/Phi Ramachandran plot obtained from PROCHECK analysis (Bhattacharya *et al.* 2007).

## Results

### Cloning and sequencing of the *cry1Ia* gene

Specific oligonucleotides were used to amplify the *cry1Ia* gene (Genbank accession number: DQ535488) in a PCR reaction using DNA from *B. thuringiensis* S1451 strain (data not shown). The amplified fragment of 2·1 kb was first cloned into the PCR cloning vector pGEM-T easy (pGemBtcry1Ia, data not shown) and three independent clones were sequenced. The sequence analysis revealed that this gene has an ORF (open reading frame) of 2196 pb, potentially coding for a protein of 731 amino acids (not shown), showing 99% identity with the *cry1Ia* gene sequence described by Tailor *et al.* (1992) (Genbank accession number: X62821). Just five nucleotide substitutions were found along its sequence (A1602T, A1793G, T1865C, C2194G, G2195C) when compared with the sequence described by Tailor *et al.* (1992). Three of the five nucleotides discrepancies resulted in alteration of three amino acids (K514N, N578S and F602S) (not shown), however, the others two mutations did not change the amino acid sequence of the protein.

### Construction and purification of recombinant virus vSynBtCry1Ia

The *cry1Ia* gene was removed from plasmid pGemBtcry1Ia and cloned into the transfer vector pSynXIVX3+ (Wang *et al.* 1991) (Fig. 1). The transfer vector was co-transfected with linearized DNA from vSynVI$^-$gal (Fig. 1) in insect cells and, 1 week later, the supernatant of transfected cells were used to purify the recombinant virus vSynBtCry1Ia (Fig. 1).

### Recombinant Cry1Ia expression in insect cells and insects

Insects were infected with recombinant virus vSynBtCry1Ia. The extracts from uninfected insects and purified crystals and virus from vSynBtCry1Ia-infected insects (120 h.p.i.) were analysed by SDS-PAGE and immunoblot with an antibody anti-Cry1Ia. Only the SDS-PAGE and immunoblot of insect extracts revealed the presence of a 65 kDa polypeptide that was recognized by the antibody raised against the Cry1Ia protein (Fig. 2).

### Bioassays with the recombinant Cry1Ia protein

Neonate *A. grandis* larvae were separately incubated with artificial diet containing different doses of the recombinant Cry1Ia protein and a week later, the $LC_{50}$ was determined (Table 1). The $LC_{50}$ for the recombinant Cry1Ia protein was significantly lower (21·5 $\mu$g ml$^{-1}$) than the Btt strain (328·0 $\mu$g ml$^{-1}$) and S811 (1500·00 $\mu$g ml$^{-1}$), when total protein was compared. Occlusion bodies (polyhedra) from wild-type baculovirus AcMNPV were also tested and did not present activity against the cotton boll weevil, proving that the virus was not the responsible for the toxicity to the insect (data not shown). The

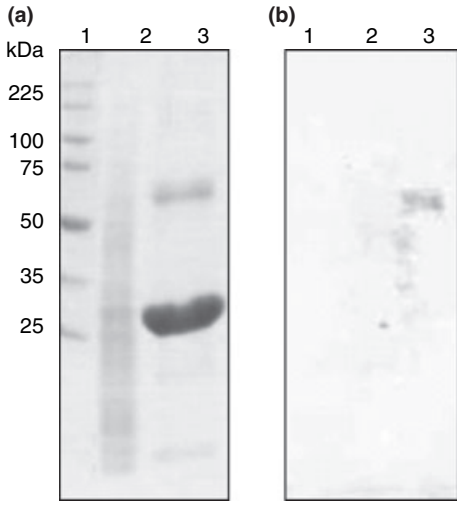


**Figure 2** Recombinant Cry1Ia protein expression: (a) 12% SDS-PAGE of extracts from mock no infected insects and purified viral occlusion bodies and crystals from vSynCry1Ia-infected insect cadavers. Lane 1 – Molecular mass marker (Promega), 2 – *S. frugiperda* mock infected and 3 – Purified crystals from S. frugiperda larvae infected with vSyn-Cry1Ia (120 h.p.i.). (b) Immunoblotting with antiserum anti-Cry1Ia produced in this work using a gel similar to the one shown in (a). The arrows indicate the protein of 65 kDa in purified crystals from *S. frugiperda* infected with the vSynCry1Ia virus. The strong band around 30 kDa is the polyhedrin protein, the main occlusion body protein.

**Table 1** Bioassay against *Anthonomus grandis*. Results for the recombinant Cry1Ia protein, S1122 (Btt) and S811 strains. The results of three different bioassays with their respective mean $LC_{50}$ values are shown. *n*: number of tested individuals per concentration each bioassay

| Sample | *n* | $LC_{50}$ ($\mu$g ml$^{-1}$)* |
|---|---|---|
| Cry1Ia | 25 | 21·5 (17·0–26·0)† |
| S1122 (Btt) | 25 | 328·0–440·0 |
| S811 | 25 | 1500·0 (0·974–2·855 |

*Mean $LC_{50}$ results.
†Number in parentheses are 95% confidence limits.

recombinant Cry1Ia toxin was also tested against lepidopteran insect *S. frugiperda* with an $LC_{50}$ of 0·289 $\mu$g ml$^{-1}$ which was around 11 times lower than the $LC_{50}$ (3·457 $\mu$g ml$^{-1}$) of the total culture from the standard strain (Btk) toxic to lepidopteran insects (Table 2).

**Table 2** Bioassay of the recombinant Cry1Ia protein and Btk strain against *Spodoptera frugiperda*. The results of three different bioassays with their respective mean $LC_{50}$ values are shown. *n*: number of tested individuals per concentration each bioassay

| Sample | *n* | $LC_{50}$ ($\mu$g ml$^{-1}$)* |
|---|---|---|
| Cry1Ia | 24 | 0·289 (0·032–3·310)† |
| S1450 (Btk) | 24 | 3·457 (0·711–15·8) |

*Mean $LC_{50}$ results.
†Number in parentheses are 95% confidence limits.

### Ultra structural analysis of putative Cry1Ia crystals

Purified viral occlusion bodies and crystals from vSynBt-Cry1Ia-infected *S. frugiperda* extracts were analysed by scanning electron microscopy (Fig. 3), showing large cuboidal crystals, probably derived from the recombinant Cry1Ia protein crystallization.

### Ultra structural analysis of *Anthonomus grandis* midguts inoculated with the recombinant Cry1Ia protein

Recombinant Cry1Ia protein proved to be highly toxic to *A. grandis* midgut cells, as it disrupted cellular integrity and microvilli of midgut columnar cells (Fig. 4).

### Molecular modelling of the Cry1Ia protein

Protein sequence comparison of Cry1Ia with PDB crystal structures made by FASTA software (Pearson 2000) revealed three possible templates, which are listed in Table 3. To increment the evaluation of compatibility between the templates and the target, the TITO score was used to better consider the choice of a template. Table 3 presents TITO scores where it is shown that the best template was the 1DLC structure (Cry3A) as the lowest score was found for that structure. The lowest value corresponds to a better compatible spatial template. The Cry1Ia structure showed a more similar and uniform distribution amino acids in the Ramachandran Plot (Lovell *et al.* 2002) when compared with the Cry3A protein (not shown) with 89·6% favourable residues, 6·7% residues in allowed locations and 3·7% no favourable residues (not shown). Although the Cry1Aa structure would share more identical residues with Cry1I (Table 3), the distribution of the amino acids for the Cry1Aa protein found only 83·4% favourable residues, 13·9% residues in allowed locations and 2·7% no favourable residues (not shown). The proposed model with the chosen template is presented in Fig. 5a. The proposed model presents the three conserved domains (I, II and III) of Cry proteins are shown in A. In Fig. 5b, domain I (in detail) is has seven $\alpha$-helices, six of them amphypatic and one hydrophobic. The domain III (in detail) can be seen in Fig. 5c.

## Discussion

In this work, we have described the cloning, sequencing, expression, possible structure and toxicity of the recombinant protein encoded by the *cry1Ia* gene from the Brazilian *B. thuringiensis* S1451 strain. The *cry1Ia* gene sequence showed 99% identity with the *cry1Ia* gene described by Tailor *et al.* (1992). In *B. thuringiensis*, the Cry1Ia protein does not crystallize, as it is secreted during

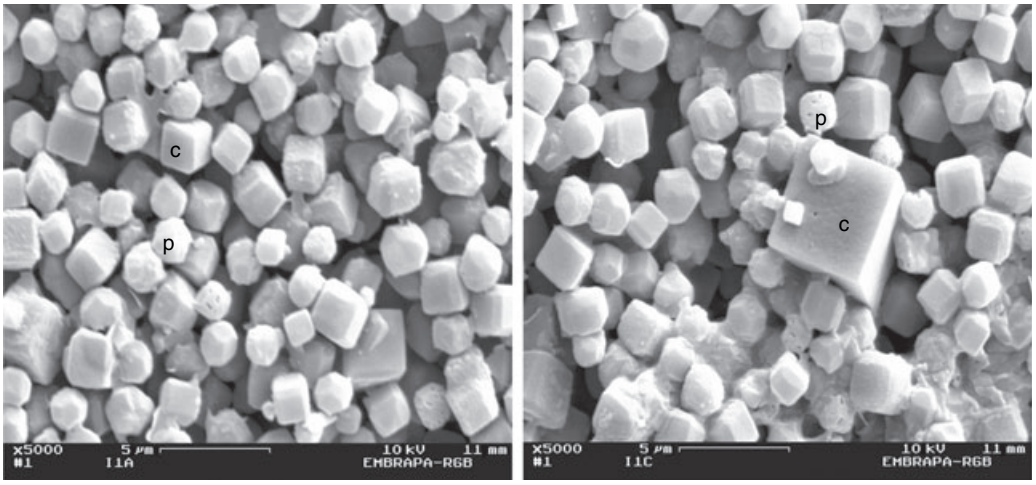


**Figure 3** Ultra structural analysis of crystals from purified viral occlusion bodies and crystals from dead *S. frugiperda* larvae infected (120 h.p.i.) with vSynBtCry1Ia. Scanning electron micrograph of crystals probably produced by the recombinant Cry1Ia protein. C (crystal) and P (viral occlusion body also called polyhedra).

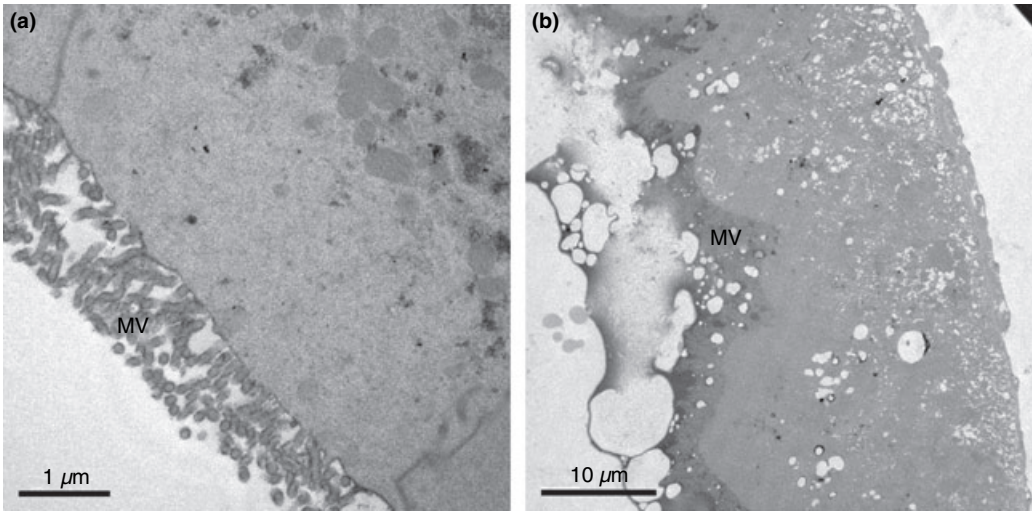


**Figure 4** Cytopathic effect of the recombinant Cry1Ia protein in *A. grandis* midgut columnar cells: (a) Midgut columnar cell of a *A. grandis* larvae without treatment with the Cry1Ia protein. (b) Midgut columnar cell of a larvae fed with artificial diet containing 115 $\mu$g ml$^{-1}$ of Cry1Ia (48 h after inoculation). Arrow indicates microvillus (MV). We can easily see the disorganization of the cell cytoplasm and the expanding of the microvillus in cells treated with the recombinant toxin (b).

**Table 3** Protein sequence comparison of Cry1Ia with PDB crystal structures made by FASTA software. The Cry3A protein showed the lowest value for the TITO scores which corresponds to a better compatible spatial template

| Template PDB ID | PDB summary | Identity (%) | E-value | TITO score |
|---|---|---|---|---|
| 1CIY (Cry1Aa) | http://www.ebi.ac.uk/thornton-srv/databases/pdbsum//1ciy/main.html | 43 | 0 | −237 045 |
| 1DLC (Cry3A) | http://www.ebi.ac.uk/thornton-srv/databases/pdbsum//1dlc/main.html | 40 | $2{\cdot}73 \times 10^{-3}$ | −264 055 |
| 1JI6 (Cry3Bb1)-chain A | http://www.ebi.ac.uk/thornton-srv/databases/pdbsum//1ji6/main.html | 39 | $2{\cdot}74 \times 10^{-3}$ | −251 813 |

the initial phase of sporulation (Kostichka *et al.* 1996). As a result, it cannot be accumulated neither detected in the bacterial cell after the T5 sporulation stage (Tounsi and Jaoua 2002; Song *et al.* 2003). In some *B. thuringiensis* strains, the *cry1Ia* gene could be detected by PCR, but neither its transcript, nor its protein could be detected. Some studies have shown that this gene can be silenced in some Bt strains because it is located downstream to

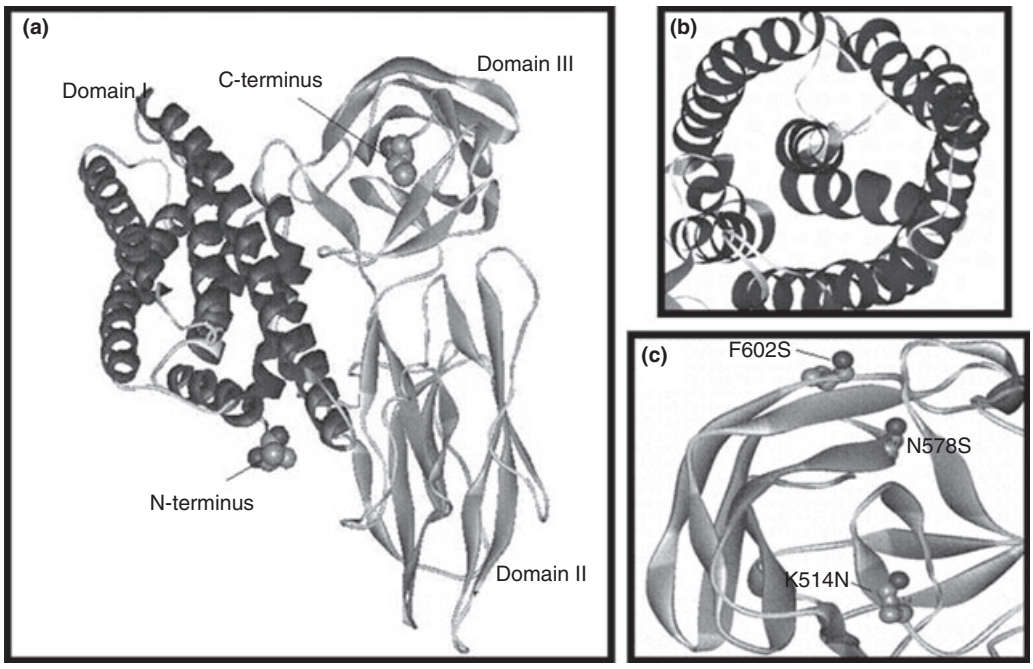


**Figure 5** (a) Theoretical tertiary structure of the Cry1Ia protein. The structure was modelled based on the known Cry3A structure (PDB code: 1DLC) and visualized with the WebLab ViewerLite 3.7 program (Molecular Simulations; Pharmacopeia Inc., Princetown, NJ); (b) Detail of domain I of the Cry1Ia protein showing seven |α-helices, six of them amphypathic and one (central) hydrophobic; (c) Detail of domain III, showing the three points of mutation (K514N, N578S and F602S) in the amino acid sequence when compared with the sequence described by Tailor *et al.* (1992) (Genbank accession number: X62821).

*cry1* genes and a strong transcriptional terminator is present between the sequences of the *cry1* and *cry1I* genes (Tailor *et al.* 1992; Gleave *et al.* 1993; Shin *et al.* 1995; Song *et al.* 2003).

The Cry1Ia protein expressed by the recombinant virus vSynBtCry1Ia in infected insect cells had the capacity of self crystallization. As the *cry1Ia* gene was under the transcriptional control of a strong promoter [a modified version of the polyhedrin promoter, PXIV, (Wang *et al.* 1991)], the high expression levels of the Cry1Ia protein may have contributed to the crystallization of the protein into cuboidal crystals. The *cry1Ia* gene encodes a protein of 81 kDa in *B. thuringiensis*. This protein is a pro-toxin and is cleaved, in the insect midgut, into an active polypeptide of around 65 kDa, capable of binding to specific receptors on the midgut of lepidopteran and coleopteran insects (Tailor *et al.* 1992; Gleave *et al.* 1993; Tounsi *et al.* 2003).

SDS-PAGE analysis of vSynBtCry1Ia-infected insects showed a polypeptide of 65 kDa, not present in mock-infected insects (Fig. 2a) and insects infected with the wild-type virus (AcMNPV) (not shown). This polypeptide probably represents the active form of the toxin that was processed by insect proteases, as the *cry1Ia* gene encodes a protein of 81 kDa (Gleave *et al.* 1993; Shin *et al.* 1995; Choi *et al.* 2000). The immunoblot using virus-infected insect extract also detected a 65 kDa polypeptide (Fig. 2b).

The recombinant Cry1Ia protein showed typical cytopatic effects of Bt toxins on the midgut cells surface. The microvilli was damaged probably by the action of the toxin (Fig. 4b) and it was possible to note endomembrane dilation and disorganization of the organelles in the cytoplasm of the cell (not shown) Bauer and Pankratz (1992) have described the effect of *B. thuringiensis* sub. *san diego* action in the midgut of *Chrysomela scripta* larvae and reported the morphologic changes in the midgut cells, very similar to the ones described in this work.

The recombinant Cry1Ia protein was shown to be highly toxic to neonate *A. grandis* larvae. When the same amount of Cry1Ia protein and the total protein of the Btt and S811 strains were administrated to the insects, the toxic activity of the recombinant protein was about 15 times higher than Btt, which has the *cry3* gene and, 69 times higher than S811, which has the *cry1Ab*, *cry1I* and *cry8* genes (Martins *et al.* 2007) (Table 1). The bioassays with the S811 strain was carried out with total culture (cells and media), as the Cry1Ia protein, in Bt strains, does not crystallize and is secreted into the culture media (Espinasse *et al.* 2003). Consequently, if the crystals from the S811 strains were purified, a fraction of the culture containing Cry proteins could be lost. The $LC_{50}$ for the recombinant Cry1Ia to *A. grandis* larvae was 21·5 $\mu$g ml$^{-1}$ (df = 0·034 and $P < 0·01$), indicating that Cry1I is a good

candidate based on its $LC_{50}$ to be used for the control of the cotton boll weevil, as it showed higher toxicity to a coleopteran insect. The Kruskal–Wallis one-way analysis of variance on ranks showed the differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P = <0{\cdot}001$) (SIGMA STAT 3.1). Furthermore, the protein also showed high toxicity to a second cotton pest, *S. frugiperda* larvae (Table 2) with a $LC_{50}$ of 0·289 $\mu g\ ml^{-1}$ (DF = 0·182 and $P < 0{\cdot}01$) (SIGMA STAT 3.1) (Kuo *et al.* 1992). Therefore, the *cry1Ia* gene is a strong candidate for the construction of transgenic cotton plants, as it is toxic to two important cotton pests.

Tailor *et al.* (1992) have shown that a dose of 195 $\mu g\ ml^{-1}$ of the Cry1Ia toxin was necessary to kill 60% of *L. decemlineata* and 16 $\mu g\ ml^{-1}$ was sufficient to kill 100% of *O. nubilalis*. Shin *et al.* (1995), also tested the Cry1Ia toxin against *Bombyx mori* (Lepidoptera, Bombycidae) and *P. xylostella* (Lepidoptera: Yponomeutidae) and obtained a $LC_{50}$ of 10·9 and 17·4 $\mu g\ ml^{-1}$, respectively. According to Choi *et al.* (2000), the Cry1Ia toxin is also toxic to *B. mori* ($LC_{50}$ of 7·08 $\mu g\ ml^{-1}$) and to *P. xylostella* ($LC_{50}$ of 2·57 $\mu g\ ml^{-1}$).

The recombinant Cry1Ia protein sequence was used for the prediction of its tertiary structure (Fig. 5), using the known tertiary structure of different Cry proteins. The Cry1Ia structure showed a more similar and uniform distribution of amino acids in the Ramachandran Plot (Lovell *et al.* 2002) when compared with the Cry3A protein (not shown) with 89·6% favourable residues, 6·7% residues in allowed locations and 3·7% no favourable residues (not shown). Although the Cry1Aa structure would share more identical residues with Cry1I the distribution of the amino acids for the Cry1Aa protein found only 83·4% favourable residues, 13·9% residues in allowed locations and 2·7% no favourable residues (not shown).

The Cry3 proteins present molecular mass of 73–75 kDa (Crickmore *et al.* 2007) and produce cuboidal or rhomboidal crystals. Krieg *et al.* (1983) described the first Bt strain that expressed a Cry3 protein. These proteins are known to be toxic to coleopteran insects, such as *L. decemlineata*. Once the protein Cry3Aa has been shown to be the best template to deduce the theoretical tertiary structure of the Cry1Ia protein, it is likely that these two proteins have a similar 3-dimensional structure. The *N*-terminal amino acid sequence of Cry3 proteins up to residue 637, corresponding to the putative toxic fragment, is homologous to other Cry proteins, like Cry1 protein (Lambert *et al.* 1992).

The theoretical tertiary structure of the recombinant Cry1Ia protein (Fig. 5) showed that the three amino acids differing from the original sequence described by Tailor *et al.* (1992) are located in the beginning of a loop region in domain III of the protein, suggesting that they may interfere in a positive or negative way in the binding of the protein to membrane receptors on the midgut cells surface and, consequently, in the protein toxicity. However, it is necessary to conduct site directed mutagenesis in these residues to confirm if there is any effect on the protein toxicity. To our knowledge, this is the first report describing a Cry protein, which is highly toxic to *A. grandis*. The data presented in this work might, therefore, help the development of transgenic plants resistant to this important cotton pest.

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## References

Aguiar, R.W.S., Martins, E.S., Valicente, F.H., Carneiro, N.P., Batista, A.C., Melatti, V.M., Monnerat, R.G. and Ribeiro, B.M. (2006) A recombinant truncated Cry1Ca protein is toxic to Lepidopteran insects and forms large cuboidal crystals in insect cells. *Curr Microbiol* **53**, 287–293.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**, 372–382.

Bauer, L.S. and Pankratz, H.S. (1992) Ultrastructural effects of *Bacillus thuringiensis* var. *san diego* on midgut cells of the cottonwood leaf beetle. *J Invertebr Pathol* **60**, 15–25.

Bhattacharya, A., Wunderlich, Z., Monleon, D., Tejero, R. and Montelione, G.T. (2007) Assessing model accuracy using the homology modeling automatically (HOMA) software. *Proteins: Struct. Funct. Bioinformatics*. EPUB ahead of print. doi: 10.1002/prot.21466.

Bozzola, J.J. and Russel, L.D. (1992) *Electron Microscopy: Principles and Techniques for Biologists*, 2nd edn. Boston, MA: Jones & Barttett.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**, 248–254.

Choi, S.K., Shin, B.S., Kong, E.M., Rho, H.M. and Park, S.H. (2000) Cloning of a new *Bacillus thuringiensis cry1I*-type crystal protein gene. *Curr Microbiol* **41**, 65–69.

Crickmore, N., Zeigler, D.R., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Bravo, A. and Dean, D.H. *Bacillus Thuringiensis Toxin Nomenclature*. Available at http://www.

biols.susx.ac.uk/Home/Neil_Crickmore/Bt/. Accessed 20 January 2007.

Espinasse, S., Chaufaux, J., Buisson, C., Perchat, S., Gohar, M., Bourguet, D. and Sanchis, V. (2003) Occurrence and linkage between secreted insecticidal toxins in natural isolates of *Bacillus thuringiensis*. *Curr Microbiol* **47**, 501–507.

Feilteison, J.S. (1994) Novel pesticidal delta-endotoxins from *Bacillus thuringiensis*. In *Proceedings of XXVII Annual Meeting of the Society for Invertebr Pathol* pp. 19–184. Montpellier, France: SIP.

Finney, D.J. (1971) *Probit Analysis*. Cambridge: Cambridge University Press.

Gleave, A.P., Williams, R. and Hedges, R.J. (1993) Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of a *cryV* gene cloned from *B. thuringiensis* subsp. Kurstaki. *Appl Environ Microbiol* **59**, 1683–1687.

Granados, R.R., Guoxun, L., Derksen, C.G. and Mckenna, K.A.A. (1994) An insect cell line from *Trichoplusia ni* (BTI-TN-5B1-4) susceptible to T*richoplusia ni* single enveloped nuclear polyhedrosis virus. *J Invertebr Pathol* **64**, 260–226.

Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Kostichka, K., Warren, G.W., Mullins, M., Mullins, A.D., Craig, J.A., Koziel, M.G. and Estruch, J. (1996) Cloning of a *cryV*-type insecticidal protein gene from *Bacillus thuringiensis*: the *cryV*-encoded protein is expressed early in stationary phase. *J Bacteriol* **178**, 2141–2144.

Krieg, A., Huger, A.M., Langenbruch, G.A. and Schnetter, W. (1983) *Bacillus thuringiensis* var. *tenebrionis*: ein neuer, gegenüber larven von coleopteren wirksamer pathotyp. *Z Ang Entomol* **96**, 500–508.

Kuo, J., Fox, E. and MacDonald, S. (1992). *Sigmastat: Statistical Software for Working Scientists. Users Manual*. San Francisco, CA: Jandel Scientific.

Labesse, G. and Mornon, J. (1998) Incremental threading optimization (TITO) to help alignment and modelling of remote homologues. *Bioinformatics* **14**, 206–211.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature* **227**, 680–685.

Lambert, B., Höfte, H., Annys, K., Jansens, S., Soetart, P. and Peferoen, M. (1992) Novel *Bacillus thuringiensis* insecticidal crystal protein with a silent activity against coleopteran larvae. *Appl Environ Microbiol* **58**, 2536–2542.

Lovell, S.C., Davis, I.W., Arendall, W.B. III, de Bakker, P.I.W., Word, J.M., Prisant, M.G., Richardson, J.S. and Richardson, D.C. (2002) Structure validation by Calpha geometry: phi, psi and Cbeta deviation. *Proteins* **50**, 437–450.

Martins, E.S, Praça, L.B., Dumas, V.F., Silva-Werneck, J.O., Sone, E.H., Waga, I.C., Berry, C. and Monnerat, R.G. (2007) Characterization of *Bacillus thuringiensis* isolates toxic to cotton boll weevil (*Anthonomus grandis*). *Biol Control* **40**, 65–68.

Monnerat, R.G. and Bravo, A. (2000) Proteínas bioinseticidas produzidas pela bactéria *Bacillus thuringiensis*: modo de ação e resistência, vol. 3, In: *Controle Biológico* ed. Melo, I.S. and e Azevedo, J.L. pp. 136–200. Jaguariúna, SP: Controle Biológico.

Monnerat, R.G., Batista, A.C., de Medeiros, P.T., Martins, É.S., Melatti, V.M., Praça, L.B., Dumas, V.F., Morinaga, C. *et al.* (2007) Screening of Brazilian *Bacillus thuringiensis* isolates active against *Spodoptera frugiperda, Plutella xylostella and Anticarsia gemmatalis*. *Biol Control* **41**, 291–295.

O'Reilly, D.R., Miller, L.K. and Luckow, V.A. (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. New York: Freeman.

Pearson, W.R. (2000) Flexible sequence similarity searching with the FASTA3 program package. *Methods Mol Biol* **132**, 185–219.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Shin, B.S., Park, S.H., Choi, S.K., Koo, B.T., Lee, S.T. and Kim, J. (1995) Distribution of *cryV*-type insecticidal protein genes in *Bacillus thuringiensis* and cloning of *cryV*-type genes from *bacillus thuringiensis* subsp. *kurstaki* and *Bacillus thuringiensis* subsp. *entomocidus*. *Appl Environ Microbiol* **61**, 2402–2407.

Song, F., Zhang, J., Gu, A., Wu, Y., Han, L., He, K., Chen, Z., Yao, J. *et al.* (2003) Identification of *cry1I*-type genes from *Bacillus thuringiensis* strains and characterization of a novel *cry1I*-type gene. *Appl Environ Microbiol* **69**, 5207–5211.

Tailor, R., Tippett, J., Gibb, G., Pells, S., Pike, D., Jordan, L. and Ely, S. (1992) Identification and characterization of a novel *Bacillus thuringiensis* delta-endotoxin entomocidal to Coleopteran and Lepidopteran larvae. *Mol Microbiol* **6**, 1211–1217.

Tounsi, S. and Jaoua, S. (2002) Identification of a promoter for the cristal protein-encoding gene *cry1Ia* from *Bacillus thuringiensis* subs. *kurstaki*. *FEMS Microbiol Lett* **208**, 215–218.

Tounsi, S., Zouari, N. and Jaoua, S. (2003) Cloning and study of the expression of a novel *cry1Ia*-type gene from *Bacillus thuringiensis* subsp. k*urstaki*. *J Appl Microbiol* **95**, 23–28.

Wang, X., Ooi, B.G. and Miller, L.K. (1991) Baculovirus vectores for multiple gene expression and for occluded virus production. *Gene* **100**, 131–137.

Yousten, A.A. (1984) *Bacillus sphaericus*: microbiological factors related to its potential as a mosquito larvicide. *Adv Biotechnol Processes* **3**, 315–343.